EBV and Systemic Lupus Erythematosus: A New Perspective

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EBV and Systemic Lupus Erythematosus: A New Perspective

Andrew J. Gross, Donna Hochberg, William M. Rand, and David A. Thorley-Lawson

We have proposed that EBV uses mature B cell biology to access memory B cells as a site of persistent infection. A central feature of this model is that EBV adopts its gene expression profile to the state of the B cell it resides in and that the level of infection is stable over time. This led us to question whether changes in the behavior or regulation of mature B cells would alter the state of EBV persistence. To investigate this, we studied the impact of systemic lupus erythematosus (SLE), a disease characterized by immune dysfunction, on EBV infection. We show that patients with SLE have abnormally high frequencies of EBV-infected cells in their blood, and this is associated with the occurrence of SLE disease flares. Although patients with SLE have frequencies of infected cells comparable to those seen in immunosuppressed patients, in SLE the effect was independent of immunosuppressive therapy. Aberrant expression of viral lytic (BZLF1) and latency (latency membrane proteins 1 and 2a) genes was also detected in the blood of SLE patients. We conclude that the abnormal regulation of EBV infection in SLE patients reflects the sensitivity of the virus to perturbation of the immune system. The Journal of Immunology, 2005, 174: 6599–6607.
the regulation of viral replication. Mechanistically, these two outcomes may have very different causes. Because the previous reports used techniques that were unable to distinguish the two possibilities, the origin of the increased viral load in SLE (more latently infected cells or more viral replication) is not known. Furthermore, these reports did not examine critical measures of viral persistence, such as viral gene expression and the phenotype of infected cells, which define more precisely the state of viral regulation.

We decided to revisit the issue of EBV perturbation in SLE, because a more thorough and complete survey of changes in viral persistence in SLE could characterize the nature of the perturbation and thereby provide new insight into the mechanisms involved in regulating the virus. In this study, we confirm definitively that EBV infection is perturbed in the setting of SLE. We now show that the increased viral load is due to an increase in the numbers of latently infected cells, particularly in patients with active disease. Furthermore, for the first time, we demonstrate that patients with SLE have aberrant expression of viral latent and lytic genes in the blood. We conclude that immune dysfunction in SLE alters the regulatory mechanisms of EBV persistence, and we discuss how B cell deregulation may impact viral persistence.

Materials and Methods
Patient populations
Patient studies were approved by the institutional review board of Tufts New England Medical Center (T-NEMC). Thirty-five patients attending the adult rheumatology clinic and the nephrology clinic at T-NEMC who met the American College of Rheumatology 1997 revised criteria for the diagnosis of SLE were recruited for this study. At the time of blood collection, patients’ medical records were reviewed, and current medications, pertinent laboratory data, SLE disease history, as well as evidence of disease activity as documented by the treating physicians were recorded. From these data, a SLE disease activity index (SLEDAI) was calculated (22) (median SLEDAI, 2; range, 0–13). A patient was considered to have a disease flare if the SLEDAI was >4 (23). The time interval since patients’ last SLE disease flare (SLEDAI >4) was also determined (months since last flare: median, 28; range, 0–122). (For patient demographics, see Table I.)

Forty-four healthy volunteers were recruited from Tufts University School of Medicine. Seventeen patients attending the rheumatology clinic who met the American College of Rheumatology 1997 revised criteria for other SAIDs were recruited as another control group. These patients generally had recent onset of these conditions (<3 mo) and were generally untreated, except for three who were treated with prednisone (5–7.5 mg/day) and three who were treated with sulfasalazine. One patient with Crohn’s disease, diagnosed by colonic biopsy, not treated with any immunosuppressive medications, was recruited for this study from the gastroenterology clinic. (For healthy and SAID patient demographics, see Table I.) (Unlike SAID patients, most SLE patients had longstanding disease (mean disease duration, 11 ± 8 years; 30 patients diagnosed with SLE ≥2 years before study), but there was no correlation between SLE disease duration and frequency of EBV-infected cells in the blood (p = 0.3; data not shown).)

Table 1. Patient demographics for the three study groups

<table>
<thead>
<tr>
<th></th>
<th>SLE</th>
<th>Healthy</th>
<th>SAID</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>35</td>
<td>44</td>
<td>17</td>
</tr>
<tr>
<td>Age, mean (range)</td>
<td>40 (21–73)</td>
<td>29 (21–61)</td>
<td>48 (24–80)</td>
</tr>
<tr>
<td>% Female</td>
<td>91</td>
<td>43</td>
<td>64</td>
</tr>
<tr>
<td>Race^c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Caucasian</td>
<td>49</td>
<td>68</td>
<td>73</td>
</tr>
<tr>
<td>% African-American</td>
<td>14</td>
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<td>20</td>
</tr>
<tr>
<td>% Asian</td>
<td>20</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>% Other</td>
<td>17</td>
<td>25</td>
<td>7</td>
</tr>
</tbody>
</table>

^a p < 0.001 for SLE and for SAID compared to healthy.
^b p < 0.001 for SLE compared to healthy; p = 0.13 for SAID compared to healthy.
^c Age and race unavailable for two patients in SAID group.

The patient and control populations were not matched. This was because we have not previously observed any relationship between age, sex, or race, and frequency of EBV-infected cells (our unpublished data). Data from the current study maintain that sex and race have no significant influence on the frequency of infected cells among healthy individuals (p = 0.4 and p = 0.14, respectively; data not shown). The current data do indicate that there is a slight but statistically significant rise in frequency of infected cells with age observed in our measurement on healthy individuals (R^2 = 0.16; p = 0.01). Whether this trend is biologically significant or an epiphenomenon of this particular data set is unclear.

Primary cells and cell lines
Sixty milliliters of patients’ blood was collected by routine venipuncture into a syringe containing 1000 USP units of sodium heparin. Blood was layered onto Ficoll-Hypaque Plus (Pharmacia) and centrifuged at 2000 rpm for 30 min at 20°C, and theuffy coats containing the PBMCs were removed. PBMCs were then kept on ice, except where indicated. PBMCs were washed twice with 1× PBS/1.0% BSA (PBSA) and then resuspended at a desired concentration in the same buffer.

EBV-negative tonsil cells were used as negative controls as well as “filler” cells for RNA preparation. Tonsils were obtained from patients undergoing routine tonsillectomies for obstructed breathing disorders at the Massachusetts General Hospital. The tonsils were minced thoroughly, and connective tissue was removed by filtering through silk screen. Cells were resuspended in PBSA and purified as described above. Tonsils were repeatedly confirmed to be EBV negative by DNA PCR (W-repeat sequence) and RT-PCR (EBER1 transcripts) (described below).

The lymphoblastoid cell lines ER, IP, and AT, generated by our laboratory, were used as positive controls for EBNA2, LMP1, LMP2, and EBER expression at the RNA level, as well as for W-repeat DNA PCR. The EBV-positive Burkitt lymphoma line AKATA (gift from E. Keif, Harvard Medical School, Boston, MA) was used as positive controls for EBNA1(Q-K) expression. The EBV-positive marmoset cell line B95.8 (gift from E. Keif) was used as a positive control for expression of the lytic cycle gene BZLF1. The EBV-negative B cell line, BJAB (gift from E. Keif), was used as a negative control for these studies. All cell lines were cultured at 37°C with 5% CO2 in RPMI 1640 supplemented with 10% FCS, 2 mM sodium pyruvate, 2 mM glutamine, and 100 IU of penicillin/streptomycin.

Cell separations
B cells were purified by negative selection using the Stem Sep System (StemCell Technologies) as described by the manufacturer. Briefly, PBMCs were resuspended to a concentration of 5 × 10^7 cells/ml and stained with 100 μl/ml B cell enrichment Ab mixture (StemCell Technologies) for 15 min at room temperature. The Ab mixture contains Abs directed against all types of peripheral blood cells except B cells. Cells were then stained with 60 μg/ml magnetic colloid labeled Ab (StemCell Technologies) for 15 min at room temperature. The sample was then passed over a column, in the presence of a magnet, which allowed the population of interest to be collected as the flow-through fraction. Flow cytometric analysis was used to assay the purity of all isolated populations. Both fractions were stained with anti-CD20 FITC (Beckman Coulter), and/or anti-CD27 PE (BD Pharmingen), and/or anti-CD22 PE (BD Pharmingen), and analyzed using a FASCalibur (BD Biosciences). Data were analyzed with WinList 4.0 software (Verity Software House). FACS analysis allowed for the determination of both the recovery and purity of the isolated populations. B cell population purity following negative selection was 65–99% and typically was >90%.

Populations of naive and memory B cells were purified by FACS separation. PBMCs, prepared as described above, were resuspended to a concentration of 2 × 10^5 cells/ml and stained for 30 min at 4°C with Abs to the desired cell surface markers, indicated above. After staining, cells were washed twice with PBSA and resuspended to a concentration of 1 × 10^6 cells/ml. Cell sorting was performed on a MoFlo FACS sorter (DakoCytomation). FACS reanalysis allowed for the determination of the isolated population purity.

Limiting dilution DNA PCR
Limiting dilution analysis was used to determine the frequency of EBV-infected cells for each patient. The details of this assay using DNA PCR have been published previously (24). It can detect the presence of a single EBV genome in a background of as many as 1 × 10^8 EBV-negative cells. Dilutions of isolated B cell populations were distributed into the wells of 96-well microtiter plates in triplicate. The cells were pelleted, the cell pellets were lysed, and the extract was subjected to DNA PCR specific to the W repeat region of the EBV genome exactly as described previously. PCR conditions were as follows: 5 μl of sample DNA,
0.2 mM dNTPs, 0.20 μM each primer, 2.0 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl, and 1 U of Taq (Applied Biosystems) in a total volume of 50 μl. The reaction was performed in a Geneamp 9600 thermocycler (PerkinElmer) for 35 cycles of 95°C for 15 s and 65°C for 1 min followed by 1 cycle of 72°C for 5 min. Specific products were resolved by electrophoresis, Southern blotted, and detected using radiolabeled purified specific PCR product from B4 cells. The frequency of infected cells was determined from the distribution of wells with positive PCR signals (as explained in Fig. 5, B and C), by applying Poisson statistics (for details of the quantification, sensitivity, and confidence limits of the analysis, see Ref. 25).

The absolute number of infected cells per milliliter of blood was calculated by multiplying the frequency of EBV-infected B cells by the fraction of CD20+ B cells in PBMCs (determined by FACS analysis) and number of PBMCs per milliliter of blood (from the number of PBMCs obtained following Ficoll extraction of blood).

**RT-PCR**

If patients had sufficient numbers of B cells isolated from the blood, EBV gene expression was determined in addition to the frequency of infected cells. RT-PCR was used to determine the expression of EBV genes indicative of viral replication and the three latency programs. The details of this assay have been published previously (7). Serial dilutions of isolated cell populations were prepared and aliquoted into Eppendorf tubes. EBV-negative tonsillar cells were added, when needed, to each tube to bring the total number of cells to 5 × 10⁶. The 10⁶ EBV+ lymphoblastoid cell line cells were used as a positive control. RNA was isolated by the TRIzol method (Invitrogen Life Technologies), and cDNA was prepared as described previously (8). PCR for the EBV genes EBNA2, EBNA1-(Q-K), LMP1, LMP2, and EBNA 3a/3b was performed by taking one-tenth of the cDNA (20 μl) for each gene. This was allowed for simultaneous PCR for all of the genes on the same sample. Primers used were as follows: EBNA1-(Q-K), TGC CCCCTCCTGACAGCATATG and AGGCGTCGGTACTCCGGAT (26); EBNA2, CATAGAAGAAGAGGAGAATGAGA and GTAGGGATTC GAGGAAATTTACGTA (27); LMP1, TTGTTGACTCTCTACTGAT GATCACC and AGTAGATCTGAGTCATCTAAGCAAGT (27); LMP2, ATCTCTTTGGCATGCGCTACTCAT and GTGTTGACGTATTGCAAA (28); EBERs, AAAACATGCGGACCACCAGC and AGGACCTACGCT ATGACTCATCTCAACATA and CATGTTAGGCAAATTGCAAA (28); BZLF1, TTCCACAGCCTGCACCAGTG and TTCCACAGCCTGCACCAGTG (28); and BZLF2, TTTCAAGACTGCAGCTCGAGTG and GCCAGCCAGCTCAGCTCAGGT (28). Reactions were performed as described previously (8). The reaction for BZLF1 was performed in a final volume of 50 μl containing 50 μM KCl, 20 mM Tris (pH 8.4), 1.5 mM MgCl2, 0.2 mM dNTPs, and 20 pM each primer under the following conditions: 40 cycles of 95°C for 15 s, annealing temperature of 59°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. Specific products were resolved by electrophoresis, Southern blotted, and detected using radiolabeled-purified specific PCR product (amplified from IB4 cells). The frequency of infected cells with advancing age in the healthy control group (Table I) was significantly different between these groups (mean ln frequency/10⁶ B cells for SLE patients = 3.54 ± 1.98; for healthy controls = 1.15 ± 1.35; p < 0.0001) (Fig. 1). Taking the antilog, these values translate into an average 10-fold increase, which is comparable to the average 12-fold increase that we have reported previously in immunosuppressed organ transplant patients (31).

Patients with SLE were significantly older than healthy controls (Table I) (p < 0.0001). Although there is no precedence for an age-related change in the frequency of infected cells, we did detect a small but statistically significant rise in the ln frequency of EBV-infected cells with advancing age in the healthy control group (R² = 0.16; p = 0.008) but not the patient groups (p = 0.7; data not shown). It is unclear whether this is a real trend or an epiphenomenon of this one data set. However, to exclude the possibility that the level of infected cells increases because of this uncertain age effect, ANCOVA was performed. This confirmed that patient group had a strong effect on the frequency of infected cells (F =

### Table II. Summary of frequencies of EBV-infected cells per million B cells in the blood of three patient groups: patients with SLE, healthy individuals, and patients with other SAIDs

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Median</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLE</td>
<td>35</td>
<td>26.0</td>
<td>1.1–1775</td>
</tr>
<tr>
<td>Healthy</td>
<td>44</td>
<td>3.3</td>
<td>0.3–30</td>
</tr>
<tr>
<td>SAID</td>
<td>17</td>
<td>9.4</td>
<td>1.5–99</td>
</tr>
</tbody>
</table>

* Because the distribution of the data in each of these groups is skewed, the medians and ranges are provided instead of the means and SDs, p < 0.0001 for all group comparisons.

**CMV DNA detection**

The 10³ PBMCs were digested with 1 mg/ml proteinase K (Sigma-Aldrich) at 55°C for at least 6 h. DNA was isolated by the phenol/chloroform/isoamyl alcohol method. One microgram of DNA was used for quantitative CMV DNA PCR assay that we have described previously (30). Because this assay detects intact infected cells, it provides a measure of the number of virus-infected cells that is independent of the number of viral genomes per cell (usually two to five copies). A limiting dilution assay prevents bias by the inclusion of even a single lytically infected cell that may contain thousands of genome copies and would therefore generate a very large increase in the viral load without affecting the frequency of infected cells.

Using this technique, we found elevated frequencies of virally infected cells in the peripheral blood of 35 patients meeting the American College of Rheumatology criteria for the diagnosis of SLE, as compared with 44 healthy individuals (Table II). Comparisons between patient groups while controlling for age and sex of the patient groups. Statistical analysis was performed using SPSS software (version 12.0; SPSS).
healthy, 14.1

fore, the high frequency of infected cells in SLE patients was not significantly more infected cells per milliliter of blood than healthy individuals (SLE, 8.8 × 10^4; healthy, 14.1 × 10^4; p = 0.01). The transformed data demonstrates that patients with SLE have significantly more infected cells per milliliter of blood than healthy individuals (SLE, mean ln infected cells per milliliter of blood, 0.51 ± 1.92; healthy, -1.12 ± 1.53; p < 0.001) (Fig. 2). Therefore, the high frequency of infected cells in SLE patients was not a consequence of B lymphopenia, but reflects an expansion in the absolute number of EBV-infected cells in the blood of these patients.

Treatment of SLE patients with immunosuppressive agents does not significantly affect the frequency of EBV-infected cells in the blood

Our laboratory and others (31, 33, 34) have shown previously that patients treated with immunosuppressive agents following organ transplant have high frequencies of EBV-infected cells in their blood. SLE patients are also treated with immunosuppressive drugs, albeit with fewer agents and at lower doses than patients who have received organ transplants. To investigate whether the high frequencies of infected cells observed in patients with SLE were a consequence of iatrogenic immunosuppression (IS), patients were stratified into three groups based on levels of IS at the time of phlebotomy: treatment with no agents other than hydroxychloroquine (no IS); treatment with prednisone, 2.5–7.5 mg/day (low IS); and treatment with prednisone, ≥10 mg/day, and/or another immunosuppressive agent such as methotrexate, azathioprine, cyclosporin, mycofenylate mofitile, or cyclophosphamide (high IS). We found no difference in the frequencies of EBV-infected B cells among these three groups (mean ln frequency/10^6 B cells: no IS, 3.27 ± 2.15, n = 13; low IS, mean, 3.35 ± 2.11, n = 10; and high IS, mean, 4.00 ± 1.75, n = 12; p = 0.6) (Fig. 3). Taking the antilog values, this represents, at best, a 2-fold increase (which is not statistically significant) between the no IS and the high IS groups, showing that the effect of SLE on the frequency of EBV-infected cells is independent of treatment with immunosuppressive medications.

Patients with SLE have higher frequencies of EBV-infected cells than patients with other SAIDs

To determine whether the effect of SLE on the frequency of EBV-infected cells in the blood is specific to SLE, we investigated EBV infection in 17 patients with other SAIDs. This included patients with rheumatoid arthritis (n = 11), spondyloarthropathy (n = 2), systemic sclerosis (n = 2), Sjogren’s syndrome (n = 1), and Crohn’s disease (n = 1). Many of these patients had been diagnosed recently with their diseases, had evidence of active inflammation, and were treated with no or very low doses of immunosuppressive medications. Although the frequencies of infected cells in these patients were higher than those of healthy individuals (p < 0.05), they were significantly lower than the frequency of EBV-infected cells in the blood of patients with SLE (SAID, mean ln frequency/10^6 B cells = 2.32 ± 1.31; p < 0.05) (Table II and

Interestingly, however, patients with SLE are not the only group with high frequencies of infected cells (Fig. 2). Our laboratory and others (31, 33, 34) have shown previously that patients treated with immunosuppressive agents following organ transplant have high frequencies of EBV-infected cells in their blood. SLE patients are also treated with immunosuppressive drugs, albeit with fewer agents and at lower doses than patients who have received organ transplants. To investigate whether the high frequencies of infected cells observed in patients with SLE were a consequence of iatrogenic immunosuppression (IS), patients were stratified into three groups based on levels of IS at the time of phlebotomy: treatment with no agents other than hydroxychloroquine (no IS); treatment with prednisone, 2.5–7.5 mg/day (low IS); and treatment with prednisone, ≥10 mg/day, and/or another immunosuppressive agent such as methotrexate, azathioprine, cyclosporin, mycofenylate mofitile, or cyclophosphamide (high IS). We found no difference in the frequencies of EBV-infected B cells among these three groups (mean ln frequency/10^6 B cells: no IS, 3.27 ± 2.15, n = 13; low IS, mean, 3.35 ± 2.11, n = 10; and high IS, mean, 4.00 ± 1.75, n = 12; p = 0.6) (Fig. 3). Taking the antilog values, this represents, at best, a 2-fold increase (which is not statistically significant) between the no IS and the high IS groups, showing that the effect of SLE on the frequency of EBV-infected cells is independent of treatment with immunosuppressive medications.

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SLE disease activity affects the frequency of virally infected cells.

SLE is a disease of flares and remissions. Although the cause of this is unknown, it is thought that disease flares represent times of greatest immune dysfunction (35). To investigate whether changes in the level of immune dysfunction could affect the regulation of EBV infection, the frequency of virally infected cells was studied in patients with active and inactive disease. Disease activity was quantified using the SLEDAI (22). A flare was defined as a score of >4 (23). Fifteen patients with recently active disease (flare within 3 mo of study) had significantly higher frequencies of infected cells than 16 patients with inactive disease (no flare within 12 mo of study) (mean ln frequency/10^6 B cells: recent flare = 4.44 ± 2.19; no recent flare = 2.60 ± 1.48; p = 0.01) (Fig. 4). (Patients with active disease were no more likely to be treated with immunosuppressive medications than patients with inactive disease (p = 0.9), because many patients with disease flares were studied before the initiation of therapy.)

To further investigate whether disease activity affects EBV infection, we measured the frequency of EBV-infected cells over time in individual patients. Unlike healthy individuals in whom the frequency of infected cells is relatively stable for years (25), we saw large fluctuations in the frequency of infected cells in the blood of seven patients over the course of 9–17 mo, and these were usually associated with changes in disease activity as indicated by the SLEDAI score (Table III). Interestingly, one patient (no. 7) had a rise in their frequency of infected cells, which was associated with a disease flare rather than a change in their immunosuppressive medications. These findings show that the effect of SLE on the regulation of EBV persistence is related to the presence of SLE disease activity and illustrates the sensitivity of the virus to changes in immune function.

There is no relationship between frequency of EBV-infected cells and specific clinical or serological features of SLE.

Because disease activity affects the frequency of infected cells, we wondered whether there was an association between serological markers of SLE disease activity and the frequency of infected cells. However, we found no correlation between the frequency of EBV-infected cells and levels of anti-dsDNA Ab (in patients who produced this Ab), complement levels, or erythrocyte sedimentation rate.

Patients with SLE can be stratified into subsets based on various clinical and immunological manifestations of the disease. We investigated whether the high frequencies of EBV-infected cells observed in many SLE patients were associated with particular disease subsets. No relationship was seen between the frequency of EBV-infected cells and the presence of nephritis (n = 14 of 35 patients with data available; p = 0.4), the presence of antiphospholipid Ab syndrome (n = 7 of 32; p = 0.9), or the production of certain autoantibodies documented at any point in patients’ disease history, including the following: anti-dsDNA Abs (n = 27 of 35; p = 0.8), anti-Smith Ab (n = 6 of 26; p = 0.8), and antiribonucleoprotein Ab (n = 8 of 18; p = 0.6) (data not shown).

Patients with SLE have aberrant latent and lytic gene expression in the blood.

Another indicator of EBV regulation in healthy carriers is the absence of viral gene expression in the blood (4). If EBV is affected...
by SLE immune dysfunction, it could result in inappropriate expression of viral genes in the blood. We investigated EBV gene expression in B cells from the peripheral blood of SLE patients using RT-PCR (Table IV). The immediate early lytic gene, BZLF1, which is involved in viral reactivation, was detected in 6 of 17 patients (35%). EBNA2 is the first latency gene activated following infection of a B cell in vitro; it was not detected in the blood of 23 SLE patients tested, indicating that newly infected cells are not present in the blood. LMP1 and LMP2a are viral genes expressed in all stages of EBV latency in tonsillar B cells and are of interest because they deliver surrogate B cell survival and differentiation signals (CD40 and BCR, respectively) (5, 36–38). LMP1 was detectable in 5 of 17 SLE patients’ blood (29%), and LMP2a was detected in 4 of 22 patients (18%). Although transcripts of LMP2a are found occasionally in the blood of healthy individuals, LMP1 is never detected (24, 27, 28, 39, 40). EBNA1 expression from the Q promoter (EBNA1(Q-K)) was also tested because this transcript is expressed along with LMP1 and LMP2a in a discrete transcription program, termed the default program, which is used in germinal center and memory B cells in the tonsils of healthy individuals as well as several tumors. Transcripts of EBNA1(Q-K) were detected in only 1 of 17 SLE patients, which is similar to what is observed in the blood of healthy people where EBNA1 is rarely detected. From these data, it can be concluded that EBV gene expression is aberrantly regulated in the blood of some patients with SLE. It is important to note that EBV regulation is not completely disrupted, because expression of the EBV growth program gene, EBNA2, was not detected.

To exclude the possibility that BZLF1, LMP1, and LMP2a expression was detected in the blood of lupus patients because of the relatively large quantities of EBV-infected cells that could be tested in these patients, limiting dilution analysis of gene expression was performed. This allows for the calculation of the frequency of infected cells expressing viral genes. Two SLE patients had sufficient numbers of samples with BZLF1 expression at various B cell dilutions to perform this analysis. Two and 0.5% of the infected cells in the blood of these patients expressed this lytic gene. Although only a small fraction of infected cells express BZLF1, such levels of expression are very abnormal. In comparison, we have never detected BZLF1 expression in the blood of healthy patients. We have detected BZLF1 expression in the blood of patients with infectious mononucleosis, but the frequency of infected cells expressing this lytic gene was considerably less (0.02 to <0.001%; n = 5) (data published in Ref. 41) than those in the two lupus patients. These findings confirm that BZLF1, and probably LMP1, are abnormally regulated in some patients with SLE.

The infected cells in the blood of patients with SLE are memory B cells

A hallmark of EBV infection in healthy individuals is the restriction of the virus to the IgD− population of memory B cells in the blood (24, 34, 42). The appearance of EBV in peripheral blood naive B cell would represent a profound deregulation of persistent infection. Therefore, we investigated whether EBV remained restricted to IgD− B cells in the blood of SLE patients. B cells (positive for the pan-B cell marker CD20) were fractionated by FACS into IgD− (which is expressed primarily on naive B cells) and IgD+ (memory cells) populations (Fig. 5A). The frequency of virus-infected cells was then assayed in these two populations (Fig. 5, B and C, and Table V). Essentially, all of the virally infected cells were in the IgD− memory B cell population. These findings indicate that the perturbations of the immune system in SLE are not so profound as to disrupt the mechanism responsible for restricting EBV to memory B cells in the blood.

CMV DNA is not present in the blood of patients with SLE

To investigate whether the immune dysfunction in SLE specifically affects EBV or whether it can affect other herpesviruses, we also studied CMV. CMV infects 70% of humans, but generally the infection is tightly controlled, and virus is not detectable in the blood. CMV can cause clinical disease in immunosuppressed patients, at which times viremia is present in the blood. We performed quantitative real-time PCR for CMV on 1.0 and 0.1 μg of

Table IV. EBV gene expression in the blood of SLE patients

<table>
<thead>
<tr>
<th>EBV Gene</th>
<th>No. Patients Positive/Tested</th>
<th>% Patients Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>BZLF1</td>
<td>6/17</td>
<td>35</td>
</tr>
<tr>
<td>LMP1</td>
<td>5/17</td>
<td>29</td>
</tr>
<tr>
<td>LMP2a</td>
<td>4/22</td>
<td>18</td>
</tr>
<tr>
<td>EBNA1(Q-K)</td>
<td>1/17</td>
<td>6</td>
</tr>
<tr>
<td>EBNA2</td>
<td>0/23</td>
<td>0</td>
</tr>
</tbody>
</table>

*In the blood of healthy individuals, BZLF1, LMP1, EBNA1, and EBNA2 expression has not been detected, and LMP2a transcripts are rarely detected (Refs. 27, 28, and 40, and our unpublished observations).
DNA from PBMCs of five SLE patients who were seropositive for CMV infection and five immunosuppressed transplant patients with known CMV viremia. SLE patients with a broad range of frequencies of EBV-infected cells in the blood (range, 2–977) were used to determine whether there was any correlation between deregulation of EBV infection and CMV infection. Although CMV DNA could be detected in the PBMCs of the five immunosuppressed transplant patients, CMV DNA could not be detected in PBMCs of any SLE patient (Table VI). Because CMV does not appear to be deregulated in patients with SLE, it can be concluded that immune abnormalities in SLE specifically deregulate EBV, further demonstrating the sensitivity of EBV to the immune dysfunction present in this autoimmune disease.

Discussion

The goal of our study was to test the prediction that diseases affecting the immune system will alter the state of EBV persistence. In agreement with this prediction, we have demonstrated a marked perturbation of EBV infection in patients with SLE, as indicated by high frequencies of infected B cells, and aberrant expression of viral genes. The logical explanation for this finding is that host factors related to SLE disrupt EBV. Because EBV dwells in and is regulated by the immune system, it is sensitive to immune dysfunction, as seen in immunosuppressed patients, e.g., organ transplant recipients. Thus, any host factor that perturbs EBV must, directly or indirectly, act on or through the immune system. We can conclude therefore that immune dysfunction associated with SLE is disrupting EBV persistence.

The alternative explanation is that the perturbed state of EBV infection causes SLE. However, this begs the question, what perturbs EBV in the first place, leading to some or all of the symptomatology of SLE? It must be either the virus or the host. It is unlikely that there is anything different about the virus, because there is no evidence for disease-specific strains, and viral perturbation has not been described in healthy individuals. Therefore, the logical conclusion is that it is a function of SLE that disrupts EBV and therefore that the altered behavior of EBV reflects the sensitivity of the virus to immune dysfunction in SLE. This concept can be generalized to state that any disease that affects the immune system may change the status of EBV persistence.

It has been suggested that EBV infection has a causative role in SLE (43). Our experiments do not directly address this possibility. The changes in EBV behavior we observe can most simply be attributed to defects in immune function in SLE patients, without the need to invoke or deny a causative role. Whether these changes in EBV infection are an intermediate and essential step in the pathogenesis of SLE or merely an epiphenomenon of impaired immune function remains to be answered (Fig. 6). What can be concluded is that deregulated EBV infection manifested as increased frequencies of infected cells, increased viral loads, and viral gene expression, cannot be interpreted per se as supporting the possibility that EBV plays a role in SLE.

To evaluate how host immune factors can affect the virus, viral biology needs to be considered. Persistent infection by EBV is characterized by a relatively stable frequency of virus-infected cells in the peripheral blood (25). To maintain this level, EBV, like all pathogens that establish chronic persistent infections, must evade immnosurveillance mechanisms. EBV achieves this by persisting in resting memory B cells where all viral protein expression is shut down (27, 28, 31, 40), thereby eliminating all potential targets for immune surveillance. The corollary, implicit in the lack of viral protein expression, is that the virus can have no influence on the behavior of the latently infected memory cells. Based on these considerations, we have hypothesized that the steady-state level of infected memory B cells in the blood is regulated by two factors. The first is the rate at which newly infected cells are produced. We assume this accrual is inversely related to the levels of immunosurveillance because cells producing infectious virus and newly infected B cells are both vigorously surveilled by CTL (1, 44). The second form of regulation is related to the homeostasis of memory B cells. Newly latently infected cells cease expression of viral proteins when they enter the memory compartment, so EBV should be responsive to the signaling mechanisms that maintain normal memory B cell homeostasis. We propose that the increased frequency of infected memory cells in the blood of SLE patients is due to a defect in the regulation of memory B cell homeostasis rather than in impaired immnosurveillance.

It is generally accepted that immunosurveillance plays an important role in regulating the levels of EBV-infected cells. This is based on studies of immunosuppressed organ transplant recipients who have high numbers of EBV-infected cells in the blood (31, 33, 45–47) and are at risk for EBV lymphoproliferative diseases (48). In this study, we have shown that patients with SLE also have elevated frequencies of infected cells in the blood, similar to those seen in immunosuppressed organ transplant recipients (31). Unlike organ transplant recipients, however, there is little evidence to suggest that this is caused by diminished immune surveillance in SLE. Kang et al. (19) have reported no change in the frequency of EBV-specific CD8 T cells in SLE patients and noted an increase in the frequency of EBV-specific CD4 T cells. Our studies indicate that CMV infection is well controlled in patients with SLE. This is consistent with the clinical observation that symptomatic CMV infection is rarely observed in SLE except in patients treated with immunosuppressive agents (49). Earlier studies of CTL function in SLE have reported debilitated cytotoxic killing, but these claims are not consistent with the findings of Kang et al. (19), possibly because those earlier studies did not measure Ag-specific, MHC-restricted responses (18).

There is also considerable clinical evidence to suggest that immune surveillance functions are relatively intact in SLE patients. Significant defects would be expected to have broad effects on a variety of infections, including herpesviruses. Although common in transplant recipients, reactivated herpesvirus infections are not usually seen in SLE. An increased incidence of reactivation by varicella zoster virus is observed, but like healthy individuals, these events are typically benign and self-limited (50). Other opportunistic infections that frequently cause clinical disease in the setting of IS, such as fungal infections, are rare in SLE patients (49).

Most directly pertinent to our assumption that immune surveillance of EBV is relatively normal in SLE is the lack of EBV-associated lymphoproliferative diseases in these patients. These diseases are usually manifested as B cell lymphomas that occur in patients who are highly immunosuppressed, either by medicines or by infection with HIV. They are thought to arise when EBV-driven cellular proliferation occurs in the absence of effective immnosurveillance (48). Strikingly, such tumors have not been reported in SLE patients, except in

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Table V. Frequency of EBV-infected naive and memory B cells in the blood of SLE patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>B Cell Population</th>
<th>Frequency/10^6 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IgD+ (naive)</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>IgD+ (memory)</td>
<td>590</td>
</tr>
<tr>
<td>3</td>
<td>IgD+ (naive)</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>IgD+ (memory)</td>
<td>4500</td>
</tr>
<tr>
<td>4</td>
<td>IgD- (naive)</td>
<td>&lt;3</td>
</tr>
<tr>
<td>4</td>
<td>IgD- (memory)</td>
<td>450</td>
</tr>
</tbody>
</table>

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In conclusion, we have demonstrated perturbation of EBV infection in patients with SLE. Future studies will need to address whether specific B cell abnormalities in SLE are associated with the changes in EBV infection observed in these patients. Regardless of the outcome of such studies, it is evident that the immune dysfunction of SLE will serve as an excellent tool for investigating the mechanisms of EBV regulation, and EBV may perhaps also be useful in investigating aspects of the immune dysfunction in SLE.

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Disclosures

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References


