

THE ROLE OF EPSTEIN-BARR VIRUS IN SYSTEMIC LUPUS ERYTHEMATOSUS

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1. ABSTRACT

Systemic lupus erythematosus (SLE) is a devastating autoimmune disease with no known cure. Lupus patients suffer from a myriad of clinical symptoms which variably include arthritis, pleuritis, pericarditis, vasculitis, and nephritis. The underlying mechanisms behind these clinical findings and the etiologic events preceding and causing disease onset, however, remain largely unknown. For many years, investigators have suspected that Epstein-Barr virus might somehow be involved in the etiology and/or pathogenesis of systemic lupus. Numerous studies have examined this possibility from various angles and have arrived at different conclusions. This work reviews these historical papers in the context of new results and presents a hypothetical role for this virus as an etiological environmental trigger for SLE.

2. INTRODUCTION

Autoimmune diseases, as a group, comprise one of the most devastating plagues of human society. Systemic lupus erythematosus, rheumatoid arthritis, myasthenia gravis, multiple sclerosis, diabetes, and thyroid disease (as well as others) serve as a reminder that the etiology and pathogenesis of many of today's most serious disease processes are still poorly understood. Systemic lupus erythematosus is a complex, multi-faceted disease which affects between 300,000 and 500,000 Americans (1). Most patients have major life-altering symptoms that are related to the involvement of essential organs or as a result of the current devastating therapy. We calculate that world-wide on the order of 400 to 800 lupus patients die per day (1). Morbidity and mortality that results from the involvement of

brain, kidney, lung, blood vessels, skin, hematopoietic, hepatic, gastrointestinal tissues, pericardium, pleura, peritoneum and synovium give lupus a well-deserved reputation as a dreaded disease (2). Albeit a disease with varied clinical presentations, one common thread unites all SLE patients - the production of large amounts of autoantibodies. These patients mount substantial, aberrant immune responses against numerous different autoantigens. These autoantibodies are detected in greater than 95% of SLE patients as a positive anti-nuclear antibody (ANA) response (3).

The origin and development of systemic lupus are still areas of relative uncertainty. Current immunologic doctrine holds that lupus may be caused, or contributed to, by a number of different factors. First, it is thought that genetics play a major role in SLE (4-5). The concordance rate for SLE in monozygotic twins is reported to be around 25%, while that in dizygotic twins is closer to 9% (4). Genome scans have identified at least 12 different regions which show genetic linkage with systemic lupus (5). Genes which have been implicated include HLA-DR2, Fas, Fas ligand, Fc-gamma-RIIA and IIIA, C1q, and many others (4-6). Several animal models also spontaneously develop lupus, a trait which can be bred into a murine line with the addition of a very few genes (7). In addition, human SLE is thought to contain a degree of hormonal dependency. Before puberty, boys and girls are afflicted almost equally; however, after menarche, females contract disease eight to nine times more often than their male counterparts (8).

Due to the 25% concordance seen in monozygotic twins, however, most investigators also suspect an environmental trigger which sets the immune

Table 1. Features of EBV consistent with an etiologic role in SLE

- EBV is nearly ubiquitous in many populations
- EBV infects primarily B cells and promotes their proliferation
- EBV produces a life-long latent infection
- The virus emerges from latency at a low frequency sufficient for continuous immune stimulation
- EBV-infected B cells are more resistant to apoptosis
- EBV is capable of promoting antibodies which are cross-reactive with self-antigens.

system on the course toward systemic autoimmunity. UV light and heavy metals have been implicated, while some drugs such as procainamide and hydralazine are also capable of causing a lupus-like phenotype in certain individuals (9). In addition, infectious agents have long been thought to be potential etiologic factors. Infectious agents are likely candidates for a number of reasons. First of all, the principal directive of the immune system is to counter invasion by microorganisms. Thus, immune responses are closely tied to, and even somewhat directed by, the presence and action of these microorganisms. There are distinct cases where infectious agents have been proven to cause autoimmunity through cross-reactivity with self-antigens, as is seen with the streptococcal M protein in rheumatic fever.

With SLE specifically, there are other reasons that such an agent fits nicely into current models of disease. A recent study has shown that autoantibodies are present in lupus patients for years before clinical manifestations of disease ever develop (Unpublished observations). This data reveals that the events which trigger autoimmunity in SLE actually begin their work long before any pathologic effect becomes apparent. The presence of a chronic, low level infection as one etiologic factor is clearly consistent with this model of disease pathogenesis. Proposed agents for lupus have included Cytomegalovirus (CMV), Hepatitis C, Parvovirus B19, JC Polyoma virus, various retroviruses, and of course Epstein-Barr virus (10-11). The most frequently studied of these potential associations, if one judges based upon sheer numbers of papers published on the subject, has been that of SLE with EBV. In fact, in the last 30-odd years, over 100 works have been published covering various aspects of this matter. Since no true epidemiologic relationship with an infectious agent has been demonstrated for systemic lupus erythematosus, it seems likely that any potential candidates must be nearly ubiquitous, rather than intermittently infecting individuals predisposed to the development of SLE. The Epstein-Barr virus handily fulfills this requirement, and has many other features which make it a promising candidate as an etiologic agent for an autoimmune disease (table 1). The studies on EBV range from simple clinical observations to

in-depth viral biology and immunology. We will attempt herein to outline the critical points and major themes of these works, and through this analysis demonstrate what data support the findings that distinct associations are present between Epstein-Barr virus and the clinical syndrome of systemic lupus erythematosus.

3. INCREASE IN ANTI-EBV TITERS IN SLE

Precipitins against EBV infected cell lines were the first available assays for previous EBV exposure developed in the late 1960s. The first information on the prevalence of anti-EBV antibodies in lupus patients comes from early work where lupus patients were used as a control population for a study of childhood lymphoma (12). Though an increased EBV titer in SLE patient sera relative to the controls was shown, the sample sizes were too small and the quantitative differences were not large.

The first serious effort to study Epstein-Barr virus in lupus came from labs headed by Alfred Evans and Naomi Rothfield who collaborated on a study published in the *Lancet* in 1971 (13). They utilized a newer method of EBV detection devised by Henle *et al.* (14) involving indirect immunofluorescence in the EB-3 (EBV infected) cell line. This study found that there were higher titers of anti-EBV antibodies in lupus patient sera than in controls. Specifically, they established that 62 of 100 lupus patients had anti-EBV titers of greater than 1:160, while less than 11% of controls had this level of anti-EBV antibodies. The mean lupus patient titer was 1:215, while that of controls was only 1:35, a statistically significant difference ($p < 0.001$). In addition, Evans *et al.* examined titers to other viruses and discovered that SLE patients also had slightly elevated titers to rubella, measles, and parainfluenza 1, while controls had higher titers to HSV-1, influenza A2, and parainfluenza 3. None of these other differences, however, were more than two-fold elevated in either direction. Thus, the observed elevation of EBV titers in SLE is clearly the most significant difference in viral antibodies between the two groups studied. They continued this work in 1973 with a somewhat larger study, finding 46 of 100 lupus patients (46%) with anti-EBV titers of greater than 1:160, while only 20 of 255 normals (8%) had similarly high levels (15). In addition, the 1973 study showed that 92% of lupus patients were positive for anti-EBV antibodies at titers of 1:40 (their cutoff for positivity), compared to only 87% of controls. Further studies from this work revealed that the anti-EBV titer in these studies varied inversely with severity of clinical symptoms in the SLE group. They also showed no correlation between EBV titers and total serum IgG levels, hinting that perhaps the higher EBV titers seen in SLE in their study were not simply due to general B-cell hyperreactivity.

In 1972, Stephens *et al.* performed a similar study on women with lupus (16). They did not find a similar increase in mean antibody titer to EBV by the Henle method of indirect immunofluorescence, nor did they see a difference in percent of seropositive individuals. As an additional assay, they examined antibody to EBV by a

Table 2. Anti-EBV antibody titers in lupus patients and normal EBV-infected individuals

Group	Year	Method	SLE Titer	Normal Titer	Ratio SLE/Normal	Significance
Dalldorf <i>et al.</i> (12)	1969	IF	48.5	11.7	4.1	Increased
Evans <i>et al.</i> (13)	1971	IF	216.8	35.3	6.1	Increased
Rothfield <i>et al.</i> (14)	1973	IF	211	65	3.2	Increased
Stephens <i>et al.</i> (16)	1972	IF	154.6	169	0.9	Decreased
Gergely <i>et al.</i> (17)	1973	IF	46.4	27.1	1.7	NS
Klippel <i>et al.</i> (18)	1973	IF	44.9	47.6	0.9	NS
Phillips <i>et al.</i> (19)	1973	IF	139.1	90.5	1.5	NS
Stancek <i>et al.</i> (20)	1979	IF	636.4	123.8	5.1	Increased
Origgi <i>et al.</i> (21)	1989	IF	351.1	123.1	2.9	Increased
Chak-Sing <i>et al.</i> (37)	1998	IF	143.5	61.8	2.3	Increased

Ratio of SLE titer to Normal titer is shown, as is Statistical Significance as determined by the original authors. Increase (in SLE), Decrease (in SLE), or Not Significant (NS).

micro-Ouchterlony diffusion assay against soluble extract from the EBV-infected cell line HR1K. Interestingly, they observed that SLE patients make antibodies against a significantly larger number of bands in this extract than do normal controls. The conclusion drawn in this work is that the overall correlation between anti-EBV antibodies and SLE is significant, but not to such a level as to implicate EBV as a universal etiologic factor.

Early in 1973, a group from Hungary tested 70 SLE patients and equal number of normal controls by the indirect immunofluorescence method for anti-EBV antibodies and determined no significant difference in rate of seropositivity to EBV between the two groups (17). They did, however, also find that the mean anti-EBV titers were significantly elevated in the lupus patient group. Later, in 1973, Evans and Rothfield combined their resources with a group headed by John Klippel (18). They examined 61 patients and 14 controls for antibodies to EBV via the Henle immunofluorescence method. This time they failed to find a difference between the mean titers of patients and controls. Another group, this time headed by Phillips and Hirshaut (19), performed a similar study with a different cell line (Jijoye, an EBV strain B infected line) as the EBV antigen source. They found that in their population, the anti-EBV titers were slightly elevated in SLE patients as compared to normal individuals, but that this mild elevation did not reach statistical significance. Phillips therefore concluded, in opposition to the original work by Evans, that this mild increase in antibody is in fact due to generalized immune hyperreactivity in lupus rather than to any specific property of the Epstein-Barr virus.

Towards the end of the 1970's, Stancek and Rovensky performed another study examining differences in EBV antibody titers (20). They similarly utilized the indirect immunofluorescence technique, although with a different source of antigen (EBV-infected P3HCR cells). They analyzed 22 SLE patients and 22 controls with infectious mononucleosis. Their data reveal that 20 of 22 SLE sera contain anti-EBV IgG at greater than a 1:160 dilution, while only 6 of 22 infectious mononucleosis (IM) patients showed similarly high levels of binding. The mean anti-EBV titer for their SLE sera was 1:696, while only 1:293 in the patients with active IM. This data, however, is difficult to interpret, as patients with active IM more commonly have an IgM response to EBV Viral Capsid

Antigen (VCA). As their time from initial viral exposure progresses, levels of anti-VCA IgG increase. The observed difference, then, may simply be due to the fact that IM patients are still at a relatively early stage of EBV infection, and thus have less anti-EBV IgG.

A group from Italy headed by Origgi later analyzed anti-EBV antibodies through indirect immunofluorescence of EBV-infected P3JHR1 cells in the late 1980's (21). Again, a significant difference in the mean titers of anti-VCA of lupus patients compared to controls was found (1:351 and 1:123, respectively). Sixteen of 18 SLE patients had titers of 1:160 or greater, while only 7 of 19 controls exhibited these high levels of anti-EBV antibody, despite comparable total IgG levels in the two groups. This study repeats the work performed in the 70's by many groups, and concluded that there is some relationship between EBV and SLE.

A new technique, flow cytometry, was employed by Yokochi *et al.* in Japan in 1989, to examine this question of anti-EBV antibodies in SLE (22). They utilized EBV-infected P3HR-1 cells, mixed them with human serum, and labeled them with FITC-conjugated anti-human IgG for flow analysis. This method is specific for the Membrane Antigen (MA) of EBV which is expressed both as part of the viral envelope and on the surface of EBV-infected cells. SLE patients produce significantly higher titers of anti-MA antibodies by this method than do normal EBV-infected individuals, even when the numbers are adjusted for total IgG concentrations. The authors postulate that since anti-MA antibodies have been shown to correlate closely with neutralizing antibody (23), it is likely that SLE patients also have high levels of neutralizing antibody which would be indicative of increased exposure to infectious EBV. They feel that it is unlikely that this neutralizing antibody remains from the primary infection of these individuals, but could suggest frequent reactivation of infectious EBV in the SLE population.

Taken as a whole, these studies leave us with a mixed impression of the possible differences in EBV antibody titers between lupus patients and normal EBV-infected individuals (table 2). Indeed, several of these investigators mention the inherent difficulty in distinguishing anti-EBV antibodies via immunofluorescence in individuals who are also known to

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be positive for anti-nuclear antibodies (ANA), as greater than 95% of lupus patients are (16-17). Consequently, the dependability of these assays remains in question. Regardless, the majority of the data seems to state that lupus patients do indeed produce higher titers of anti-EBV antibodies. However, since several studies have failed to describe the same association, it appears premature to ascribe any role for the Epstein-Barr virus in lupus in the face of such conflicting data. Thus, we must examine other aspects of the interaction between virus and host in order to correctly ascertain whether or not EBV plays a part in this devastating autoimmune disease.

4. DIVERSE ANTI-EBV ANTIBODY SPECIFICITY IN SLE

The initial attention to EBV and SLE arose largely as a result of the aforementioned potential differences seen in antibody titers. It was only natural, then, that interest spread to other aspects of the anti-EBV immune response. The question arose as to whether or not the humoral response being measured by titer assays was in fact identical in different individuals. The first major work to demonstrate differences in antibody specificities among lupus patients and controls was published in the *Journal of General Virology* in 1986 (24). They found that lupus patient sera recognized specific viral peptides more commonly than normal control sera. This group examined anti-EBV immune responses through Western blots against EBV-infected cell lysates in a number of different clinical groups including SLE, IM, and rheumatoid arthritis (RA). They revealed that 64% (14/22) of their lupus patients bound 36 and 38 kD viral peptides (EBV Early Antigen – EA) not recognized at all in normal EBV-infected individuals and only rarely in patients with IM. Half of the SLE sera also recognized a 44-48 kD EA complex of proteins which were not bound by normal sera and infrequently in the other clinical groups. Roughly half of normal EBV-infected individuals, however, make antibodies against a 50kD EBV protein not recognized at all by lupus sera. These were the most significant differences seen between any of the three affected groups and normals, and suggest that lupus patients mount a different immune response against the Epstein-Barr virus than do normal individuals or individuals with IM or RA.

Following closely on the heels of this breakthrough, Kitagawa *et al.* in Japan performed a similar study examining the antibody responses against EBV antigens via Western blot (25). They utilized a different EBV-infected cell line (Raji) than Sculley *et al.* (24), which gave much clearer results concerning the higher molecular weight viral proteins EBNA-1, EBNA-2, and EBNA-3 (70, 90, 140 kD respectively). They discovered that SLE sera recognize all three of these proteins significantly more often than do sera from normal, EBV-infected individuals. Ninety-nine percent (64/65) of SLE patients versus 79% (52/66) of normals were positive for binding EBNA-1, while the differences for the other two proteins were even more striking. Sixty-nine percent and 77% of lupus patients bound EBNA-2 and EBNA-3, respectively, while only 8% to 9% of normals' sera contained antibodies to

these peptides. Previous work had shown that EBNA-2 is involved in the transforming potential of the virus and EBNA-3 is important for growth transformation in B cells and maintenance of the viral genome (26-27). The authors postulate that the copious amounts of antibody to these peptides in SLE indicates the likelihood that these proteins are expressed at high levels in these individuals, thus implying that the latently infected B cells are continuously expanding in lupus patients.

Studies were later performed by Ngou *et al.* in 1990 examining the differences in anti-EBV Early Antigen antibodies by immunoblot among individuals with Nasopharyngeal carcinoma (NPC), IM, SLE, and MCTD (28). They found that SLE patient sera recognizes numerous EBV antigens ranging from 33 to 134 kD significantly more often than normals or patients with MCTD (70% of SLE vs. 34% of MCTD patients and 34% of normal individuals). Interestingly, in their study, patients with IM and NPC have similar binding profiles to SLE patients, recognizing the same EBV antigens in roughly similar frequencies.

Vaughan *et al.* in 1990 analyzed the binding of antibodies from normals and patients with RA or SLE to various peptides of the EBNA-1 protein (29). They built short (11-18 aa) peptides from EBNA-1 and tested sera for reactivity via standard ELISA. Lupus patients make statistically higher levels of antibody against three major peptides including amino acids 368-381, 451-463, and 461-474. Normal EBV-infected individuals' sera do not significantly bind these regions. Instead, normal individuals react strongly with an epitope (aa 145-167) containing part of the glycine-alanine repeat of EBNA-1. RA patient sera bind the first two peptides at greater levels than controls, but not nearly as significantly as do the patients with SLE. Two of these three regions of SLE reactivity (368-381 and 451-463) contain primarily glycine-arginine rich regions. These regions would later prove to be of great importance in potential mechanisms of autoimmunity induction.

5. ASSOCIATIONAL STUDIES WITH EBV AND SLE

In order for Epstein-Barr virus to serve as a potential etiologic agent in SLE, a number of predictions must be true. Among the foremost of these is the prediction that there must be an association between EBV infection and the incidence of lupus. The majority of the previous studies have formed important observations about antibody titers and specificities, but few have been able to examine an association on a large enough scale to allow significant statistical analysis. One of the major difficulties in comparing infection rates of EBV is its nearly ubiquitous presence in adult populations. An excellent example of this problem is seen in the 1995 work by Tsai *et al.* (30). They found that 13/16 lupus patients (81%) and 15/20 controls (75%) were positive for anti-VCA antibodies, while only 3/20 and 0/20 were positive for EBV by PCR of peripheral blood samples. The EBV antibody and DNA rates are different for SLE patients and controls, but the sample size is simply too small to determine significance when dealing

Table 3. Anti-EBV antibody prevalence in lupus patients and normal EBV-infected individuals

Group	Year	Method	SLE	% Positive	Controls	% Positive	Significance
Tsai <i>et al.</i> (30)	1995	ELISA	13/16	81%	15/20	75%	NS
James <i>et al.</i> (31)	1997	ELISA	116/117	99%	107/153	70%	Increased
James <i>et al.</i> (34)	2001	ELISA	195/196	99.50%	370/392	94.40%	Increased

Percent positive by standard ELISA is shown, as is Statistical Significance as determined by the original authors. Increase (in SLE), Decrease (in SLE), or Not Significant (NS).

Table 4. EBV DNA positivity rates in lupus patients and normals

Group	Year	Method	SLE (ELISA)	% Positive	Controls (ELISA)	% Positive	Significance
Tsai <i>et al.</i> (30)	1995	PCR	3/20 (16/20)	15%	0/20 (15/20)	0%	NS
James <i>et al.</i> (31)	1997	PCR	32/32 (32/32)	100%	23/32 (21/32)	72%	Increased
Chak-Sing <i>et al.</i> (37)	1998	PCR	20/34 (34/34)	59%	16/22 (22/22)	73%	NS
Incaprera <i>et al.</i> (35)	1998	PCR	8/15 (NA)	53%	6/28 (NA)	21%	Increased

Number of individuals positive by EBV PCR is followed by the number of seropositive individuals (by ELISA) in parentheses. Percent positive by DNA analysis is also listed. Statistical significance is indicated in the right-most column, with Increased (in SLE), Decreased (in SLE), or Not Significant (NS).

with such a small effect, thus their differences were determined to be insignificant.

Therefore, in 1997, we performed an association study looking at infectivity rates in children and teenagers with systemic lupus (31). In addition to finding a sample population in which this hypothesis could be tested, we also had at our disposal powerfully sensitive techniques not available to previous studies. In the first portion of this analysis, we examined EBV seroconversion utilizing commercially available anti-EBV VCA ELISAs. It was found that 116/117 lupus patients (age 4-19) were seropositive for EBV VCA, while only 107/153 age and sex-matched normal controls had seroconverted (table 3). These data yield an impressive odds ratio of 49.9. Similar assays were run to test for seroconversion to other Herpes viruses, but no similar associations were discovered. In order to reaffirm this data, 32 of these lupus patients and their matched controls were tested for the presence of EBV genomes in their peripheral blood mononuclear cells (PBMCs). The method of detection was PCR, although altered from that performed in previous studies as additional Southern blotting, coupled with the use of multiple reactions for each sample, was also performed on these samples to increase the assay sensitivity. All 32 lupus cases had EBV DNA present, while only 23/32 matched controls were positive (table 4). This served to confirm the serologic data, and demonstrate that Epstein-Barr virus infection is clearly statistically associated with systemic lupus in this younger population. In addition, with an odds ratio of near 50, this association is nearly twice as great as any risk factor delineated in a recent review of potential risk factors in SLE (32). As further substantiation of this work, we have now collected DNA and sera from 24 additional pediatric lupus patients and 24 matched controls. Seropositivity to EBV is 100% in this patient group and 68% in controls (odds ratio ≥ 14 , $\chi^2=7.0$, $p<0.01$) and fundamentally confirms our earlier work (33).

In order for this association to be applied to adult lupus patients, additional work was required. Designing these experiments, however, led to several serious

problems, as the EBV infection rate is considered to be close to 95% in the U.S. adult population. Thus, the expected numbers needed to give power to a statistical association are very large. In order to test for this association, we utilized a large collection of adult lupus patients and controls (34). Actually, 196 lupus patients, along with 392 age, sex, and race-matched controls, were tested via commercially available ELISAs for antibodies to the major common herpes viruses EBV (VCA), Herpes simplex-1 (HSV-1), Herpes simplex-2 (HSV-2), CMV, and Varicella zoster virus (VZV). This study found that 195/196 (99.5%) of the adult lupus patients had seroconverted to EBV, while 370/392 (95%) of the controls had also seroconverted (table 3). This difference yielded an odds ratio of 9.35 ($p<0.015$) when corrected for potential confounders of familial clustering. No truly significant differences were noted with any of the other Herpes viruses tested.

The major differences between these three newer studies (31, #34-35) which have demonstrated an association between EBV and SLE and those previous studies which have not (30, #36-37) lie in the sensitivities of the assays and the numbers and nature of individuals enrolled in the studies (tables 3 and 4). All of the work performed before 1997 relied upon serological assays which had demonstrated less than 90% sensitivity. In the adult population, the seronegative rate for EBV in adults is only expected to be approximately 5%. Therefore, the potential of these studies to demonstrate the power required to prove statistical association was greatly hindered by the limits of these assays. In addition, only one of these previous studies (30) took the statistically beneficial path of examining younger lupus patients. All three analyses, however, utilized small cohorts (15, 16, and 34 patients respectively) which had little possibility of demonstrating significance given the small expected difference. In addition, they utilized methods of EBV DNA detection which were not maximized to detect one infected B cell per million cells. In fact, one of these groups (30) was able to identify EBV DNA in fewer than 10% of their serologically EBV positive individuals, while another (37) detected EBV DNA in only 67% of their seropositive individuals (table 4). Finally, the post-1997 studies have utilized much larger

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cohorts of patients and controls than previous work, vastly increasing the potential for determining statistical significance.

Several case reports linking SLE to EBV infection have also been reported (38-39). In one such case, written by Bhimma *et al.*, the course of autoimmune disease closely followed that of the IM in a 10-year old girl, with the symptoms and serology remitting and flaring in tandem (38). However, none of the case reports has provided other than circumstantial evidence that the two were directly related and not simply coincidental occurrences.

6. POTENTIAL MECHANISMS OF VIRAL PREDISPOSITION TO AUTOIMMUNITY

Strong statistical associations, however, can only go so far in explaining the role of a ubiquitous infectious agent (EBV) in a much less common autoimmune disorder (SLE). Specific mechanisms must be at play, if the interaction between virus and host is to predispose an individual to the development of an autoimmune disorder. In order to explore this possibility, several investigators have looked at the potential for the Epstein-Barr virus to affect cellular function in lupus patients and normal individuals. One interesting approach to this arena of investigation was taken in the early 1980's by Aya *et al.* in Japan (40). They examined the mechanism by which immune surveillance against EBV-transformed cells is propagated. To do this, they isolated peripheral lymphocytes from EBV-positive individuals and SLE patients. They then transformed these lymphocytes with the B95-8 strain of EBV. They next tested for cytotoxicity against these EBV-infected cells utilizing autologous effector lymphocyte populations. In normal individuals, cytotoxicity occurs when EBV-infected cells are mixed with uninfected autologous lymphocytes and serum from the EBV-positive individual, but not in the absence of the EBV-positive serum. This indicated that antibody-dependent cellular cytotoxicity (ADCC) is one of the major mechanisms of action in clearance of EBV-infected cells. Interestingly, they also noticed that this process is severely impaired in lymphocytes from SLE patients, which were consistently as much as 90% less effective than normal lymphocytes. Defects in the lymphocytes of SLE patients were not a new observation, but this was the first example of a defect specifically applying to immune surveillance of the Epstein-Barr virus (40).

Another look at the lupus patient response to EBV came shortly after, as Tsokos *et al.* examined both B and T cell responses to the virus (41). They found that B cells from lupus patients infected in vitro with the B95-8 strain of EBV generate a comparable amount of proliferation relative to controls in response to this T-independent stimulus. However, it had also previously been shown that addition of purified T cells from normal individuals inhibited this mitogen-induced proliferation. This proved to be the case in the normals studied by Tsokos as well. The T cells from SLE patients in this study, however, failed to show a similar inhibitory effect on

mitogen-induced B cell proliferation whether autologous or normals' B cells were used. Despite this observation, however, lupus patient T cells responded normally to inhibition of concanavalin A-induced stimulation of mononuclear cells. It was therefore proposed that lupus patients have some defect in T-cell mediated suppression of EBV-induced B-cell proliferation. Whether this failure to properly suppress proliferation leads to an increase in EB viral loads in SLE patients or has some other, as yet unknown, effect is not entirely clear. Based upon these studies it is clear that lupus patients demonstrate different, perhaps defective, cellular responses to the Epstein-Barr virus.

A fascinating study from Newkirk and Tsoukas in Canada, 1992, remarked on the effects of Epstein-Barr virus infection on the levels of autoantigens in the cell (42). They demonstrated that cells infected with EBV have, on average, a 3-fold increase in the cytoplasmic levels of the 48kD La and 60 kD Ro autoantigens. Unfortunately, none of the other common lupus autoantigens were assessed in this study. Anti-La antibodies are more common among patients with primary Sjögren's syndrome, but anti-60 kD Ro is a common lupus autoantigen, present in as many as 50% of patients with SLE. This obviously raises the question of whether or not an EBV-related increase in the amount of specific autoantigens present in cells could play a role in lupus autoimmunity. Ro and other autoantigens have also been shown to cluster on the surface of apoptotic blebs (43). This data, coupled with the fact that many lupus patients show defects in clearing of apoptotic bodies, has led to the theory that increased (or differential) expression of autoantigens on the surface of apoptotic bodies could be involved in the breakdown of tolerance to these self proteins seen in SLE. The capability of Epstein-Barr virus, then, to increase the expression of autoantigens in the cell could potentially magnify this effect.

In 1993, Henderson *et al.* from the UK noticed that Epstein-Barr virus infection can enhance the survival of B cells through up-regulation of Bcl-2 (44), a cell signaling protein known to be involved in the prevention of apoptosis. They identified sequence homology between Bcl-2 and BHRF-1, an EBV-encoded early lytic cycle protein. BHRF-1 transfected cells and Bcl-2 transfected cells exhibit a similar protection against *in vitro* induced apoptosis. The investigators hypothesize that BHRF-1 provides a Bcl-2-independent method of increasing cell survival that may operate during the viral lytic cycle. This protection from apoptosis could potentially provide for a method of survival for autoreactive B lymphocytes, thus supplying another prospective means for EBV to promote autoimmunity.

Another interesting correlation between EBV and SLE lies in the cytokine profiles of lupus patients and EBV-infected individuals. Interleukin 10 (IL-10) levels have been shown to be significantly higher in lupus patients than in normals. Park *et al.* demonstrated in 1998 that in fact the difference was on the order of 7-10 fold (45). They also found that levels of IL-10 correlated fairly well ($r=0.51$, $p<0.01$) with SLE Disease Activity Index

(SLEDAI) scores in a cohort of 41 lupus patients. Interestingly, Kimberly *et al.* have recently demonstrated that there is a strong genetic association between the IL-10 promoter and systemic lupus erythematosus, especially in African-Americans (46). Of particular interest to this work, it has also been shown that EBV infection induces human B cells to produce IL-10 (47). In fact, EBV infection causes human B cells to secrete 10-20 fold more IL-10 than B cells stimulated through surface immunoglobulin or CD40 triggering. The authors of this work (47) propose that this virus-induced IL-10 production serves to decrease the anti-viral immune response as well as to promote proliferation of the infected B cells. The question remains, however, as to whether or not this stimulation of IL-10 production also contributes to autoimmunity or disease activity in SLE, although evidence for the role of EBV is mounting.

Incaprera *et al.* in 1998 examined the possibility that patients with autoimmune disorders are actually experiencing reactivation of the Epstein-Barr virus. This reactivation would increase the viral load as well as B-cell proliferation, thus perhaps providing a nidus for the initiation of autoimmunity. They performed a study examining the prevalence of infectious Epstein-Barr virus in the oropharyngeal secretions of lupus patients and controls (35). The presence of viral DNA in cells from this location is generally held to be indicative of viral reactivation, often subclinical in nature. Previously, EBV DNA has been shown to be present in oropharyngeal secretions of 10-20% of normal EBV-infected persons (48). In this study, EBV DNA was found to be present in 21% of normals, but in 56% of lupus patients and an impressive 92% of infectious mononucleosis patients. In addition, infectious virus was cultured from the oropharynx of 44% of lupus patients, 42% of IM patients, and only 7% of normals. This increase in IM patients is not unexpected, as their high levels of active viral secretion are well documented. The increase in SLE patients, however, is remarkable. It does, however, seem to fall in line with previous data showing higher levels of anti-EBV antibodies in SLE. Perhaps more importantly, though, it suggests high levels of viral activity (or more specifically reactivation) in lupus patients, on a level that is significantly higher than normal EBV-infected individuals and even somewhat comparable to patients with active IM, a disease state defined by enormous viral reactivation. This data would seem to be in agreement with the work by Incaprera (25) describing high levels of antibody against EBV antigens known to be involved in viral expansion and reactivation.

7. MOLECULAR EVIDENCE SUPPORTING AN ETIOLOGIC ROLE FOR EBV IN SLE

In addition to general properties of EBV infection, there is strong immunologic evidence that the Epstein-Barr virus can be directly involved in the induction of autoimmunity. Vaughan *et al.* demonstrated this in 1995 when they discovered several novel hematopoietic autoantigens in individuals with infectious mononucleosis (49). Autoantibodies recognizing these proteins were primarily of the IgM class, cross-reacted with the EBNA-1 protein of EBV, and were inhibited by addition of (GA)_x

rich peptides from the sequence of EBNA-1. The identification of anti-EBNA-1 antibodies which cross-reacted with cellular antigens led Vaughan *et al.* to examine the presence of these antibodies in non-IM patients with autoimmune disease (50). They discovered that while IgM class antibodies of this type are present in IM patients, and less commonly in normals, IgG class antibodies recognizing these autoantigens are primarily found in patients with primary Sjögren's syndrome (SS) and systemic lupus erythematosus. Thus, it appears that mimicking epitopes from the Epstein-Barr virus can trigger IgM autoantibodies in normal individuals, but that T-cell help and a subsequent switch to IgG autoantibodies of this specificity only occurs in individuals who develop autoimmune diseases such as SS and SLE. Unfortunately, there is no data to date which reveals whether this virus-induced autoimmunity precedes the onset of SS or SLE, thus giving it a possible role in the etiology of these diseases, or is simply another example of an immune system gone awry through systemic autoimmunity.

A breakthrough study was performed in late 1993 by Sabbatini *et al.* at the University of Pisa (51). One of the major autoantibody specificities in SLE, and one of the diagnostic criteria for disease, is anti-Sm. The Sm proteins are polypeptide components of the spliceosome, which is involved in the splicing of heterogeneous nuclear RNAs into messenger RNAs. The major Sm proteins which are common antigens in SLE are Sm B/B', D1, D2, and D3. The authors noticed significant sequence homology between amino acids 95-119 of the Sm D1 protein, a major lupus autoantigen, and amino acids 35-58 of EBNA-1. Both of these regions are glycine-arginine rich areas. They demonstrated that SLE patient antibodies, affinity-purified with the Sm D1 95-119 peptide, are capable of binding native Sm D1, the EBNA-1 35-58 peptide, and whole EBNA-1 from viral extracts. They proceeded to immunize animals with the EBNA-1 35-58 peptide, and discovered that these animals made antibodies which recognized whole EBNA-1, Sm D1 95-119, and whole recombinant Sm D1. This was the first strong evidence that perhaps a molecular mimicry mechanism could be involved in the induction of the autoimmune response in systemic lupus, and that this mimicry might occur between proteins from the Epstein-Barr virus and spliceosomal autoantigens. Similar confirmatory studies were performed later in several independent laboratories. We demonstrated that one of the major epitopes recognized by anti-Sm D1 antibodies is the carboxy-terminal (GR)_x rich portion of the protein (52). Peptides consisting of this sequence are also bound by sera from lupus patients with serologies other than anti-Sm, but not by sera from the normal individuals in this study. We have also shown that antibodies to this region of Sm D1 from SLE patients also cross-react with other common spliceosomal autoantigens, most notably Sm D3 (53). Secondly, Rivkin *et al.*, pulled Sm D1 homologues from a mouse cDNA library via phage display with human anti-Sm serum (54). These Sm D1 homologues contained the (GR)_x portion of the c-terminal end of Sm D1. Affinity-purified antibodies to these (GR)_x regions also cross-reacted with EBNA-1 in viral lysates, reaffirming the data from Sabbatini.

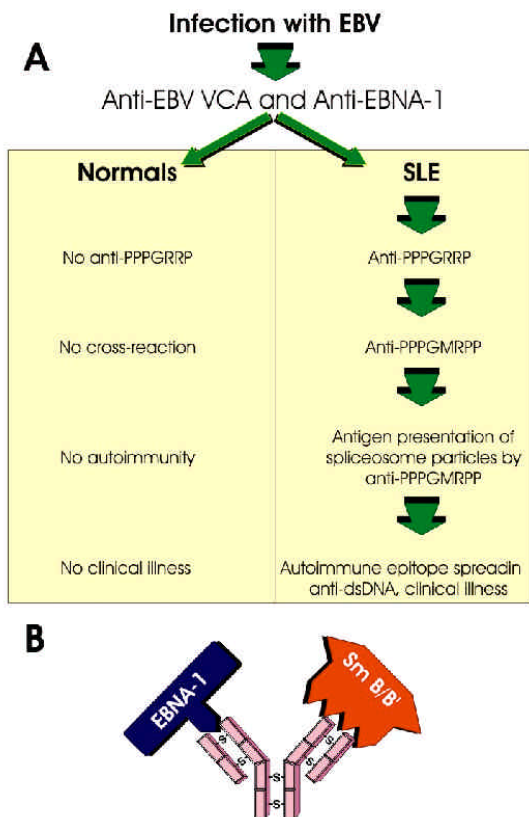


Figure 1. Model for possible pathogenesis of lupus for patients who develop disease through an anti-Sm B/B' initial autoimmune response. Panel A represents the postulated sequence of events and panel B presents one of the postulated critical antibodies for the induction of lupus. In this scenario, PPGRRP and PPGMRPP are both postulated to be bound by antibody and thereby mediate an antigenic cross-reaction between EBNA-1 and the spliceosomal protein Sm B/B' (60).

Sabbatini *et al.* further characterized this cross-reactive response in the *Journal of Autoimmunity* in 1994 (55). They demonstrated that anti-EBNA 35-58 antibodies are present in some non-lupus EBV-infected individuals. Of great interest, however, was that affinity-purified anti-EBNA 35-58 antibodies from non-lupus sera fail to cross-react with the Sm D1 95-119 peptide and that some non-SLE patients bind the EBNA peptide but not the Sm D1 peptide. The cross-reactivity, therefore, seems to be due not just to the structure of the antigen, but also to the nature of the antibody.

Previous work has demonstrated that lupus patient sera with both anti-Sm and anti-nRNP precipitins have a very homogeneous pattern of binding to the overlapping octapeptides of Sm B/B' (56). All anti-B/B' antibody positive patient sera tested also bind four nearly identical peptides from Sm B/B', with the sequence PPPGMRP(G)P. These anti-PPPGMRP(G)P responses account for a significant percentage of anti-Sm reactivity in several sera tested by affinity absorption. Thirty-five to 60% of the anti-Sm autoantibody response was directed

against these sequences. This result was confirmed by the dramatic depletion of anti-Sm antibodies as measured by Western blot and Ouchterlony analysis after anti-PPPGMRPP absorption (57). Finally, these proline-rich carboxyl terminal sequences of Sm B/B' appear to be the very first targets of anti-Sm antibodies during the course of disease (58).

As an accidental observation, it was discovered that rabbits immunized with the earliest observed B cell epitope of Sm B/B' in human lupus patients, PPPGMRPP, developed a lupus-like illness (59). Surprisingly, these rabbits not only develop anti-peptide antibodies, but also break tolerance to the whole spliceosomal complex. Some develop anti-Sm, anti-nRNP, anti-dsDNA, and anti-nuclear antibodies, as well as thrombocytopenia, elevated creatinine, proteinuria and/or glomerulonephritis. This animal model of SLE suggested possible etiologies that could initiate SLE spliceosomal autoimmunity through molecular mimicry. Molecular mimicry is the breakdown of self tolerance to autoantigens occurs through cross-reactive antibodies that are initially targeted toward foreign entities (figure 1). Candidate protein sequences that possess primary structural similarity to the peptide PPPGMRPP were identified as possible inciting antigens for anti-spliceosomal autoimmunity. It was discovered that Epstein-Barr Virus Nuclear Antigen 1 (EBNA-1) contains the peptide, PPPGRRP, which was found as the sequence most similar to PPPGMRPP in the sequence databases. This viral peptide was commonly recognized by antibodies from lupus patients, but not by anti-EBV positive normal individuals. Further studies revealed that immunization of animals with this viral peptide (PPPGRRP) on a MAP backbone was capable of triggering lupus autoimmunity in a fashion similar to that seen before with the Sm B/B' peptide. This viral peptide-induced autoimmunity included cross reactivity of these antibodies to the PPPGMRPP peptide of Sm B/B', followed by the development of autoantibodies to other spliceosomal proteins. This antibody "spreading" followed patterns originally observed both in our animal model described above and in some anti-Sm positive patients. Thus, clear links have been discovered between anti-viral antibodies and lupus autoantibodies. Whether these links are indicative of a role for EBV in the etiology of systemic lupus, or are simply a by-product of autoimmunity has yet to be discovered; however, the specificity of these responses and uniformity with which they appear makes the latter appear highly improbable.

8. PERSPECTIVE

Systemic lupus has been described as a clinical entity for over 150 years. This disease currently affects an estimated 2-4 million individuals worldwide and is a significant cause of morbidity and mortality. Existing therapies consist of steroids and other powerful immunosuppressives which predispose to opportunistic infections and have serious, even life-threatening side effects. Clearly, better strategies for management and prevention of this devastating disease are required. The best avenue available would likely be one of disease

prevention. Convincing evidence has been provided by a number of investigators that there is some type of association between the Epstein-Barr virus and systemic lupus erythematosus. This evidence includes powerful associational studies as well as the defining of distinct molecular mechanisms which provide potential links between anti-viral immunity and lupus autoimmunity. These associations and links are much too potent and specific to merely define coincidental events. If indeed this virus is acting as an etiologic trigger for the onset of lupus, it provides fascinating opportunities for halting the disease process before it becomes all-consuming autoimmunity. If the work reviewed herein is upheld in future studies, the potential for a vaccine against the Epstein-Barr virus to prevent the onset of systemic lupus (even if only in a subset of individuals) cannot be ignored.

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