Detection of autoimmunity in early primary Epstein-Barr virus infection by Western blot analysis

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Abstract
Objectives
The Epstein-Barr virus (EBV) represents a potentially important factor in the pathogenesis of certain autoimmune disorders such as systemic lupus erythematosus (SLE), and Sjögren’s syndrome, probably through a molecular mimicry mechanism. Several studies have focused on the relationship between previous EBV infection and clinically overt connective tissue diseases (CTDs), while the aim of this study was to investigate the immunological alterations during the early phase of primary acute EBV infection by means of ENA Western blotting (WB) analysis. This technique is able to detect a wide spectrum of anti-ENA autoantibodies, potentially directed against diverse epitopes of the same antigen.

Methods
Sera from 54 subjects (F/M=24/30, mean age 17±6 SD years) with primary acute EBV infection were analysed using indirect immunofluorescence (IF) on Hep-2 cells for ANA, and both ELISA and WB for ENA.

Results
Only 8 ANA+ and no ENA+ were found by means of IF and ELISA techniques, respectively; however, one or more ENA autoantibodies were detected in 24/54 (44%) sera using WB. The autoantibodies were no longer present at the second evaluation. Subjects with immunological alterations had not developed any significant clinical manifestations at a 5-year follow-up.

Conclusions
This study demonstrated the appearance of autoantibody production in a high proportion of individuals with primary acute EBV infection; interestingly, the observed serological subsets are quite similar to clinical SLE clusters. Moreover, the absence of immunological disorders during the follow-up reinforces the role of multiple genetic and/or environmental co-factors in the pathogenesis of CTDs.

Key words
EBV infection, SLE, Western blot.

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Introduction

Virus infections have been implicated in the origination of multiple human autoimmune syndromes, for example, infectious mononucleosis, inclusion body hepatitis, Guillain-Barré syndrome, Sjögren’s syndrome, rheumatoid arthritis, chronic hepatitis C, SLE after Epstein-Barr virus infection and the extra-hepatic manifestations of hepatitis virus (HBV, HCV). Besides, some autoimmune manifestations can be revealed after a live virus vaccination (measles, chickenpox, smallpox). Moreover, many types of virus (above all, RNA) have been used for inducing autoimmune disorders that reproduce human pathologies, such as type 1 diabetes mellitus, myocarditis, polymyositis and other syndromes.

In this report, we have focused on the lymphotropic Epstein-Barr virus (EBV) (EBV) that represents one of the most important factors potentially involved in the pathogenesis of systemic autoimmune disorders (1-3). This herpes virus is the etiological agent of infectious mononucleosis in young adults; it may remain latent in B cells and, through a series of complex molecular mechanisms, it may be involved in the pathogenesis of both Burkitt’s lymphoma and nasopharyngeal carcinoma. The ability of the virus to induce autoimmune disorders also emerges from the potential complications observed during infectious mononucleosis (4-6). The evaluation of the etiological role of EBV in human autoimmune diseases is, however, problematic, since the virus is ubiquitous, it can persist in a latent state with periodic reactivation in all infected subjects, and the seroconversion occurs in the majority of cases within the second decade of life. So the association between EBV and autoimmune diseases could be merely coincidental or might be due to an increased susceptibility of infection of these patients (7).

However, the hypothesis of a causal relationship between EBV and systemic lupus erythematosus (SLE), in particular, may be strongly supported by many investigations: anecdotal observations of SLE after EBV infection (8,9,10); common auto-antigenic targets in SLE, including Sm B’ (PPGMRPP) and Sm D1 (GR), cross-react with the sequences PPGRRP and (GR), respectively, from Epstein Barr nuclear antigen (EBNA-1) (11, 12); appearance of lupus-like reactivity in animals after immunization with EBV sequences (13-16). Experimental studies on animals do not help to clarify the possible mechanisms due to the difficulties experienced in reproducing animal models; James et al. showed that epitope spreading occurred in normal NZW rabbits immunized with the peptide derived from SmB/B’ PPGMRPP that cross-react with the sequences from EBNA-1(15,16). Mason et al., instead, were not able to confirm the appearance of autoimmunity in the same model by Elisa or with immunofluorescence but did find autoab by Western blot (the same technique used by James) in some animals (17).

Recent research has provided convincing evidence that EBV infection may play a major role in SLE, including B-cell aberrations and apoptosis that are responsible for a perpetual heightened immune response (18).

Observational studies showed that SLE patients have elevated levels of viral DNA in their saliva and an altered antiviral response (19); EBV seroconversion is present in 100% of juvenile SLE cases 20). Moreover, in serial serum samples available before the clinical onset of SLE, anti-Ro antibodies invariably followed anti-EBNA-1 antibody production (21, 22). It is noteworthy that in these patients the initial antigenic epitope cross-reacts directly with an EBNA-1 peptide.

Previous studies described direct relationships between EBV and lupus autoimmunity focusing only on antibodies to Sm and Ro (12, 16, 22), usually found in about 40% of all SLE patients. It is possible that various cross reacting regions of EBV could be the triggering factors in different SLE subsets or that EBV-induced molecular mimicry is limited to only Sm- and Ro-positive SLE. On the other hand, some bias, including an inappropriate immunological window, cannot be excluded in retrospective studies (21).

Acute viral infections in children and in adults can induce only transient autoimmune responses with low title of serum autoantibodies (22, 23); we...
therefore attempted to evaluate the role of EBV in the initiation of autoimmune phenomena by reversing the approach to this intriguing matter. In particular, we investigated the appearance of EBV-related non-organ specific autoantibodies during the early acute phase of infection—namely before the appearance of EBNA-1—in a narrow immunological window, by means of Western blotting (WB) analysis that in experimental models proved capable of detecting autoantibodies not otherwise found by ELISA (17). Moreover, WB can detect a wide spectrum of anti-extractable nuclear antigen (ENA) autoantibodies, including those directed against rare antigens, and can characterize individual antigenic responses, whereas ELISA can detect only immune response when predefined antigens are used in the system (24).

Materials and methods
Sera from 54 subjects (24 F, 30 M, mean age 17±6 SD years) with a diagnosis of primary EBV infection were collected at the Laboratory of Virology at the University-based Hospital of Modena; in particular, the sera were characterized by the presence of anti-early antigen (anti-EA) seropositivity, both IgM and IgG types, and the absence of anti-Epstein Barr nuclear antigen-1 antibodies (anti-EBNA-1). Anti-EA-IgM preside the synthesis of viral nucleic acids and indicate the presence of replicating EBV, while anti-EBNA-1 are absent during acute infection and appear during convalescence. Using these criteria, only acute primary EBV infections, without any clinical-serological findings suggestive of other diseases, were analysed. Moreover, the presence of IgG anti-EA was necessary to evaluate the appearance of autoantibodies to extractable nuclear antigens (ENA) antibodies (anti-EBNA-1). Anti-EA-IgM preside the synthesis of viral nucleic acids and indicate the presence of replicating EBV, while anti-EBNA-1 are absent during acute infection and appear during convalescence.

Sera analysis
Sera were analysed for autoantibodies against nuclear antigens in a blinded fashion using three different methods: 1) ANA detected by indirect immunofluorescence on Hep-2 cells (Alphadia, Belgium): ANA titres ≥1:80 were considered positive; 2) ENA ELISA screening, which measures anti-ENA antibodies: namely, antibodies anti-Sm, -RNP, -SSA, -SSB, -Jo1, -Jo-1, -Scl-70 using a Microplate autoimmune ENA plus instrument (Bio-Rad, USA).

Fig. 1. Western blot Strip 1 is a reference strip showing the position of the labeled ENA bands.
Strip 2: positive control.
Strip 3: 38 kd (antiRNP).
Strip 4: 52Kd (SSA) e 43Kd (SSB).
Strip 5: 38 Kd (RNP), 52 Kd (SSA), 70 Kd (RNP).
Strip 6: 22 Kd (RNP-C), 28 e 29 Kd (Sm) 38 Kd (RNP), 55 Kd (Jo-1).
Strip 7: negative control.

Subjects                  20      4           10      15

into strips and packaged. These ready-to-use strips were incubated with diluted patient serum (1:50). If ENA specific antibodies are present, they will bind to the corresponding ENA antigen bands. After washing the unbound serum from the strip, the bound ENA specific antibody is reacted with alkaline phosphatase conjugated anti-human IgG. The strip is then washed to remove the unbound conjugated antibody. Finally, the strip is reacted with a precipitating colour developing solution which deposits a purple precipitate onto the antibody reacted antigen bands. Results were assessed against the control strips provided showing the position of Ag bands. The system contains a Specimen Reactive Control (SRC) stripe on each strip that assures the application of the test sample on each test strip. A positive and a negative control are included in each run.
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Follow-up study
Five years later, 20 WB-positive patients were interviewed by phone to verify the presence of symptoms suggestive of CTDs; in 10 of these patients, anti-ENA was re-evaluated.

Results
Of the 54 individuals investigated, the screening by immunofluorescence on Hep-2 cells revealed 8 ANA-positive sera (15%), with speckled nuclear and cytoplasmic fluorescence patterns, at titres varying from 1:80 to 1:640. Using the immunoenzymatic ENA methodology, no serum was positive, while WB analysis allowed the detection of ENA in 24/54 (44%) sera with a great variety of autoantibodies (Fig. 1). The percentage of positive sera was significantly higher in males compared to females (17/30, 57% vs. 7/24, 29%; $p=0.029$; Fisher’s exact probability test). Among 24 ENA WB-positive sera, the most frequent finding was the positivity for 38 kDa anti-RNPrribosomal (RNPr), marker of SLE, detected in 11 (44%); while 9 sera were positive for SmB-29 (36%), and 8 for SmB-28 (32%) (Table I). Of these latter, 4 were positive for SmD-15 (16%). Regarding the positivity for anti-SSA and anti-SSB antibodies, the overall prevalence of these antibodies was quite high (15/24, 62%), including 7 SSB-43 (29%), 4 SSA-52 (16%), and 4 SSA-60 (16%). RNP seropositivity was found in 14/25 (56%): 22kDa in 7 (28%), 70kDa in 5 (20%), and 33kDa in 2 (8%).

Three patients also had a 55-kDa band that is normally associated with the presence of anti-Jo-1. Finally, anti-centromere 15 kDa antibodies were detected in 2 sera, while 2 others showed unidentified bands.

Overall, the results of the present study permit the identification of the following serological subsets (Table I):
- with anti-SSA and/or -SSB (n=9; 16.6%);
- with anti-Sm and -RNP (n=7; 13%);
- with anti-RNPr alone (n=3; 5.5%);
- sera with autoimmune response against many different structures (n=3; 5.5%);
- with unidentified antibodies (n=2; 3.7%);
- without any autoantibody response (n=30, 55.5%).

Table I. Autoantibodies detected by Western blot analysis in 25 EBV infected subjects: serological subsets.

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Overall, the results of the present study permit the identification of the following serological subsets (Table I):
- with anti-SSA and/or -SSB (n=9; 16.6%);
- with anti-Sm and -RNP (n=7; 13%);
- with anti-RNPr alone (n=3; 5.5%);
- sera with autoimmune response against many different structures (n=3; 5.5%);
- with unidentified antibodies (n=2; 3.7%);
- without any autoantibody response (n=30, 55.5%).
In the 10 WB-positive patients with serological follow-up, the autoantibodies were no longer present at the second evaluation. The telephone interview 5 years after the infection revealed that the 20 WB-positive patients did not refer the presence of any symptom suggestive of CTDs. The follow-up of the 20 Western blot positive patients was performed after 5 years by telephone interview (20 patients) and by repeat serologic testing by Western Blot (10 patients). The autoantibodies were no longer present. Among the questions posed during the telephone interview, the patients were asked explicitly if they had had any painful or swollen joints in the past 5 years. None of the study subjects admitted to having had any symptoms suggestive of CTD.

Discussion
In non-autoimmune individuals with various (bacterial, viral, parasitic, and rickettsial) acute infections, elevated titers of ANA and/or anti-annexin-V, prothrombin, laminin, Saccharomyces cerevisiae, phospholipids antibodies were most frequently detected, but never anti-ENA. (25).

Even in our subjects ENA are negative in ELISA essay, while, on the contrary the most interesting finding in this study is the presence of anti-ENA autoantibodies, detected by means of WB analysis, in a high percentage of sera from subjects with early primary EBV infection. The difference in ENA positivity between ELISA and Western Blot methods is very interesting. WB allows to evaluate the “fine specificity” of autoantibody response to multiple subunits of the spliceosome complex, such as the recognition of the U1 RNP A, B, and C proteins and the Sm B, B’, D, and E proteins. WB bands also appear for minimal amounts of autoantibody while with Elisa the autoantibody is considered positive only above a cut-off. Moreover, the difference might be due to different binding of the antibody to a particular conformation of the antigenic structure. We know, for example, that sera from patients with autoimmune disease recognize conformational determinants on the 60-kD Ro/SS-A protein (26); while WB analysis may detect only linear epitopes, as the target autoantigens are denatured by SDS-PAGE. (27). Also in Mason’s research, after immunization with PP-PGMRPP the NZW rabbit sera were negative when tested by Elisa for anti Sm, Sm-RNP, aRo; however, the development of ANAs in the sera from three of the rabbits was revealed by WB of nuclear antigen extracted from HeLa cells, namely a SmB/B’, RNP-C, RNP r, RNP-A band. No ANA binding was observed by WB of sera from control rabbits (17)

In this phase in our subjects, characterized by both IgM and IgG anti-EA seropositivity and absence of anti-EBNA-1, it was possible to detect a wide panel of different autoantibodies typically found in SLE patients. The WB analysis might reveal one or more autoantibodies even in the general population, but in our laboratory only SSA-52 or SSA-60 were occasionally and transiently present.

The more prevalent is the 38 kDa anti-RNPr, highly specific for SLE and occasionally reported in other CTDs (28) while in our experience it is invariably absent in a series of 150 CTDs or chronic inflammatory arthritis (personal communication).

In this study, the WB technique shows one or more typical SLE-related autoantibodies, or some markers of primary and secondary Sjögren’s syndrome, often associated with SLE. It is worthwhile noting that SSB-43, detected in 7 cases, is usually correlated with severe salivary and lacrimal gland involvement, but rarely found in SLE (29).

Three patients also had a 55-kDa band (always associated with anti-RNPand/or Sm) that normally associates with polydermatomyositis complicated by pulmonary involvement, an EBV-associated condition described only in immunodeficient patients (30, 31).

The autoantibody response due to the cross reactivity mechanism between EBV and nuclear antigens in a percentage (45%) of our infected subjects is difficult to explain. This variable response might be attributed to the presence of different viral subtypes, i.e., EBV-1 and EBV-2, which present different epitopes. The stable percentage of EBV-related autoimmunity in the sera collected over a lengthy period does not seem to confirm the above hypothesis, even if all 52 kDa antigen-positive samples were collected during 1998 while the 60 kDa-positives were collected during 1999. It is not easy to understand this latter observation and it could be merely incidental.

The significantly higher prevalence of EBV-related autoantibodies in males is quite intriguing. Since autoimmune disorders are usually more frequent in women, supposedly other genetic and endocrine co-factors may be necessary for the development of clinically overt autoimmune diseases.

A clinicopathological assessment was not available in the majority of the subjects. Five years after the EBV infection, the telephone interview excluded the presence of any systemic autoimmune disease in the follow-up of 20 WB-positive subjects; moreover, the re-evaluation of sera in 10 patients documented the disappearance of all ENA antibodies.

Our study shows that, even in its early stage, an EBV infection is capable of inducing a transient autoantibody response similar to that found in autoimmune diseases in a significant subgroup of individuals. This initial event may be caused by cross-reactivity between the virus and several autoantigens, in addition to the subsequent EBNA-1 response. This autoimmune mechanism may lead to overt disease in some subjects with the contribution of defective response to EBV and/or genetic factors (32). Patients with SLE have, indeed, a 40-fold increase in EBV viral loads in peripheral lymphocytes, which probably stem from inadequate CD8+ T cell responses against EBV (33, 34). Genetic factors might be decisive for early autoantibody responses to EBV infection, reproducing different SLE clusters. The X-linked lymphoproliferative syndrome, a rare primary immunodeficiency disease characterized by an abnormal response to EBV infection, occasionally related to transient autoimmune phenomena (35), may support this hypothesis.
Conclusions

On the whole, the results of this study suggest the following considerations: EBV may cause a polyclonal activation of B lymphocytes, which could explain the considerable autoantibody production observed in individuals with primary EBV infection; this B-cell activation could be comparable to that observed in other chronic viral infections. The autoantibody production might not be due to EBNA-1-associated molecular mimicry alone. The appearance of potential cross-reactive antibodies as early as during the primary infection may suggest that multiple reactivation is not necessary for the induction of autoantibodies. The diversity of autoantibodies and the presence of various clusters in SLE patients could suggest that different triggers are important in individual patients. In the literature, the etiologic role of EBV in SLE is postulated only in Sm and Ro positive (60kd) patients. In our subjects, many autoantibodies (SSA 52, SSA 60, SSB 43, Sm-28, Sm-29, RNP-22, RNP 70, RNP-r 38) appeared in the same period during early EBV infection. The variable responses among infected individuals reproduce different immunological patterns of CTDs, such as SLE (36) or Sjögren’s syndrome. The appearance of different autoantibody clustering during primary EBV infection may support the role of different etiopathological co-factors, mainly host immune reactivity. The crucial relevance of this latter is supported by the high percentage of negative sera as well as the gender-related different response.

In the majority of subjects, the appearance of autoantibodies can be merely transitory; the fact that no symptoms developed could be taken as a hint for the irrelevance of these autoantibodies. The factors that influence the appearance, persistence, or recurrence of these autoantibodies after EBV infection hold great promise for a better understanding of lupus.

References