Molecular diversity of IgG responses to Epstein–Barr virus proteins in asymptomatic Epstein–Barr virus carriers

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Abstract

The Epstein–Barr virus (EBV) is a ubiquitous pathogen that infects over 90% of adults. EBV is the primary etiological agent of infectious mononucleosis and is closely associated with nasopharyngeal carcinoma, gastric carcinoma, Hodgkin lymphoma and Burkitt lymphoma. Clinical serological assays for EBV diagnosis only survey a small portion of the viral proteome, which does not represent the total antigenic breadth presented to the immune system during viral infection. In this study, we have generated an expression library containing the majority of EBV ORFs, and have systematically evaluated IgG responses to those EBV proteins in sera from EBV carriers. In addition to confirming previously recognized dominant EBV antigens, this study has identified additional immunodominant antigens, and has revealed a more expansive antigenic profile of the humoral responses to EBV in asymptomatic carriers. This EBV expression library will be deposited in a public repository with the goal of disseminating this new research tool for the application of identifying potential new biomarkers for EBV-associated diseases.

INTRODUCTION

EBV seroconversion rates worldwide are reaching approximately 95% by adulthood [1]. While the majority of these infections are clinically asymptomatic, in certain segments of the population EBV infection causes infectious mononucleosis (IM), and also contributes to development of several malignancies, such as nasopharyngeal carcinoma (NPC), gastric carcinoma (GC), Hodgkin lymphoma (HL), and Burkitt lymphoma (BL) [1].

Clinical diagnostics of humoral responses to EBV infections include tests for heterophile antibodies and antibodies targeting specific EBV antigens [2–4]. The technology used to detect EBV-specific antibodies has progressed from immunofluorescence assays (IFAs) and enzyme immunoassays (EIAs) to chemiluminescence immunoassays (CLIA) and newer multiplex flow immunoassays (MFIs) [5] and all the newer tests use recombinant EBV antigens. When confirmatory tests are warranted, immunoblotting using lysates of EBV-transformed cells is often the method of choice [6]. However, what has not changed in the last 20 to 30 years is the selection of EBV antigens used in all of these analyses. The screening tests exclusively rely on a few antigens from the viral capsid antigen (VCA) complex, the early antigen (EA) complex, and the EBV nuclear antigen (EBNA) complex identified in early studies [7–12]. More specifically, most tests use combinations of products of BFRF3 (p18), BMRF1 (EA-D) and BKRF1 (EBNA1) genes expressed in and purified from heterologous systems [5, 13]. Notably, none of these antigens seems to be useful in diagnostics of the EBV-associated malignancies. Recognizing the limitations of the current approaches, additional viral antigens have been considered as markers of EBV infection and diseases: EBNA2 and EBNA3s [14], LMP1 [15], BZLF1 and BRLF1 [9, 16], BMRF2, BXLF1 and BGLF5 [17, 18], BclF1, BALF2 [18, 19], and BLLF1 (gp350/220) [20]. But these studies only illustrate a lack of consensus on the use of defined EBV proteins or combination of proteins for EBV serological testing. Moreover, the majority of studies published thus far could not resolve the molecular complexity of the anti-EBV humoral responses, which extends well beyond the few tested antigens. We have identified three limitations that hinder our ability to analyse the full repertoire of IgG responses to EBV infection. First, virtually all published
studies use lysates from EBV-infected cells as the source for EBV antigens. While a robust lytic programme can be induced in certain cell lines, the diversity of cell lines used in these studies and the dynamic nature of the EBV lytic programme make it an unpredictable and inconsistent source for EBV proteins. The proteins in such lysates could be expressed at different levels and their antigenicity cannot be compared or, if they are not expressed at all, they will be eliminated from the analysis. Second, only a limited number of highly specific monoclonal antibodies against EBV proteins are available, which restricts our ability to confirm detection of a particular protein in the lysate. Third, even when the monoclonal antibodies are available, their sensitivity and specificity differ significantly and thus levels of detection of different proteins cannot be compared. The only comprehensive study available to date used a protein microarray with purified GST-tagged EBV proteins expressed in yeast and screened against plasma from 15 HIV+/KS patients, 10 HIV−/B-cell lymphoma patients, and 10 healthy donors [21]. This study proved the feasibility of systematic screens against a complete viral proteome and emphasized the importance of investigating not just individual antibody titres but also patterns of the humoral immune responses. However, the study was limited to a small collection of sera, and was lacking sera from dedicated EBV-negative controls or from patients with EBV-associated diseases. In addition, the source proteins were expressed in yeast, which could create a distorted antigenic profile [22]. A similar systematic study of humoral responses to Kaposis’s sarcoma-associated herpesvirus (KSHV) using ELISA and multiplex flow immunoassay has also been recently reported [23].

In order to resolve the molecular complexity of the IgG response to EBV proteins, we have cloned and expressed the majority of apparent EBV ORFs in EBV-negative mammalian cells and quantified responses to these proteins in sera from EBV-seropositive and -seronegative individuals. The comprehensive library of EBV ORFs generated as a part of this study can serve in the future as a valuable tool for identifying currently unavailable prognostic and diagnostic markers for EBV-associated diseases and malignancies.

RESULTS

EBV antigens detected by human sera in lysates from EBV-infected cells

Fifteen sera from EBV-seropositive individuals and eight sera from EBV-seronegative individuals (as defined by current clinical laboratory tests) were screened by immunoblotting against whole-cell lysates prepared from EBV-negative BL cell lines, DG75 and BJAB, and EBV-positive BL cell line, Akata, which were treated with goat anti-human IgG to induce the viral lytic programme. The EBV− sera did not detect any EBV-specific antigens and weakly detected several cellular proteins in all lysates (Fig. 1). In contrast, the EBV+ sera consistently detected a pattern of 9±2 EBV antigens (per visual count) in lysates from lytically induced Akata cells and only one EBV antigen in uninduced Akata cells. Importantly, the identity of these proteins were unknown. Similar to EBV− sera, several cellular proteins were weakly detected in all lysates by the EBV+ sera (Fig. 1). The single EBV antigen detected in lysates from uninduced Akata cells may represent the only latent viral protein expressed in these cells, EBNA1 [24]. These data are comparable to previously reported patterns of EBV detection [7, 18], therefore validating conditions of the assay. However, the information obtained by this approach is inadequate due to the limitations outlined earlier.

Cloning and expression of the EBV genes

Seventy-five EBV ORFs were cloned in-frame with 3×FLAG-tag. Sixty-two proteins could be expressed in HEK293 cells at levels detectable in our experimental conditions. The 13 ORFs that could not be expressed or detected include secreted proteins (BARF1 and BCRF1), very small proteins (<10 kDa) (BBLF1, BDLF3.5, BLRF1, BSRF2), and putative proteins (BARF0, BNLF2a and b). The library will be deposited in a public repository (Addgene) and made available as one complete expression library.

Serum IgG responses to individually expressed EBV antigens in EBV-seropositive and EBV-seronegative individuals

To systematically evaluate and compare the antigenicity of the various EBV antigens, we measured IgG responses by immunoblotting in three sets of human sera: (a) from EBV-seropositive (EBV+) individuals (n=15), (b) from EBV-seronegative (EBV−) individuals (n=10) and (c) from asymptomatic individuals with unknown EBV status (n=10). The positivity or negativity in regard to EBV status were defined based on

![Fig. 1. Human EBV+ serum IgG responses to viral antigens in lysates of EBV-infected cells. The EBV lytic programme was induced in Akata cells by incubation with anti-human IgG. At 24 h post-induction, lysates from uninduced (U) and induced (I) Akata cells were subjected to SDS-PAGE and probed with a 1:500 dilution of EBV+ or EBV− human sera. Lysates from DG75 and BJAB cells served as a negative control. Bands marked with the (#) sign indicate proteins detected specifically in induced cells; bands marked with the (*) sign indicate proteins detected in both induced and uninduced cells. Molecular marker weights in kDa are shown.](image-url)
current clinical laboratory tests. The sera were screened against all expressible EBV antigens and the ratios between the detection levels of the antigens by human sera and expression levels of the antigens were calculated (see Methods). These ratios were arranged in the heatmap (Fig. 2), where their values are represented by colour intensity. Higher ratios correspond to better detection of an antigen by human sera.

All the sera from asymptomatic individuals were EBV+ as evident by their detection pattern being very similar to the pattern exhibited by the sera from EBV+ individuals. Therefore, for future analyses these sera were combined and referred to as EBV+ sera (n=25). The EBV+ sera detected, on average, 17.4±1.3 antigens, which was significantly greater than detection by the EBV− sera, which detected, on average, 1.5±0.5 proteins (Fig. 3a). The EBV+ sera detected both virion and non-virion viral proteins with almost equal frequencies. Notably, by using this approach we identified almost twice as many proteins as were detected (but not identified) in cell lysates from EBV-infected Akata cells (Fig. 1).

One of the advantages of our approach is the ability to measure the efficiency of detection for each of the EBV antigens and compare these efficiencies across all EBV antigens to identify dominant antigens with higher levels of detection by EBV+ human sera. To this end, the detection ratios were calculated for each of the EBV antigens and compared on the volcano plot (Fig. 3b). Ten EBV proteins were detected significantly more frequently (P<0.01) by EBV+ sera than by EBV− sera. These were BFRF3, BRRF2, BRLF1, BZLF1, BPLF1p3, BSLF2/BMLF1 (SM), BKRF1 (EBNA1), BKRF4 and BLLF3 proteins. BFRF3 and EBNA1 proteins, which are currently used in clinical diagnostics, were detected most frequently, but detection of the former was significantly better (detection ratio 1.07 vs 0.13) (Fig. 3b, red letters). Several other proteins (BZLF1, BRLF1 and BRLF2) previously proposed as biomarkers for EBV
The data obtained in this study also reveal an interesting phenomenon: more than half of the sera designated as EBV negative detected at least one EBV protein (Fig. 2). In fact, one EBV− serum detected five proteins, two sera detected three EBV proteins, another one detected two proteins, and the remaining two sera detected one protein each. However, none of the proteins detected by these sera is included in the current clinical diagnostic kits. These data suggest two possibilities: (1) the observed detection represent a false-positive result, or (2) almost a third of the clinically diagnosed EBV− cases are misdiagnosed due to the limitations of the current clinical kits. To distinguish between these two possibilities, we have isolated the total DNA from the sera and attempted to detect EBV DNA by PCR (Fig. 4). Despite a reasonably low limit of detection (down to one copy), we were unable to detect EBV DNA even in two seropositive samples (served as positive controls). This lack of detection is consistent with previous studies which established that cell-free body fluids such as serum or plasma contain negligible amounts of EBV DNA, suggesting that EBV DNA is detectable in biofluids only in association with reactivated infection or EBV-related disease, or from cell-associated virus [25, 26]. Thus, in order to definitively address this question, a prospective study is warranted in which donor PBMCs will be collected along with serum and both EBV DNA and serology will be analysed in parallel.

**DISCUSSION**

Historical survey of studies on immune responses to EBV infection shows that a lot of work has been done at the time when our knowledge of EBV biology and genetics was fairly limited. The discoveries made by several groups served as a foundation for development of clinical EBV tests currently used in clinical laboratories. While significantly more studies have been performed between the early 1970s and now, a comprehensive, systematic and unbiased analysis of immune responses to EBV has not been performed. In the last 20–25 years, the majority of studies were focused on cellular immune responses (reviewed in [27]), whereas analyses of humoral immune responses lagged behind. Perhaps more importantly, these studies focused on a small group of EBV proteins based on their perceived significance in pathogenesis of EBV-associated malignancies or in EBV replication. Only one study to date attempted a systematic and comprehensive survey of humoral responses to EBV antigens against proteins expressed in yeast [21]. The study demonstrated feasibility and value of such analyses and reiterated the need for additional systematic studies. Hence, our study has specifically focused on IgG responses in human sera against EBV proteins expressed in mammalian cells. The study aimed to systematically characterize and quantify anti-EBV IgG responses in sera from EBV carriers.

Classical analyses of humoral responses to EBV infection are usually defined by responses to three antigenic complexes: viral capsid antigens and membrane antigens (VCA/MA), early and immediate-early antigens (EA/IEA), and Epstein–Barr nuclear antigens (EBNA). The previously reported immunoreactive components of the VCA/MA complex are the major capsid protein (BclLF1) [28], the tegument protein p23 (BLRF2) [19], the small capsid protein (BFRF3), and the scaffold protein (BdRF1) [9, 11]. Our data clearly support the earlier observations that BFRF3, BLRF2, and BdRF1 were also detected frequently and their detection ratios ranged between 0.33 and 0.46 (Fig. 3b, green letters). Most importantly, we have identified novel markers (BRRF2, BKRF4 and BPLF1p3) that were frequently and efficiently detected by the EBV+ sera (detection ratios 0.52, 0.08 and 0.33, respectively) (Fig. 3b, blue letters).

![Fig. 3. Dominant EBV antigens detected by the sera from EBV carriers. (a) Total numbers of antigens detected by EBV+ and EBV− sera (mean±SEM). (b) Dominant antigens eliciting strong IgG response in majority of sera from EBV carriers. The detection/expression ratio is plotted on the horizontal axis; the corresponding P-value is plotted on the vertical axis. Red signifies antigens currently used in clinical serology tests, green shows antigens previously proposed as biomarkers for EBV diagnostics, and blue displays the dominant antigens identified in this study. Dashed lines represent cut-off values for the ratio (0.04) and P=0.01.](image-url)
and BdRF1 are the dominant EBV antigens, which were detected by the majority of sera from EBV carriers (Fig. 2). Likewise, in line with previous observations [29], the BcLF1 protein was detected with lesser frequency and intensity (Fig. 2) and was not included in the dominant antigen group. The MA component of the complex is typically represented by BALF4 and BLLF1 proteins [19, 30]. While the BALF4 protein was represented in our panel, the detection of it was fairly infrequent and weak (Fig. 2). This is not surprising considering that membrane proteins are notoriously refractive to analyses by immunoblotting [31] and thus were not well represented in our EBV antigen panel. However, we have identified two ‘structural’ antigens that can be added to the VCA/MA complex – BPLF1-p3 and BRRF2. Both proteins are components of the tegument, a proteinaceous layer between the capsid and the envelope of the virion. In our analyses, both proteins were detected with high frequency and intensity (Figs 2 and 3). Moreover, several serum samples from the EBV-negative group detected BdLF1, BLRF2 and BPLF1-p3 proteins, suggesting that these individuals’ infection status could have been misdiagnosed (Fig. 2). The EA/IEA complex consists of the following immunoreactive components: BMRF1 [32–34], BALF2, BALF5, BGLF5, BZLF1, and to some extent BGLF4, BXLF1 and BRLF1. Some authors add BHRF1 and BORF2 proteins to that group [35, 36]. Of these, only BZLF1 and BSLF2/BMLF1 (SM) were designated as dominant in our study (Figs 2 and 3). Interestingly, the BMLF1 protein has been initially proposed as a dominant antigen [37], but this observation had not been confirmed in later studies [32, 34]. The BZLF1 proteins have been previously shown to be detected by ~60 % of healthy EBV carriers [18, 38, 39], but in our study this number is close to 100 % (Fig. 2). From the components of the EBNA complex, only EBNA1 was detected by all tested sera from asymptomatic EBV carriers (Fig. 2). However, its detection efficiency (or immunoreactivity) was significantly weaker than that of several other proteins (BRRF2, BLRF1, BPLF1-p3), which might reflect the levels of exposure of these proteins to the components of the immune system. Finally, the IgG response to malignancy-associated EBV antigens such as latent membrane proteins (LMPs) was very low (12 % for LMP1 and 0 % for LMP2A) supporting several previous reports [39–42]. Overall, our study revealed the antigenic breadth of the humoral response against EBV proteins, confirming several previously reported EBV-dominant antigens and adding several previously unrecognized immunodominant antigens. We also found that almost half of the sera designated as EBV-negative by the current clinical diagnostic test detected up to five EBV antigens. Attempts to correlate these results with EBV DNA analysis in the available serum samples were unsuccessful (Fig. 4) and the retrospective analysis of corresponding B-lymphocytes was not possible. While the detection of EBV proteins by the EBV– sera in our panel may simply represent a false-positive result, we argue that it also raises a possibility that current clinical diagnostic tools may be inadequate and that expansion of the EBV antigen panel could be explored to improve accuracy of diagnosis. Future prospective studies involving a comparison of IgG responses to an extended panel of antigens and cell-associated viral DNA need to be performed to estimate the frequency of misdiagnosed cases.

In conclusion, in this study we described a comprehensive library of EBV proteins. A screen of sera from EBV carriers confirmed previously identified immunodominant EBV antigens and added several novel antigens. The library will be deposited in a public repository with goal of disseminating this new research tool to identify potential new biomarkers of EBV-associated diseases.

**METHODS**

**Serum samples and antibodies**

Serum samples were obtained under institutional review board (IRB)-approved protocols. Fifteen (n=15) serum samples were designated as EBV-seropositive (EBV+) and ten (n=10) serum samples were designated as EBV-seronegative (EBV–) based on the results of clinical tests performed in the Pathology Laboratories at the Memorial Medical Center (Springfield, IL) (Bioplex 2200 EBV IgG kit; Biorad). Three EBV+ and two EBV– serum samples were positive for anti-VCA IgM. Ten (n=10) additional serum samples were obtained from asymptomatic individuals through a tissue bank maintained by the Simmons Cancer Institute of the SIU School of Medicine. All the sera were pre-absorbed for 24 h with formaldehyde-fixed HEK293 cells, aliquoted and stored at −80 °C until use. Anti-FLAG M2 monoclonal antibodies were purchased from Sigma-Aldrich (St. Louis, MO).

**Plasmids**

Seventy-five EBV ORFs were PCR- amplified using sets of primers based on the GenBank sequences V01555 and AJ507799.2. The bacterial artificial chromosomes B95.8/F (generously provided by H.-J. Delecluse) and total genomic DNA from lytically induced Akata cells were used as templates. The PCR products were cloned into the pCMV-3 × FLAG (Sigma-Aldrich, St. Louis MO) or pcDNA3.1-Myc-
3×FLAG vectors. The latter was generated by inserting a linker (GAAATGGAAACAAAACTCATTCTGAAGAG-GATCTGAAAGCTTGCGGCCGATATCGGTACGAC TCAAAAGACCATTGCAGTATTATAAGAGCTGAG ATCGATTACAAAGACGATGACGACAAGTGA) into Nhel and XbaI sites of the multiple cloning site of the pCDNA3.1+ vector (Thermo Fisher Scientific, Waltham, MA). Several ORFs could not be cloned or expressed as full-length genes and were cloned as fragments. Detailed cloning information is summarized in Table S1 (available in the online Supplementary Material). The EBV expression library generated in this study will be made available to the research community through Addgene.

Cell lines
EBV-negative BL cell lines DG75 and BJAB [43, 44] and the EBV-positive BL cell line Akata [45] were maintained in RPMI-1640 medium supplemented with 10 % FBS, 1× anti-biotic-antimycotic mixture, non-essential amino acid mixture, and 1 mM sodium pyruvate (BioWhittaker, Cambrex Bio Science). Human epithelial kidney 293 cells (ATCC CRL1573) were grown at 37 °C and 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium supplemented with 10 % FBS and antibiotic/antimycotic mixture. The tissue culture media and supplements were purchased from Thermo Fisher Scientific (Waltham, MA).

Transfections and EBV lytic programme induction
Transfections of the HEK293 cells were performed using Turbofect (Thermo Fisher Scientific, Waltham, MA) by following the manufacturer’s protocol. The EBV lytic programme was induced in Akata cells by treatment with 50 µg ml⁻¹ of goat anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) for 24 h as previously described [24].

Immunoblotting
Whole-cell lysates were prepared by boiling in Laemmli sample buffer [46]. An amount of lysate equivalent to 2×10⁵ cells lane⁻¹ was separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were blocked with blocking buffer (5 % non-fat milk in TBS and 0.2 % Tween-20) and then incubated overnight with anti-FLAG mAB diluted 1:3000 in the blocking buffer. The membranes were briefly washed in TBST and then incubated overnight with human sera diluted 1:500 in the blocking buffer. After washing, the membranes were incubated with a mixture of IR-dye-800CW-conjugated goat anti-mouse and IR-dye-680LT-conjugated goat anti-human antibodies (Li-Cor Biosciences, Lincoln, NE) diluted 1:10 000 in the blocking buffer. The membranes were then scanned on a Li-Cor Odyssey scanner, and the scans were exported for analyses. The levels of detection in both channels were quantified using ImageJ [47] and the ratio between values in the 700 nm channel (representing protein detection levels by human sera) and values in the 800 nm channel (representing protein expression levels) were calculated and arranged in a heatmap using Prism 7.0 (GraphPad Software, La Jolla, CA). Higher ratio values indicate more efficient detection of a particular antigen by human sera. Ratio values below the exclusion threshold (<0.04) were designated as negative.

PCR detection of EBV DNA
Total genomic DNA was isolated from 200 µl of serum using phenol-chloroform extraction without DNase-treatment to isolate both free and virion-associated DNA, and resuspended in 50 µl water. All seronegative samples (no. 7, 13, 23, 32, 35, 37) except for sample 27, and two seropositive control samples (no. 10, 11) yielded measurable DNA concentrations in the range of 1.8–5.3 ng µl⁻¹ sufficient for qPCR analysis. Twelve µl of template was analysed by PCR in a 25 µl reaction using the BALF5 (pCMV-3X-FLAG2) plasmid (7782 bp) as detection standards (1–10⁶ copies). The PCR product (66 bp) was analysed by agarose gel electrophoresis with a limit of detection of ≥1 copy. PCR reactions were assembled with 0.1 µM qBALF5f and qBALF5r primers (5’ GAGCGATCTTGGAATCTCT 3’ and 5’ TGGTCATGGATCTGTAACCC 3’) and 1X Taq polymerase PCR master mix (Thermo Scientific) using the following cycle conditions: 95 °C 3 mins, 40 cycles of (95 °C 10 mins final

Statistical analysis
Differences in proportion of serum samples detecting each EBV protein were evaluated by Fisher’s exact test with a two-tailed P-value.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

References


