Peripheral Blood Lymphocytes Express Recombination-Activating Genes 1 and 2 during Epstein-Barr Virus–Induced Infectious Mononucleosis

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Implicit in the persistence of Epstein-Barr virus (EBV) in B lymphocytes is the successful circumvention of ongoing cell selection for competence of B cell receptors (BCRs). Because the EBV infection of B cells in vitro induces enzymatic machinery that is responsible for secondary immunoglobulin gene rearrangement, we examined the expression of the recombination-activating genes (RAGs) in peripheral blood mononuclear cells (PBMCs) from 26 patients with infectious mononucleosis (IM). RAG1 and/or RAG2 RNA was detected in PBMCs from 42% of patients with IM but not from healthy control subjects. EBV may usurp the cellular mechanism that diversifies the BCR, to guarantee a level of survival signaling sufficient for its own persistence.

Epstein-Barr virus (EBV) is a human γ-herpesvirus that establishes persistent infection in 95% of the population by adulthood. The EBV carrier state is characterized by intermittent shedding of virus from the oropharynx, with quiescent memory B lymphocytes providing the reservoir for chronic, latent infection. Despite the efficiency with which EBV sets up this life-long carrier state, early events that culminate in such a stable virus-host equilibrium are only beginning to be understood [1].

An appreciation of the persistence of EBV in the B lymphocyte requires viewing that accomplishment in the context of B cell selection, where signaling through the B cell receptor (BCR), or surface immunoglobulin, has major implications for the fate of any potential host cell [2]. Ligation of the BCR may trigger survival signals, leading to cell proliferation and differentiation, or, conversely, may trigger negative signals, leading to apoptosis. The factors that determine a positive or negative signaling response are diverse and involve the maturation state of the cell, tissue location, affinity of the BCR for a ligand, and binding of additional cell-surface molecules in sequence. In the infected cell, cross-linking of the BCR may also stimulate the cytolytic reactivation of EBV from latency [3]. Thus, for EBV to achieve a rapport with randomly targeted naive and memory B cells that is adequate to ensure its long-term survival, it must have evolved the means to subvert this most fundamental process of B cell biology.

Evidence for such a stratagem is the expression by EBV of 2 ligand-independent signaling molecules, latent membrane protein (LMP)–1 and LMP2A, which act as surrogates for cellular CD40 and BCR, respectively [4, 5]. When they are expressed in naive B cells, these viral molecules drive the infected lymphoblast through the normal differentiation pathways engendered in a germinal center reaction, which leads to virus-infected memory B cells and, ultimately, the nonpathogenic carrier state [1, 6]. In addition to providing the tonic survival signal normally furnished by an intact BCR, LMP2A is thought to negate the reactivation of

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EBV that occurs with BCR cross-linking [3, 5]. Whether these virus-encoded molecular mimics are sufficient to render signaling through the BCR entirely superfluous to cell fate, regardless of the stage of cell differentiation or tissue microenvironment, remains in question [7].

Because B cell selection presents a formidable challenge to the persistence of EBV, the virus may have evolved redundant mechanisms to address the issue. Earlier reports of the induction of recombination-activating genes (RAGs) by EBV at the time of infection of mature B cells [8, 9] raise the possibility that cellular machinery capable of BCR alteration may also be enlisted in the establishment of the virus carrier state. Receptor editing and revision through RAG reexpression and secondary immunoglobulin gene rearrangement, when they occur in the natural context of bone marrow and, perhaps, germinal centers, has been advanced as a means to salvage B cells with autoreactive, low-affinity, or nonfunctional BCRs [10, 11]. That a persistent lymphotropic virus should avail itself of innate biological mechanisms that effectively enhance the odds for host cell survival appears to be consistent with the modus operandi described for EBV [1]. To ascertain whether in vitro observations have parallels in a clinical setting, we analyzed serial samples of peripheral blood mononuclear cells (PBMCs) from patients with infectious mononucleosis (IM), to determine whether the reexpression of RAG is a feature of acute EBV infection.

PATIENTS, MATERIALS, AND METHODS

PBMCs from 26 patients (12 men and 14 women; median age, 19 years; age range, 17–38 years) with a clinical (fever, pharyngitis, and cervical lymphadenopathy) and serological (heterophile antibody and IgM to viral capsid antigen) diagnosis of EBV-associated acute IM were studied. Patient demographic, clinical, and psychological features have been reported elsewhere [12]. Blood samples were obtained within 2 weeks of symptom onset (diagnosis) and then 1, 2, and 6 months later. From 22 of 26 patients, a fifth sample was obtained at a median of 4 years after diagnosis (range, 1.9–5.4 years). Five healthy, seropositive adults (2 men and 3 women) served as control subjects. PBMCs used for RNA and DNA extraction were isolated by density centrifugation (Ficoll; Amersham Pharmacia), resuspended in medium that contained 10% dimethyl sulfoxide (Sigma), and frozen in liquid nitrogen before nucleic acid extraction. Informed consent was obtained from patients, parents, or guardians. Human experimentation guidelines of the US Department of Health and Human Services and the Louisiana State University Health Sciences Center were followed.

Total RNA was isolated by use of the RNeasy Mini Kit (Qiagen). For cDNA synthesis, ~5 μg of RNA was first incubated with dNTPs (1 mmol/L; Amersham Pharmacia) and random hexamers (0.5 μg/mL) in 50 μL total volume at 65°C for 15 min, followed by 1 min on ice. Reverse transcription was then performed by adding 50 μL of the reaction mixture (400 U Moloney murine leukemia virus reverse transcriptase [RT; Invitrogen], 80 U of RNasin [Promega], 10 μL of dithiothreitol [0.1 mol/L; Invitrogen], 20 μL of 5× RT buffer [250 mmol TRIS/L {pH 8.3}, 375 mmol KCL/L, and 15 mmol $MgCl_2$/L], and diethyl pyrocarbonate water) and incubating at 37°C for 1 h. RNase H (1 U; USB) was added for 30 min, to remove RNA that might interfere with the PCR.

For the gene-expression analysis of EBV nuclear antigen (EBNA)–1, RAG1, and RAG2, specific assays were developed by use of a 5′ nuclease-based real-time quantitative polymerase chain reaction (RQ-PCR) and the ABI PRISM 7700 Sequence Detection System, in which specific amplification of the target sequence is measured by the increase in fluorescence emission of a nonextendable dual-labeled internal probe (Applied Biosystems). Primer and probe sets were designed by use of the GenBank Sequence Database (accession numbers V01555 for EBV, M29474 for RAG1, and M94633 for RAG2). Forward and reverse primers were located on distinct exons, with probes spanning the exon-exon junction. Primers were 5′-AGACCTGGGAGCAGATTCACC-3′ and 5′-GTACCTGGCCCCTCGTCAG-3′ (EBNA1 exons U–K), 5′-TCAACACTTTGGCCAGGCA-3′ and 5′-CCAAGGTGGGTGGGAAAGA-3′ (RAG1 exons 1A–2), and 5′-AGCAGCCCCTCTGGCC-3′ and 5′-GAGAAGCCTGGCTGAATTAAGG-3′ (RAG2 exons 1–2). FAM/TAMRA dual-labeled probes were 5′-CCGCGGCCGTCTCCTTTAAGATG-3′ (EBNA1), 5′AGGTACCTCAGCCAGCATGGCAGC-3′ (RAG1), and 5′-TCAGACAAAAATCTACGTACCATCAGAAACTATGTCTCTG-3′ (RAG2). As a positive control, a VIC-labeled probe to 18S rRNA (*Taq*Man Ribosomal RNA Control Reagents; Applied Biosystems) was used. PCR amplification was performed as per the manufacturer's protocol, with 2× *Taq*Man Universal Master Mix (Applied Biosystems) adjusted to 50 μL with primers (300 nmol/L), probe (200 nmol/L), template (10 μL of cDNA), and water. Real-time measurements were taken, and C_t values (the cycle number where the signal crosses the threshold, set 10-fold above background) were calculated for each sample. Each sample was run in duplicate with multiple template–negative controls.

For the standardization of each RQ-PCR assay, 2-fold dilutions of plasmids that contained RAG1 (gift from R. M. Goorha, St. Jude Children's Research Hospital, Memphis, TN), RAG2, EBNA1, and the *Eco*RI B fragment of the rRNA locus were used to establish a linear relationship between the log starting copy number of template and the corresponding C_t for each RQ-PCR assay. A correlation coefficient $r > .99$ was found for each assay across 5 orders of magnitude of template, with the ability to detect at least 10 copies of each target sequence. In RQ-PCRs that contained either Reh (RAG positive) or Raji (EBNA1 positive) cells diluted in 10^6 Burkitt lymphoma (BL)–

2 cells (negative for both parameters), as few as 10 cells expressing EBNA1, RAG1, or RAG2 were detected.

For DNA isolation, PBMCs lysed in digestion buffer (100 mmol NaCl/L, 10 mmol TRIS/L [pH 8.0], 10 mmol EDTA/L [pH 8.0], and 0.5% SDS) were digested with proteinase K (0.5 mg/mL; Sigma). DNA extracted with phenol/chloroform/isoamyl alcohol was precipitated with 150 mmol sodium acetate/L and ethanol. DNA pellets were resuspended in TRIS-EDTA buffer and stored at −20°C until use. The EBV DNA load in PBMCs was determined by RQ-PCR; primers and probes are described elsewhere [13]. In brief, the EBV copy number was determined by amplification of the single-copy EBNA1 gene (the *Bam*HI K fragment). To confirm the number of cells present in each sample, a second amplification measured genomic C-reactive protein (CRP) DNA (2 copies/cell). The number of EBV copies per cell was determined by dividing the number of copies of the EBNA1 gene by the number of cells. DNA from the BL cell line Namalwa (ATCC CRL-1432), which contains 2 copies of EBV/cell, was used as a standard for both assays.

Select experiments to show RAG expression after in vitro infection used the EBV-negative BL cell line, BL-2, exposed to recombinant B95-8 virus expressing green fluorescent protein (GFP; a gift from R. Longnecker, Northwestern University, Chicago, IL). B95-8 cells that contained recombinant virus were induced by 4 days of exposure to 12-0-tetradecanoylphorbol 13-acetate (TPA; 20 ng/mL; Sigma). The cell supernatant containing virus was either concentrated at 20× and frozen or first purified over a sucrose gradient, resuspended at 10×–20× concentration from the original volume, and stored at −70°C. RAG determinations were made if ⩾30% of the BL-2 cells expressed GFP.

RESULTS

To confirm the EBV-induced expression of RAG1 and RAG2 in mature B lymphocytes [8] and to validate the results of RQ-PCR assays for use on patient-derived PBMCs, we infected the BL-2 cell line and established a time course for RAG induction. Because TPA has been reported to down-regulate RAG1 and RAG2 in pre–B cell lines [14], we first tested the effects of TPA-induced virus stocks on the pre–B cell line Reh, which is RAG positive but is not susceptible to infection by virtue of lacking the EBV receptor, CD21. TPA-generated EBV stocks produced a 10-fold down-regulation of RAG1 transcripts in Reh cells, which is equivalent to suppression from TPA alone. By contrast, intrinsic RAG expression was unaffected when Reh cells were exposed to virus preparations that were first purified over sucrose gradients (data not shown).

In the experimental infection of BL-2 cells with gradient-purified EBV, the induction of RAG1 and RAG2 transcripts was detected as early as 24 h after infection (figure 1), with expression persisting for up to 15 days in select experiments. No amplification signal was present in RT-negative or mock-infected controls. EBNA1 RNA, which was measured as an internal control for infection, was detected concurrent with RAG expression (figure 1), consistent with previous reports that EBNA1 alone is sufficient for the induction of RAG [8]. Indeed, at 6 h after infection, which is before the time required for viral genome circularization and gene expression, RAG1 and RAG2 transcripts were not detected (data not shown).

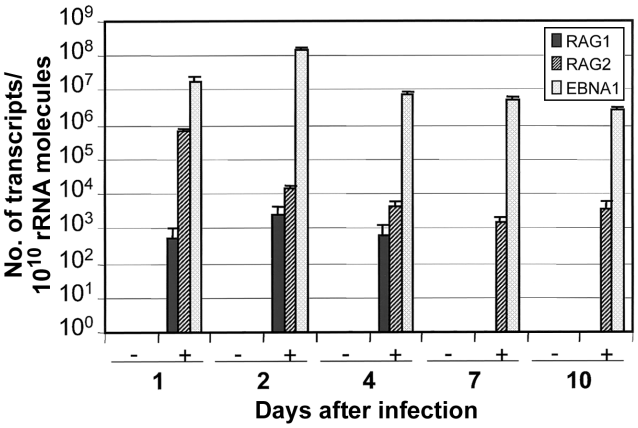

Figure 1. In vitro Epstein-Barr virus (EBV) infection up-regulates the expression of recombination-activating gene (RAG). EBV-negative Burkitt lymphoma (BL)–2 cells were infected with recombinant EBV expressing green fluorescent protein and total RNA harvested at days after infection, as indicated. RAG1, RAG2, and EBV nuclear antigen 1 (EBNA1) transcripts were determined by real-time quantitative polymerase chain reaction and normalized to levels of rRNA in each reaction. +, EBV-infected BL-2 cells; −, mock-infected BL-2 cells.

To assess the significance of RAG induction in vitro to the biology of primary EBV infection, we isolated PBMCs from 26 patients with IM at the time of diagnosis and 1, 2, 6, and ⩾24 months after diagnosis. First, the virus load in each sample was determined to provide virologic confirmation of acute EBV infection, as well as an estimate of infected cell numbers per sample. Normalizing EBV DNA to the cellular gene CRP by RQ-PCR, the median number of EBV copies was 265 copies/10^5 PBMCs (range, 9–9890 copies/10^5 PBMCs) at the time of diagnosis, 245 copies/10^5 PBMCs (range, 0–18,108 copies/10^5 PBMCs) 1 month after diagnosis, and 125 copies/10^5 PBMCs (range, 0–6478 copies/10^5 PBMCs) 2 months after diagnosis (figure 2*A*). During the acute phase, all patients showed a virus load ⩾125 EBV copies/10^5 PBMCs for at least 1 sampling time point. On the basis of estimates of 5 EBV genomes per infected peripheral blood B cell [15], 125 copies/10^5 PBMCs would equate to some 25 infected cells, a number that is within the range of sensitivity of our RQ-PCR assays for RAG expression (see Patients and Methods). Should circulating lymphocytes be lytically rather than latently infected, this estimate of the number of infected cells would be exaggerated. Six months after diagnosis, the EBV load declined to 34 EBV copies/10^5 PBMCs (range, 0–7154 EBV copies/10^5 PBMCs). Follow-up samples

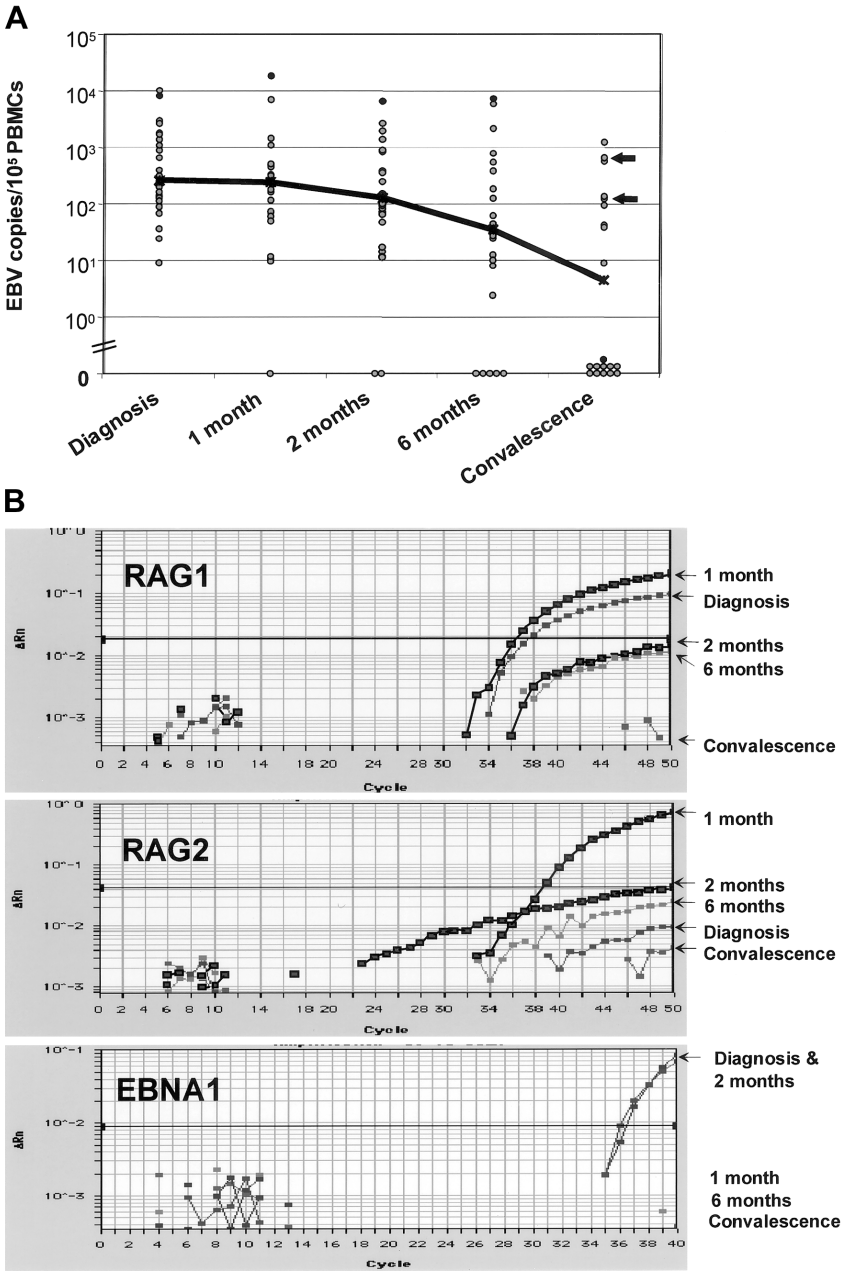

Figure 2. Recombination-activating gene (RAG) expression in peripheral blood mononuclear cells (PBMCs) during infectious mononucleosis. *A,* No. of copies of Epstein-Barr virus (EBV) DNA/10^5 PBMCs, as analyzed by real-time quantitative polymerase chain reaction (RQ-PCR) for each patient sample *(circles),* with median values plotted over time *(line). Black circles,* EBV DNA levels in a patient whose PCR amplification profile for RAG is shown in panel B. *Arrows,* EBV DNA levels in 2 convalescent samples that were also positive for RAG. *B,* RQ-PCR amplification profile for RAG1, RAG2, and EBV nuclear antigen 1 (EBNA1) RNA in PBMCs from 1 patient within 2 weeks of symptom onset (diagnosis); 1, 2, and 6 months after diagnosis; and at convalescence. *Horizontal line,* threshold setting (10 SDs above baseline) at which a sufficient no. of amplicons have accumulated to be statistically significant.

taken after 1.9–5.4 years showed a median EBV load of 4 EBV copies/10^5 PBMCs (range, 0–1211 EBV copies/10^5 PBMCs), a level that is consistent with that in healthy carriers [15].

RQ-PCR analysis of PBMCs from the patients with IM demonstrated RAG1 expression in 11 (42%) of 26 patients and RAG2 expression in 2 (8%) of 26 patients (table 1). Five patients with IM had RAG transcripts at >1 time point. RAG transcripts were generally detected early (within 2 months of diagnosis) during the course of IM, correlating with the acute phase of disease and high virus load. However, convalescent samples from 2 patients with an elevated EBV load (see figure 2*A*) also showed RAG expression. A representative amplification plot for serial samples

Table 1. Analysis of Epstein-Barr virus (EBV) load and recombination-activating gene (RAG) expression in peripheral blood mononuclear cells (PBMCs) from patients with infectious mononucleosis (IM).

		Months after diagnosis			
Patients with IM	Diagnosis	1	2	6	Convalescence
Patient 1					
Virus load	8198	18,108	6478	7154	0
RAG status	RAG1	RAG1 and RAG2	…	…	…
Patient 2					
Virus load	138	72	70	184	120
RAG status	…	…	…	RAG1	RAG2
Patient 3					
Virus load	36	161	379	61	0
RAG status	…	…	RAG1	…	…
Patient 4					
Virus load	653	50	354	383	NA
RAG status	…	…	RAG1	…	…
Patient 5					
Virus load	9890	314	+[a]	+[a]	0
RAG status	…	RAG1	…	RAG1	…
Patient 6					
Virus load	200	1454	878	182	38
RAG status	RAG1	…	RAG1	…	…
Patient 7					
Virus load	319	250	64	0	120
RAG status	RAG1	…	…	…	…
Patient 8					
Virus load	12	1078	246	44	NA
RAG status	…	RAG1	RAG1	…	…
Patient 9					
Virus load	140	273	246	24	649
RAG status	…	…	…	…	RAG1
Patient 10					
Virus load	389	10	82	2	0
RAG status	…	…	…	RAG1	…
Patient 11					
Virus load	127	+[a]	+[a]	+[a]	0
RAG status	…	RAG1	…	…	…

NOTE. Virus load is expressed as no. of copies of EBV DNA/10^5 PBMCs. NA, no sample available for analysis.

[a] EBV positive, as determined by Southern blot of polymerase chain reaction product.

from a single patient is shown in figure 2*B*. PBMCs from 5 healthy EBV carriers did not express RAG1 or RAG2.

DISCUSSION

Neither RAG1 nor RAG2 mRNA is routinely found in normal PBMCs [16]. During primary EBV infection, the acquisition of virus is followed by a 30–50-day incubation period that precedes the clinical onset of IM. Because the induction of RAG1 and RAG2 by EBV is immediate under in vitro conditions of infection (figure 1), both the length of the natural incubation period and variability in the time course of disease may have affected our ability to demonstrate either transcript in many of the patients tested. Although the concurrent expression of RAG1 and RAG2 necessary for V(D)J recombination was demonstrated in 1 patient, functional activity in the setting of acute IM is as-yet unproved. However, the detection of RAG in PBMCs during a common viral infection puts into a human context the controversial issue of the reinduction of RAG expression in mature lymphocytes, which has hitherto been addressed largely in experimental systems [10, 11, 17–19].

Our finding of RAG expression in PBMCs from 42% of patients during acute EBV infection lends itself to 3 interpretations, each of which is supported by evidence from animal model systems but is without confirmation, to date, in humans. First, it may represent an accumulation of RAG-expressing immature B cells that have entered peripheral circulation as a nonspecific response to infection [17]. Second, because total PBMCs were analyzed, our finding may reflect the reexpression

of V(D)J recombination machinery in $CD8^+$ and $CD4^+$ subsets of peripheral T lymphocytes comprising the exuberant cellular immune response that accompanies acute IM [18, 19]. Third, it may denote a reinduction of RAG in peripheral B lymphocytes as a consequence of EBV gene expression. Although further patient-based studies will be required to distinguish among these possibilities, results derived from experimental infections of B cells, together with evidence for RAG induction after transient EBNA1 transfection [8] or EBNA1 transgene expression [20], support the latter alternative.

To date, attempts to implicate the EBV induction of RAG in chromosomal translocations found in virus-associated B cell tumors have been unproductive [20, 21]. However, the findings presented here provoke an entirely different interpretation of the potential biological ramifications of RAG induction. The activation of RAG during primary EBV infection suggests its participation in the establishment of the virus carrier state. Transient RAG induction may serve to diversify the BCR, concurrent with virus-driven cell expansion. Secondary immunoglobulin gene rearrangements by EBV-induced RAG would generate, from an otherwise limited B cell repertoire, new cell progeny with BCR specificities that are sufficiently diverse to maximize survival signaling in at least some portion of the infected population. Diversification of the BCR in an expanding cell clone would also modulate the viral reactivation incidental to BCR cross-linking—this would minimize, on the one hand, the likelihood for generalized cytolytic EBV replication in memory B cells uniformly bearing a high-affinity BCR, while ensuring, on the other hand, the capacity for virus release from naive B cell targets maintaining an otherwise low-affinity receptor. This notion also integrates the biology of EBV persistence with possible mechanisms for viral pathogenesis. Editing machinery used to rescue dysfunctional BCRs may also create autoreactive B cells. Indeed, a high proportion of patients with EBV-induced IM show transient levels of autoantibodies. Whereas a strategy of receptor diversification within a host-cell lineage could diminish fortuitous viral elimination, it comes at the risk of autoreactive antibody production and possible exuberant B cell proliferation driven by self-antigen.

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