

# Acute Infection with Epstein-Barr Virus Targets and Overwhelms the Peripheral Memory B-Cell Compartment with Resting, Latently Infected Cells

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**In this paper we demonstrate that during acute infection with Epstein-Barr virus (EBV), the peripheral blood fills up with latently infected, resting memory B cells to the point where up to 50% of all the memory cells may carry EBV. Despite this massive invasion of the memory compartment, the virus remains tightly restricted to memory cells, such that, in one donor, fewer than 1 in 10<sup>4</sup> infected cells were found in the naive compartment. We conclude that, even during acute infection, EBV persistence is tightly regulated. This result confirms the prediction that during the early phase of infection, before cellular immunity is effective, there is nothing to prevent amplification of the viral cycle of infection, differentiation, and reactivation, causing the peripheral memory compartment to fill up with latently infected cells. Subsequently, there is a rapid decline in infected cells for the first few weeks that approximates the decay in the cytotoxic-T-cell responses to viral replicative antigens. This phase is followed by a slower decline that, even by 1 year, had not reached a steady state. Therefore, EBV may approach but never reach a stable equilibrium.**

The B-lymphotropic herpesvirus Epstein-Barr virus (EBV) is a ubiquitous human virus (reviewed in references 23 and 45) that establishes a lifelong persistent infection in memory B lymphocytes (3). It is an important pathogen because of its association with several human neoplasias (reviewed in references 38 and 45). However, no good animal model exists for the study of EBV. The murine gammaherpesvirus 68 (MHV68) is a related virus which has tropism for B lymphocytes (44) and also persists in memory B cells (12, 50). However, it appears to lack the specific latency transcription programs of EBV and persistence is not dependent on B lymphocytes (49). Primate homologues of EBV are known (7, 11, 42), but these are not useful because of financial constraints and the lack of virological and immunological reagents for these systems. Despite this limitation, EBV has emerged as an excellent system for studying persistent infection in humans. There are two primary reasons for this. First, EBV infects resting B cells in culture and transforms them into continuously proliferating, latently infected lymphoblasts (35, 36). This provides a readily manipulable *in vitro* system for studying the functions of the latency-associated proteins. Second, the major sites of viral persistence, the peripheral blood and Waldeyer's ring (29), are relatively accessible for study. Taking together information from both *in vitro* and *in vivo* studies we have developed a unified model of how EBV establishes and maintains a persistent infection (46) (Fig. 1). The key underlying theme of the model is that EBV uses its latent proteins to provide signals to the infected B cell that cause it to become activated and then differentiate, through a mechanism analo-

gous to the germinal center reaction (30, 31), into a resting memory cell. We have provided evidence that these events occur in the lymphoid tissue of Waldeyer's ring (4). The cells then enter the peripheral circulation. As a consequence, EBV in the blood is tightly restricted to latently infected (9), resting (32), memory B cells (3, 19). These cells circulate back to Waldeyer's ring (29), where occasionally they differentiate into plasma cells and release infectious virus (L. L. Laichalk and D. A. Thorley-Lawson, submitted for publication). The striking feature of the model is that the virus uses virtually every aspect of normal mature B-cell biology to establish (activation and differentiation) and maintain (long-term memory) persistent latent infection and then be released (terminal differentiation).

The activated proliferating blasts produced when EBV first infects B cells could potentially be pathogenic if their proliferation *in vivo* was unchecked. One tenet of the model is that the virus must produce these activated blasts to allow the latently infected cell to differentiate into the memory compartment. It follows that these cells will not be pathogenic because they are only produced in the lymph nodes, are transient, and only enter the peripheral circulation by becoming resting memory cells. Support for these ideas came from studies in allograft patients where immunosuppression causes the levels of infected cells to increase, on average, 50-fold, but the virus remains restricted to resting memory cells in the blood (2). These patients are at risk for developing EBV-positive lymphoma (17); however, most patients do not develop lymphoma despite the large numbers of infected cells present. This suggests that EBV is predisposing rather than causative in the development of these lymphomas and that the oncogenic event is rare. Consequently, we concluded that the primary effect of the immune response is not to change or regulate the forms of infection that occur in the blood and Waldeyer's ring but

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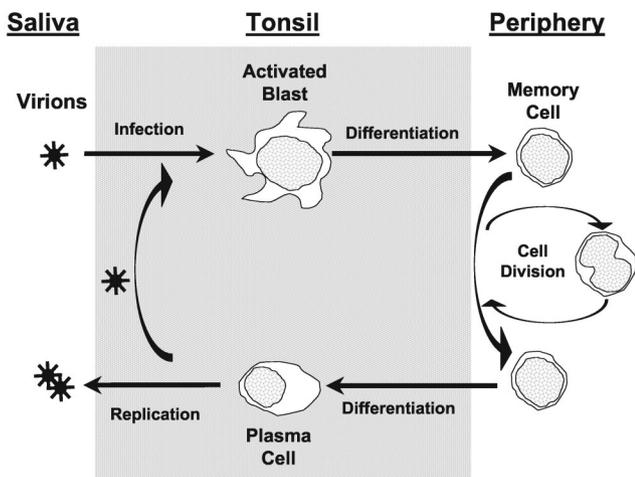


FIG. 1. Schematic drawing of a model for EBV persistence. Virions enter the mucosal surface of the nasopharynx through saliva. The virus enters the lymphoid tissue of Waldeyer's ring, where it infects resting naive B cells and drives them to become proliferating lymphoblasts through expression of nine latent proteins (the growth program) (46). These blasts can then differentiate into resting memory cells by a process analogous to the germinal center reaction by using the default program (46). Once in resting memory cells, all viral protein expression ceases (the latency program) (16, 46). Latently infected memory cells circulate in the periphery and return to the lymphoid tissue, where at some unknown rate, they differentiate into plasma cells and release infectious virus to initiate a new round of infection (Laichalk and Thorley-Lawson, submitted). Each stage of the cycle is subject to immunosurveillance (CTL against infected cells and antibody against virions), with the exception of the memory cells, which are invisible because they express no viral proteins (16). In the absence of an immune response, the cycle will amplify until the memory B compartment is filled with latently infected cells. Once the immune response is activated, the cycle is dramatically reduced or perhaps even completely blocked and the reservoir of latently infected memory cells decays.

rather to reduce the overall load of infection. As a result, infected memory cells are found in the blood of healthy carriers at extremely low levels, around 1 in 10<sup>4</sup> to 1 in 10<sup>6</sup> infected cells (3).

One criticism that has been directed at this model is that, in the absence of an immune response, there is nothing to stop the cycles of infection, differentiation, and reinfection from spinning ever faster, causing the memory compartment to fill up with latently infected cells. This should be the case for newly infected individuals. Primary infection of adults and adolescents with EBV frequently gives rise to acute infectious mononucleosis (AIM), a self-limiting lymphoproliferative disease (34). The infected cells are reported to be lymphoblasts (39, 47), although a contradictory study reported that they were small lymphocytes (8). Viral replication has also been reported (38). This implies that unregulated virus production, infection, and transformation are occurring in the blood of AIM patients. This is a very different situation from long-term persistent infection. In the present study, we set out to identify the types of cell harboring EBV in AIM and to describe how the reportedly unregulated acute condition resolves into tightly regulated persistent infection. We found that the memory compartment does indeed fill up with latently infected cells to the point where up to 50% of all memory B cells in the blood can carry

the virus. Despite this phenomenal invasion and contrary to most earlier reports, the specificity remains. The infection is tightly regulated, the majority of the infected cells are resting, not lymphoblastoid, and no significant viral replication or infection of the naive B-cell population is detected.

**MATERIALS AND METHODS**

**Cell lines and primary cells.** The EBV-negative cell line BJAB (gift of Elliot Keiff) was used as a negative control. The lymphoblastoid cell line IB4 (gift of Elliot Keiff) was used as a positive control for W repeat DNA PCR. The B958 EBV-positive marmoset lymphoblastoid cell line was used as a positive control for expression of the lytic cycle gene BZLF1. All cell lines were cultured at 37°C with 5% CO<sub>2</sub> in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM sodium pyruvate, 2 mM glutamine, and 100 IU of penicillin-streptomycin.

Adolescents (ages 17 to 24 years) presenting to the clinic at the University of Massachusetts at Amherst Student Health Service (Amherst) with clinical symptoms consistent with AIM were recruited for this study. Following the obtainment of informed consent, blood was collected at presentation with symptoms and at 3 weeks, 6 weeks, 6 months, and 1 year following presentation. Diagnosis at the time of presentation to the clinic required a positive monospot test and the presence of atypical lymphocytes (15). Confirmation of primary EBV infection required the detection of immunoglobulin M (IgM) antibodies to the EBV viral capsid antigen in patient sera (14). These studies were approved by the Human Studies Committee at the University of Massachusetts Medical School (Worcester). All blood samples were diluted 1:1 in 1× phosphate-buffered saline (PBS).

Tonsillar cells were provided by Massachusetts General Hospital. Specimens were obtained from otherwise healthy individuals with obstructive problems. The tonsils were minced thoroughly with forceps and scissors, and connective tissue was removed by filtering through a silk screen. Cells were then resuspended in 480 ml of 1× PBS-0.5% bovine serum albumin (PBSA).

Cell suspensions prepared as described above were layered in 30-ml aliquots onto 20 ml of Ficoll-Hypaque (Amersham-Pharmacia) in 50-ml conical tubes. Samples were then centrifuged for 30 min at 2,000 rpm at 25°C. Buffy coat cells were removed from the interface, washed twice with PBSA, counted, and resuspended to the desired concentration.

**Cell separations.** Mononuclear cells, prepared as described above, were resuspended to a concentration of 2 × 10<sup>7</sup> cells per ml and stained for 30 min at 4°C with antibodies to the desired cell surface marker(s). The antibodies and dilutions used were as follows: anti-CD20 fluorescein isothiocyanate (FITC) (DAKO), 1:200; anti-CD5 phycoerythrin (PE) (Pharmingen), 1:200; anti-CD20 Cy5 (DAKO), 1:100; anti-CD27 PE (Pharmingen), 1:100; anti-IgD PE (Southern Biotech), 1:2,000; anti-IgG, -IgA, and -IgM FITC (Jackson), 1:125. After staining, cells were washed twice with PBSA and resuspended to a concentration of 10<sup>8</sup> cells/ml. Cell sorting was performed on a Cytomation MoFlo fluorescence-activated cell sorter (FACS).

Negative selection for purifying B cells was performed by using the Stem Cell Technologies Stem Sep system as described by the manufacturer. Briefly, peripheral blood mononuclear cells (PBMCs) were resuspended to a concentration of 5 × 10<sup>7</sup> cells/ml and stained with 100 µl of antibody cocktail for 30 min on ice. Antibody cocktails contain antibodies directed against all types of PBMCs except the population of interest, in this case B cells (Stem Cell Technologies). Cells were then stained with 60 µl of magnetic colloid (Stem Cell Technologies) for 30 min on ice. The sample was then passed over a column in the presence of a magnet. The population of interest was collected as the flowthrough fraction. Purified populations were analyzed for purity on a Becton Dickinson FACSCalibur. The population purity was always greater than 90% and often greater than 95%.

**Cell cycle analysis.** Cells were resuspended to a concentration of 10<sup>6</sup> cells/ml. Hoechst 33342 (Sigma) was added to a final concentration of 10 µg/ml and incubated for 30 min at 37°C in the dark. Samples were washed once in PBSA, followed by costaining with anti-CD20 FITC for 30 min at 4°C. Cells were washed once in PBSA and analyzed on the MoFlo FACS (Cytomation) within 1 h of staining. Cells with a 2 N DNA content were collected as G<sub>0</sub>/G<sub>1</sub> cells, and cells with a >2 N DNA content were collected as S/G<sub>2</sub>/M cells.

**Limiting dilution analysis.** Limiting dilution analysis was used to determine the frequency of EBV-infected cells for each AIM patient. The details of this assay with DNA PCR have been published previously (3). It can detect the presence of a single EBV genome in a background of as many as 10<sup>6</sup> EBV-negative cells (33). PCR conditions were as follows: 5 µl of sample DNA, 0.2 mM concentrations of deoxynucleoside triphosphates (dNTPs), 20 pM concentrations of each primer, 2.0 mM MgCl<sub>2</sub>, 50 mM KCl, 10 mM Tris-HCl, and 1 U of *Taq*

(Applied Biosystems) in a total volume of 50  $\mu$ l. The reaction was performed in a GeneAmp 9600 thermocycler for 35 cycles of 95°C for 15 s and 66°C for 1 min followed by 1 cycle of 72°C for 5 min. Products were visualized by Southern blotting, and the frequency was determined by Poisson statistics (33). Phenol-chloroform-extracted DNA from 10<sup>5</sup> IB4 cells was used as a positive control for the W repeat PCR.

Virus-infected cells were also quantitated by testing for expression of EBER 1 RNA by real-time PCR. The same dilution series were set up in 0.2-ml PCR tubes (MicroAmp) arrayed in a 96-well format. Eight replicates of 2, 5, 10, and 20 single memory cells (CD20<sup>+</sup>, IgD<sup>-</sup>) were sorted into 10  $\mu$ l of 1 $\times$  first-strand buffer (Invitrogen) and immediately frozen on dry ice. Single-cell cDNA synthesis was performed according to the protocol of Wang and Stollar (48) by substituting 5 pmol of EBER1-specific primer (AGGACCTACGCTGCCCTAGA) (10) for the Ig constant region-specific primers. EBER1 *TaqMan* PCR was performed in a volume of 25  $\mu$ l on the ABI PRISM 5700 sequence detection system (Perkin Elmer). For each run, a master mix was prepared containing 12.5  $\mu$ l of Universal master mix (PE Applied Biosystems), primers (450 nM) and fluorogenic probe (125 nM). The primers used were ACCGAAGACGGCAGA AAGC and CCTACGCTGCCCTAGAGGTTT, and the probe was 6-carboxy-fluorescein-ACAGACACCGTCTCACCACCCG-6-carboxymethylrhodamine. To each well of an optical 96-well plate (PE Applied Biosystems), 5  $\mu$ l of cDNA and 20  $\mu$ l of master mix were added. Each well was capped with an optical cap (PE Applied Biosystems). Thermal cycling was initiated with an incubation step at 50°C for 2 min, followed by a first denaturation step at 95°C for 5 min, and continued with 3 cycles of preamplification at 95°C for 15 s and 55°C for 1 min, followed by 60 cycles of amplification at 95°C for 15 s and 60°C for 1 min.

**RT-PCR.** Serial dilutions of isolated cell populations were prepared and aliquoted into Eppendorf tubes. EBV-negative tonsillar cells were added, when needed, to each tube to bring the total number of cells to 5  $\times$  10<sup>6</sup>. EBV IB4 cells (10<sup>6</sup>) were used as a positive control. RNA was isolated by the Trizol method (Invitrogen) as detailed previously (18). The entire RNA sample was used for cDNA synthesis. Random primers (250 ng; Invitrogen) were added to each sample and incubated for 8 min at 68°C. This was followed by a 2-min incubation at -20°C. Samples were quickly centrifuged to collect the sample to the bottom of the tube. First-strand master mix (7  $\mu$ l) was then added to each tube, mixed gently, and incubated for 10 min at room temperature. The first-strand master mix for one reaction mixture consists of 4  $\mu$ l of 5 $\times$  first-strand buffer (375 mM KCl, 250 mM Tris [pH 8.4], 15 mM MgCl<sub>2</sub>), 2  $\mu$ l of dithiothreitol, and 1  $\mu$ l of 10 mM dNTPs. Superscript II reverse transcriptase (1  $\mu$ l; Invitrogen) was then added to each tube, and the tubes were incubated for 10 min at room temperature. Reverse transcription (RT) was carried out at 42°C for 50 min, followed by inactivation of the enzyme at 68°C for 15 min. The cDNA was then brought to a final volume of 200  $\mu$ l with water and used for PCR. PCR was performed on 1/10 of the cDNA (20  $\mu$ l). The primers used were as follows (47): BZLF1, TTCCACAGCCTGCACCAGTG and GGCAGCAGCCACCT CACGGT; EBER1, AAAACATGCGGACCACCAGC and AGGACCTAC GCTGCCCTAGA. Reactions were performed in a GeneAmp 9600 thermocycler in a 50- $\mu$ l final volume of 50 mM KCl, 20 mM Tris (pH 8.4), 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, and 20 pM concentrations of each primer under the following conditions: 40 cycles of 95°C for 15 s, annealing temperature for 30 s, 72°C for 30 s, and a final extension at 72°C for 5 min. Products were visualized by Southern blotting, and frequencies were determined by use of Poisson statistics (33).

## RESULTS

**EBV infection in blood of AIM patients is restricted to memory B cells.** The cardinal feature of persistent infection with EBV is the strict tropism of the virus for resting memory B cells in the peripheral blood. This property defines the highly regulated nature of EBV infection in vivo. To test whether the same regulation holds in AIM or whether the infection is unregulated, we analyzed peripheral B lymphocytes from AIM patients. Memory and naive populations of B cells were separated based either on CD27 or IgD expression. CD27 expression is a specific marker for memory B cells (25). Lack of surface IgD is a marker for isotype-switched memory B cells but does not include the small IgD<sup>+</sup> memory subset (25). However, lack of IgD is a very specific marker for B cells la-

tently infected with EBV because the virus is excluded from the IgD<sup>+</sup> memory subset in the blood of healthy carriers (3, 19).

B cells were identified based on expression of the pan-B-cell marker CD20 and then separated into CD27-positive and -negative fractions by FACS. Figure 2 shows an example of such an experiment. FACS reanalysis of whole PBMCs and the separated CD20<sup>+</sup> CD27<sup>+</sup> and CD20<sup>+</sup> CD27<sup>-</sup> fractions are shown at the top of the figure. In the lower panels are shown representative dilutions from the limiting dilution DNA PCR assay. In this assay, the cells were serially diluted and then multiple replicates of each dilution were tested for the presence of the virus by a DNA PCR assay that can detect a single copy of the viral genome (22). Poisson statistics were then used to calculate the absolute frequency of infected cells. It is apparent from this experiment that the virus is highly enriched in the memory compartment. The frequency of infected naive B cells is <1% of that found in the memory cells. The same result was obtained when IgD was used to fractionate the memory cells (Fig. 3). In this experiment only ~0.01% of the infected cells reside in the IgD<sup>+</sup> compartment. This is the highest level of specificity for the memory compartment that we have ever been able to measure.

A summary of the results from two separate patients studied with CD27 and four patients studied with IgD are presented in Table 1. These results confirm that in AIM the virus remains tightly restricted to the memory cell compartment.

**The phenotype of latently infected cells in blood of AIM patients is the same as in healthy carriers.** The analysis in the previous section shows that infection in the blood of AIM patients is not unregulated but it is tightly restricted to memory B cells. To confirm and substantiate this conclusion, we performed further cell surface characterization of the infected cells. It was shown previously that the cell surface phenotype of latently infected cells in the blood of healthy carriers is surface Ig<sup>+</sup> (sIg<sup>+</sup>), and CD5<sup>-</sup> (3, 19). When these markers were used to fractionate B cells from the blood of AIM patients, we found the same phenotype. The results of studies on two separate patients are shown for both markers in Table 1. The presence of sIg confirms that the latently infected B cells are bona fide B cells and not aberrant cells that are IgD<sup>-</sup> because they lack sIg. The lack of CD5 indicates that the virus is excluded from the B1 subset. This is a subset of B cells that, like memory cells, are long lived but they have not been through a germinal center reaction (20, 51).

In conclusion, EBV infection in the blood of AIM patients is not chaotic but tightly regulated. Just as with persistent infection in healthy carriers, the phenotype of the latently infected cells is restricted to IgD<sup>-</sup>, sIg<sup>+</sup>, CD27<sup>+</sup>, CD5<sup>-</sup>, and CD20<sup>+</sup>.

**EBV-infected cells proliferate with the bulk population of B cells.** One characteristic of latently infected cells in the blood of healthy carriers is that they are in a resting state. This contrasts with lymphoblastoid cells expressing the growth program in culture which are aggressively proliferating. To test whether the latently infected memory cells in AIM blood were also resting, we checked their cell cycle distribution. Figure 4A shows FACS analysis of an EBV-transformed lymphoblastoid B-cell line, and Fig. 4B shows purified B cells from the blood of an AIM patient. Typically, approximately half of the cell line cells are in S/G<sub>2</sub>/M, whereas only a very small number (2.9% in this case) of the AIM blood B cells are in this fraction. We

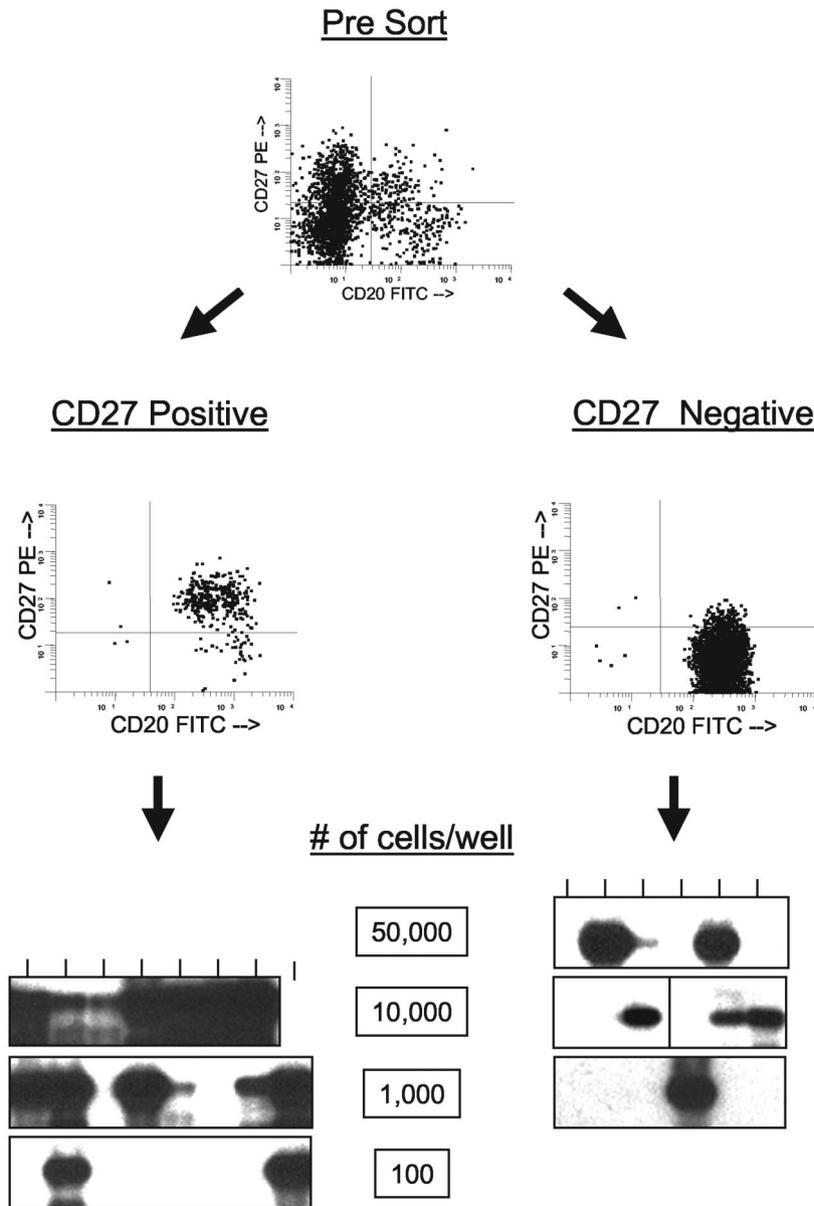


FIG. 2. EBV-infected cells in the periphery of AIM patients are CD27 positive. PBMCs were stained for expression of CD20, a pan-B-cell marker, and CD27, a memory B-cell marker (upper panel). The naive and memory B cells were then sorted by FACS and reanalyzed for purity (middle panels). The purified populations were then tested for the presence of the virus by limiting dilution DNA PCR. In this technique, serial dilutions of each population are prepared and then multiple aliquots of each dilution are tested for the virus by DNA PCR. The PCR products are separated on a gel and detected by Southern blotting.

sorted the AIM B cells into the  $G_0/G_1$  and  $S/G_2/M$  populations and estimated the frequency of infected cells in each by limiting dilution DNA PCR (Fig. 4C). Since >90% of the B cells in the blood of the AIM patient are resting, we would expect to see a dramatic enrichment of EBV-infected cells into the  $S/G_2/M$  fraction if the EBV-infected cells were proliferating. The results of such measurements for three patients are summarized in Table 2. It is apparent that the EBV-infected cells do not have the same cell cycle distribution as the cell line but closely resemble the bulk population of B cells: 3.4 to 10% of the infected cells and 2.2 to 9% of the bulk B cells are in  $S/G_2/M$ . These numbers are similar to what have been reported for

healthy carriers (32) but are not consistent with previous claims that the infected cells in AIM blood are transformed, proliferating lymphoblasts (39, 40, 47). We conclude that the latently infected B cells in the peripheral blood of AIM patients are resting cells that divide with the bulk B cells presumably as part of the homeostatic regulation of B-cell levels.

**Viral replication does not occur in blood of AIM patients.** Previous studies have claimed (37), and it is generally accepted, that EBV replication is ongoing in the blood of AIM patients (38). Because, in vitro, EBV infects naive and memory B cells indiscriminately, the production of infectious virus in the blood would lead to promiscuous infection and prolifera-

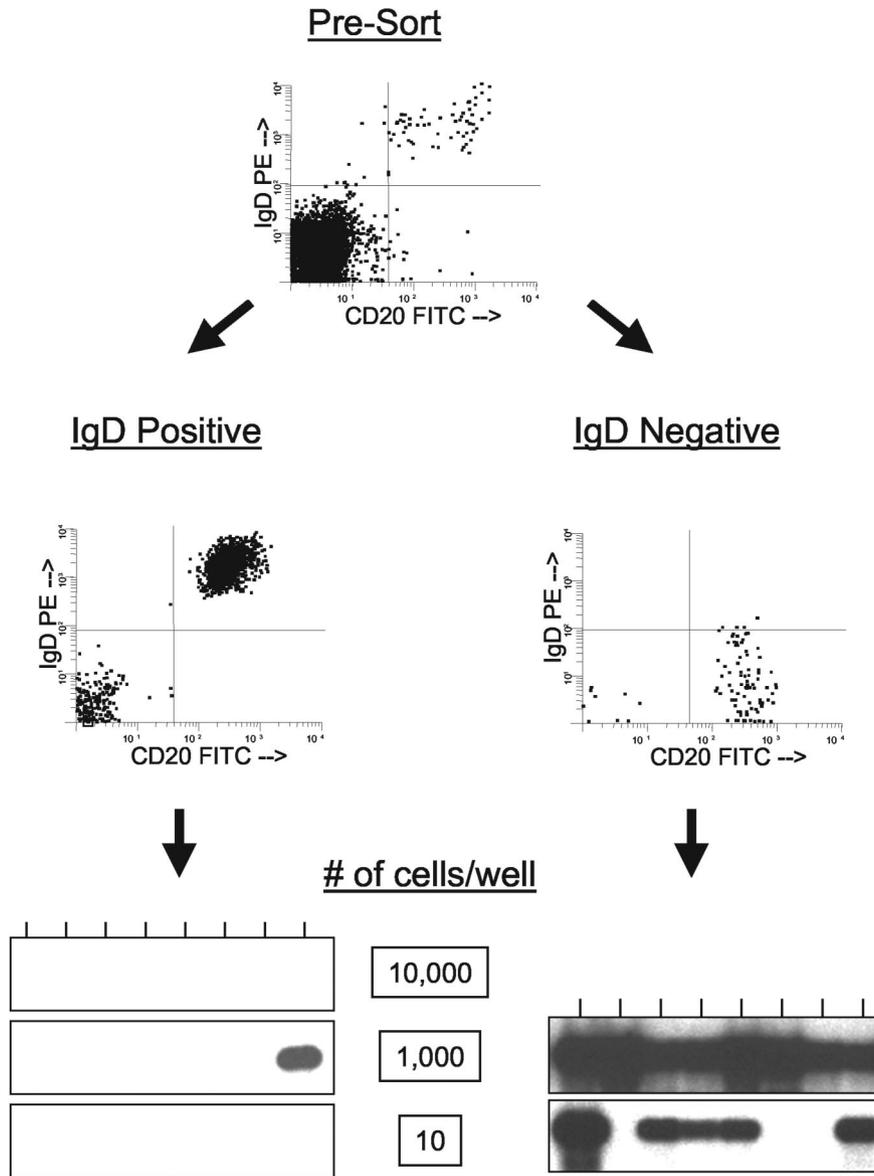


FIG. 3. EBV-infected cells in the periphery of AIM patients are IgD negative. PBMCs were stained for expression of CD20, a pan-B-cell marker, and IgD (upper panel). The naive (IgD positive) and memory (IgD negative) B cells were then sorted by FACS and reanalyzed for purity (middle panels). The purified populations were then tested for the presence of the virus by limiting dilution DNA PCR. In this technique, serial dilutions of each population are prepared and then multiple aliquots of each dilution are tested for the virus by DNA PCR. The PCR products are separated on a gel and detected by Southern blotting.

tion of B cells. This would be inconsistent with our conclusions above that the virus is restricted to resting memory B cells. Previous researchers have identified viral replication by performing RT-PCR analysis on bulk populations of cells; however, these studies may be misleading because they do not estimate the actual number of cells replicating the virus. As shown below, the levels of infected cells in AIM are very high; therefore, even if viral replication was an extremely rare event, it may be picked up by very sensitive RT-PCR techniques. To check this possibility, we quantitated the exact number of infected cells expressing the viral BZLF1 gene in the peripheral blood B cells from AIM patients. BZLF1 is an immediate-early gene that initiates the cascade of viral gene expression that ultimately leads to the production of infectious virus (13). As a

TABLE 1. Phenotype of EBV-infected cells in blood of AIM patients

Patient no.	Marker	Frequency/10 <sup>5</sup> cells <sup>a</sup>	
		Enriched	Depleted
1	CD27	250	5.0
2	CD27	170	2.0
3	IgD	7.4	2,500
4	IgD	15	1,400
5	IgD	120	12,000
6	IgD	1.1	9,800
7	Ig	51	<0.7
8	Ig	4,500	170
9	CD5	1.1	220
10	CD5	100	770

<sup>a</sup> Enriched, marker-positive population; Depleted, marker-negative population.

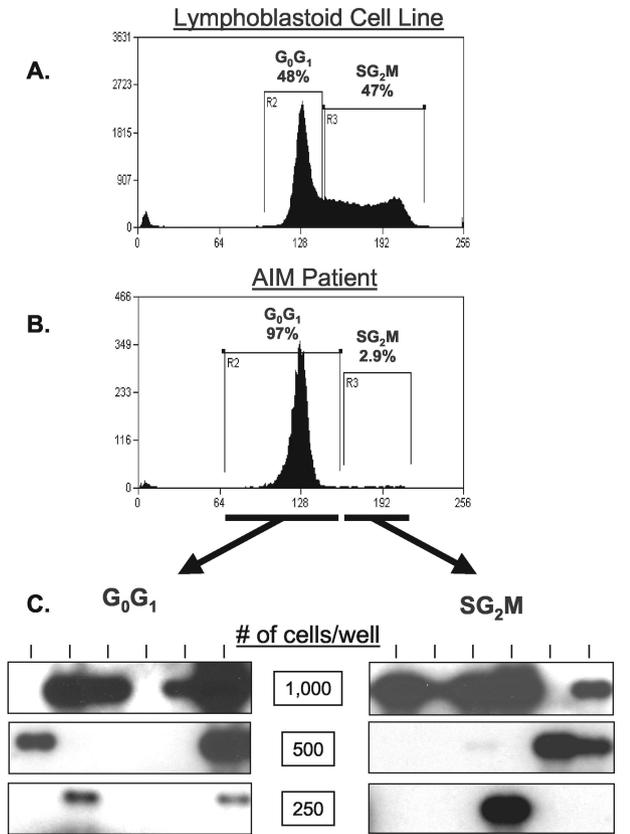


FIG. 4. Cell cycle stage of EBV-infected cells in the blood of AIM patients. B cells from an EBV-transformed cell line (A) or from the blood of AIM patients (B) were stained with the vital DNA dye Hoechst 33342. The AIM B cells were then separated into the G<sub>0</sub>/G<sub>1</sub> and S/G<sub>2</sub>/M fractions by FACS, and the frequency of infected cells in each population was measured by limiting dilution DNA PCR (C).

positive control we also tested for EBER1 expression (1). EBER1 is a small untranslated RNA that is believed to be widely expressed in infected cells. Serial dilutions of B cells were aliquoted into multiple replicates and then tested for BZLF1 and EBER1 expression by RT-PCR analysis that can

TABLE 2. Cell cycle distribution of latently infected cells

Patient no.	Cell cycle stage	Frequency of infected cells/10 <sup>6</sup> cells	% of B cells	% of infected B cells <sup>a</sup>
1	G <sub>0</sub> /G <sub>1</sub>	14,000	97	95
	S/G <sub>2</sub> /M	26,000	2.8	5
2	G <sub>0</sub> /G <sub>1</sub>	900	98	97
	S/G <sub>2</sub> /M	1,400	2.2	3.4
3	G <sub>0</sub> /G <sub>1</sub>	14,000	91	90
	S/G <sub>2</sub> /M	16,000	9	10

<sup>a</sup> The percentage of infected B cells was calculated from the data in columns 3 and 4, e.g., for patient 1, for every 10<sup>6</sup> B cells; 97% are in G<sub>0</sub>/G<sub>1</sub> and the frequency of infected cells is 14,000/10<sup>6</sup> cells. This is equal to 0.97 × 10<sup>6</sup> G<sub>0</sub>/G<sub>1</sub> B cells with 13,800 infected cells, and 2.8% are in S/G<sub>2</sub>/M and the frequency of infected cells is 26,000/10<sup>6</sup> cells. This is equal to 0.028 × 10<sup>6</sup> S/G<sub>2</sub>/M B cells with 730 infected cells. The total number of infected cells is 13,800 plus 730, which equals 14,530 cells. The percentage of cells in G<sub>0</sub>/G<sub>1</sub> is 13,800 × 100/14,530, which equals 95%, and the percentage of cells in S/G<sub>2</sub>/M is 730 × 100/14,530, which equals 5%.

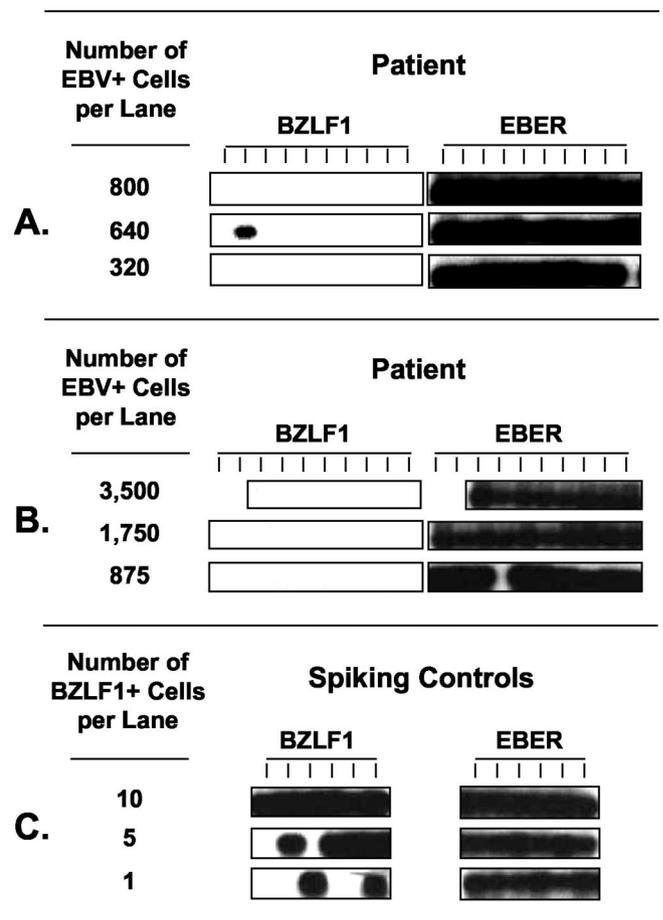


FIG. 5. B cells, replicating EBV, are rare in the blood of AIM patients. PBMCs (A) or purified B cells (B) were isolated, and the frequency of virus-infected cells expressing the BZLF1 or EBER1 genes was measured by performing a limiting dilution RT-PCR assay. Serial dilutions of cells were prepared, and multiple samples of each dilution were tested for expression of each RNA by RT-PCR. BZLF1 is the gene that initiates viral replication, and EBER1 is believed to be widely expressed in EBV-infected cells. The number of infected cells tested was predetermined by limiting dilution DNA PCR. (C) A control experiment with an EBV-positive cell line demonstrating that the technique can detect a single infected cell expressing BZLF1.

readily detect a single cell expressing either gene (Fig. 5C). In parallel, the dilutions were also tested for the presence of viral DNA to measure the frequency of virus-infected cells. This allowed the RT-PCR data to be expressed in terms of the number of infected cells expressing BZLF1. The RT-PCR for two AIM patients are shown (Fig. 5A and B), and a summary of the results is given in Table 3 along with data from three

TABLE 3. Percentage of EBV-infected cells in blood of AIM patients replicating EBV

Patient no.	% Memory B cells infected	% Infected cells expressing BZLF1
1	17	0.003
2	5	0.021
3	4.8	<0.001
4	0.27	<0.01
5	0.13	<0.01

TABLE 4. Frequency of infected memory cells and total B cells in blood of AIM patients

Patient no.	% of memory B cells	% of total B cells
1	46	7.6
2	36	6.2
3	25	4.0
4	17	3.7
5	12	2.4
6	9.1	2.4
7	5.6	2.1
8	4.8	1.3
9	3.6	1.2
10	3.3	0.83
11	2.5	0.63
12	1.4	0.43
13	1.3	0.33
14	0.83	0.15
15	0.56	0.078
16	0.25	0.070
17	0.22	0.053
18	0.18	0.032
19	0.10	0.026
20	0.06	0.007

other patients. Consistent with previous studies, it was possible to detect cells expressing BZLF1 in the five patients, but the frequency was extremely low,  $\leq 2$  in 10,000 infected cells (Table 3). Therefore, viral replication is extremely rare in the blood of AIM patients, and essentially all of the cells are latently infected memory cells. This is consistent with the observation that the virus-infected cells are restricted to a single subset, the memory cells, and is in agreement with our model that latently infected cells in the blood are not derived through direct infection but through differentiation of latently infected cells in the tonsils.

**EBV-infected memory B cells overwhelm the peripheral memory compartment during acute infection.** From the exper-

iments described in the previous sections, it was apparent that the levels of virus-infected cells are extremely high in acute AIM patients. This was consistent with previous studies that have reported up to 20% of B cells infected based on immunofluorescence detection of viral nuclear antigens (21, 24, 39–41). However, in a recent study, employing immunofluorescence and quantitative RT-PCR, we were unable to confirm these high numbers of cells expressing nuclear antigens. The previous studies used a nonquantitative technique that employs low-titer human sera and complement-mediated amplification of the signal. This makes the assay prone to false-positive (nonspecific staining) and false-negative (low sensitivity of the assays and/or failure of the infected cells to express the antigen) artifacts. To measure the actual number of infected cells present in AIM blood, we used our quantitative limiting dilution DNA PCR technique that detects infected cells solely on the basis of the presence of one or more viral genomes in the cell and is independent of viral gene expression. The results from 20 patients are summarized in Table 4. It is apparent that the range in levels of infected memory B cells (0.1 to 46%) is wide and that the absolute numbers can be extremely high. One-quarter of the patients had  $\geq 10\%$  and two-thirds of the patients had  $\geq 1\%$  of their peripheral memory B cells infected. This is much higher than previously described for healthy carriers or immunosuppressed organ transplant patients (Fig. 6). One concern we had was that the DNA PCR was detecting viral fragments attached to the B cells rather than truly infected cells. This is unlikely because it was previously shown that it is not true for healthy carriers (33) and we would not expect the fragments to stick specifically to memory cells. Nevertheless, to eliminate this concern we also used a newly developed limiting dilution analysis that depends on detecting EBER1 RNA (1) with similar results. Examples for high-frequency patients with either DNA PCR or EBER1 RT-PCR are shown in Fig. 7.

For some of the AIM patients, we were able to measure the

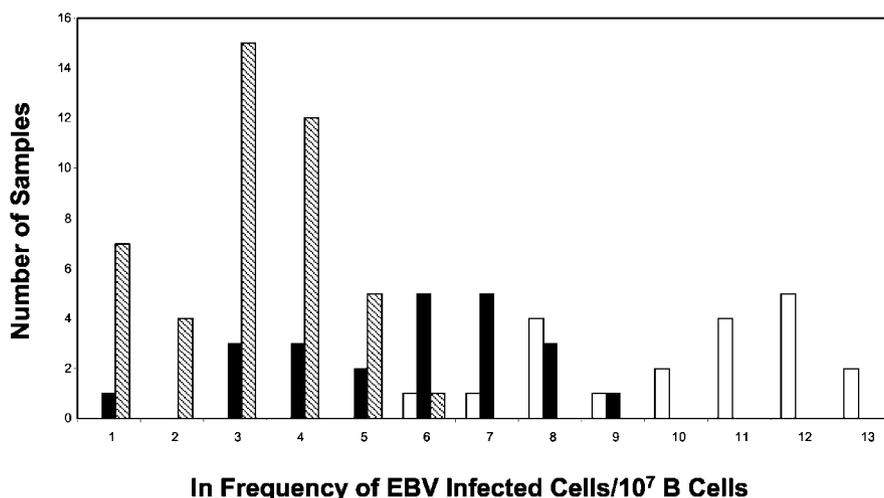


FIG. 6. Comparison of frequencies of EBV-infected B cells in blood of AIM patients, immunosuppressed allograft patients, and healthy carriers. The AIM data ( $n = 20$ ) (open bars) are from the patients listed in Table 4, the data from the immunosuppressed allograft patients ( $n = 25$ ) (filled bars) have been published previously (2). The healthy carriers ( $n = 46$ ) (hatched bar) are a new cohort, but similar data have been published previously by our laboratory (22, 29, 33) and are not detailed here. The frequencies of infected cells in this figure are expressed as the natural logs of the number of infected cells in  $10^7$  total B cells, not memory B cells.

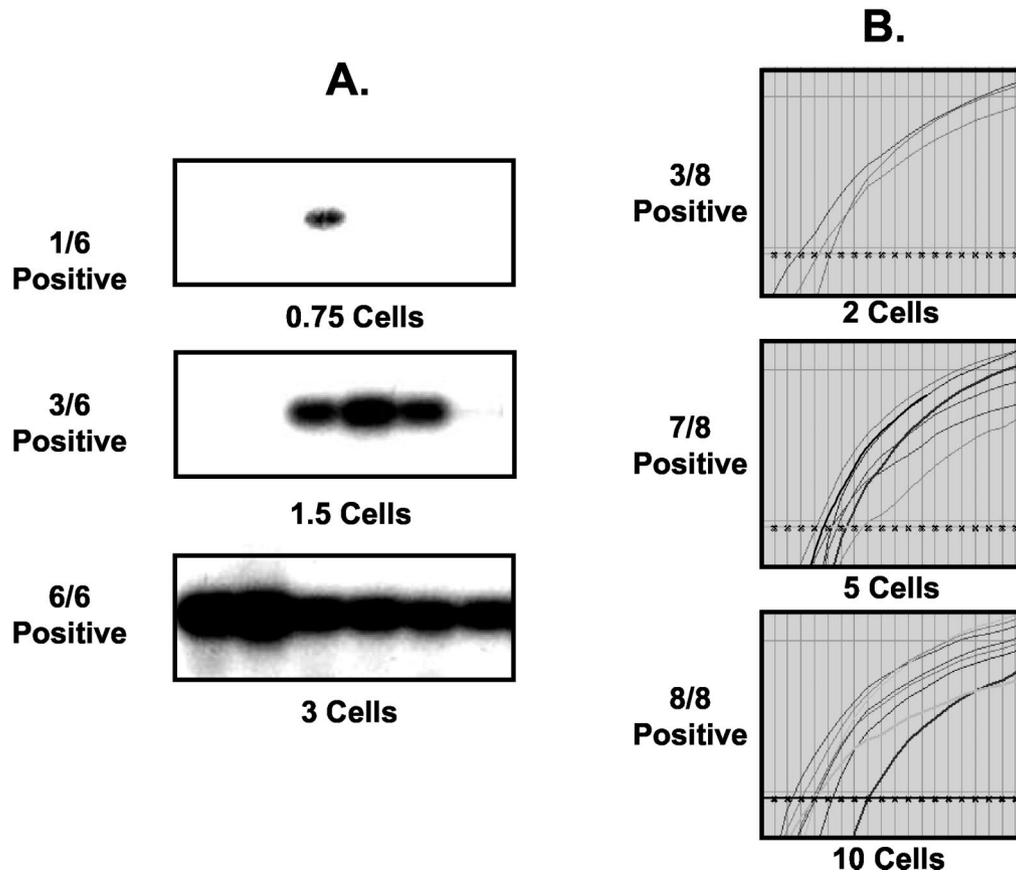


FIG. 7. Detection of high levels of EBV-infected memory B cells in blood of AIM patients. IgD-negative memory B cells were isolated as described in the legend to Fig. 3. Serial dilutions of the cells were then prepared (e.g., 0.75 means that, on average, 7.5 of 10 replicates will have 1 cell), and multiple replicates of each dilution were tested for the presence of EBV either by DNA PCR and Southern blotting for the viral DNA (A) (patient 1 in Table 4) or by real-time PCR for EBER1 RNA (B) (patient 2 in Table 4).

frequency of infected memory cells at several time points over the course of 1 year. The results are presented in Fig. 8. The time course is characterized by a very rapid decline for the first few weeks followed by a much slower decline that extends out for at least 1 year. Several features of this study are striking. First, we never saw an increase in the level of infected cells when the patients returned for a second visit, usually 1 to 2 weeks later. This suggests that, at the time of presentation in the clinic, the levels of infected memory cells are already falling. Second, by 1 year postinfection, the levels of infected memory cells are within or approaching the range seen for healthy carriers but are still significantly higher. The mean number of infected cells per  $10^7$  memory B cells was 1,140 for the 1-year AIM patients ( $n = 4$ ) and 430 for the healthy carriers ( $n = 34$ ). Although the population of AIM patients is small, the differences are nevertheless significant by Student's  $t$  test ( $P = 0.048$ ). This might imply that acute infection associated with AIM results in persistently higher levels of infected cells than subclinical infection. Alternatively, it may reflect that the virus infection has not reached a steady state. This is evident from the third feature, which is that, even by 1 year postinfection, the levels of infected cells are still decreasing.

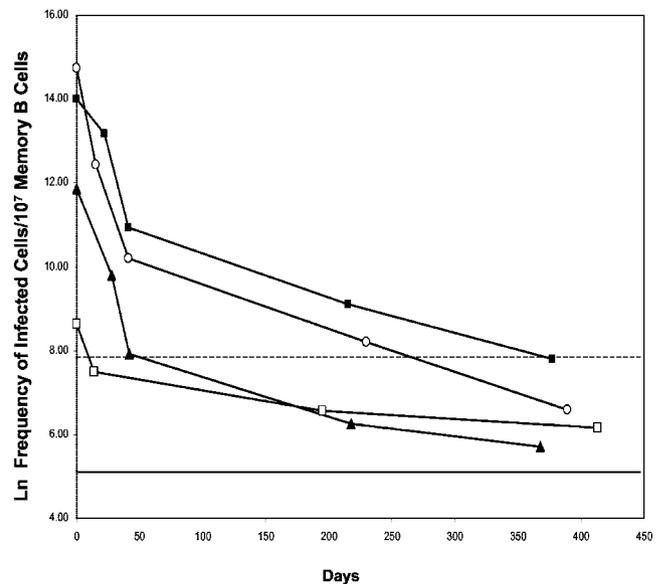


FIG. 8. Time course of resolution of EBV infection in blood of AIM patients. The frequencies of infected cells are expressed as the natural logs of the number of infected cells per  $10^7$  memory B cells. The dashed horizontal line represents the upper limit, and unbroken line represents the mean of the number of infected memory B cells detected in healthy carriers.

## DISCUSSION

In this study we demonstrated that during acute infection with EBV the peripheral blood fills up with latently infected, resting memory B cells to the point where up to 50% of all of the peripheral memory cells may carry EBV. At this time, the number of virus-infected cells is already falling, suggesting that the levels of infected cells may peak at even higher percentages than those reported here. Despite this massive level of infection, the virus maintains a highly stringent regulation, such that it remains restricted to resting memory B cells.

These experiments were carried out to test a prediction of our model that EBV persistence is a circle of latent infection, activation, and differentiation into memory cells, the major site of long-term persistence, followed by sporadic terminal differentiation and release of infectious virus to initiate a new round of infection (Fig. 1). In this model, the role of the cellular immune response is to modulate the overall level of infection, not the type or location of infected cells. Consequently, during acute infection, before the immune response is effective, the memory compartment of B cells should fill up with latently infected cells. Our results support this prediction of the model and demonstrate that EBV infection is not indiscriminate in the blood but is tightly restricted to resting memory cells even during acute infection. In a separate study (16), limiting dilution RT-PCR was used to quantitate the numbers of infected cells expressing viral latent genes in the blood of AIM patients. It was found that >99% of the cells express no detectable latent proteins (16), consistent with our conclusion here that the cells are resting.

Previous studies reported that 0.01 to 20% of B cells in the blood of AIM patients expressed EBNA (21, 24, 39–41). We are unable to reproduce these findings (16). In our studies, we find EBNA expression only in a very small fraction of B cells ( $\ll 0.1\%$ ), even in patients with very high levels of infected cells ( $>1\%$ ). Our data confirm the earlier studies of Crawford et al. (8), who showed that the vast majority of infected cells in AIM were EBNA negative. The other estimates are probably incorrect because they were based on an old immunofluorescent staining protocol for EBNA proteins. This assay was notoriously difficult and prone to false positives because it employed low-titer human sera and a large amplification step involving complement. The previous claims that the infected cells are proliferating lymphoblasts (39, 47) also appear to be incorrect, since we have shown that the cells are not proliferating (this study) and do not express EBNA2 (16). Again, this is in agreement with the earlier studies of Crawford et al. (8), who showed that the infected cells in AIM were small lymphocytes not lymphoblasts.

It is well known that AIM patients have highly elevated levels of cytotoxic T cells (CTL) specific for EBV lytic antigens (43). The rate of decay in the levels of these cells approximates that of the latently infected memory cells in the periphery (6). The memory cells are not expressing viral proteins (16); therefore, they are not disappearing through CTL-mediated lysis. We suspect that the latently infected cells are decaying by circulating back to the tonsil to produce infectious virus and die. This would provide the antigenic load to stimulate the CTL response, thus explaining the parallel decay in the levels of CTL to lytic antigens. This would mean that the rapid early

decay of infected memory cells is a function of the rate at which they initiate viral replication. This phase is followed by a slower decline that, even by 1 year, had not reached a steady state. This poses a fundamental question about the nature of EBV persistence, namely, does it come to a long-term equilibrium, as is generally believed, or does it never reach a stable equilibrium but continue to decay more and more slowly over time. Previous measurements have not been precise enough (22) to unequivocally resolve these two outcomes which would involve very different biologies. Clarification of this issue will be an important question to address in the future.

One interpretation of our data is that the specificity of EBV for memory B cells in the blood is not a consequence of regulation by the biology of the virus but the elimination of all other infected cell types by CTL. This interpretation cannot be correct, however, for two reasons. First, immunosuppressed organ transplant patients have impaired CTL function that has been shown previously to result in significantly higher (on average 50-fold) levels of EBV infection (2) (Fig. 6), with no evidence for the emergence of other infected cell types. The virus in the blood remains restricted to the memory B cells. Second, despite active CTL responses in healthy carriers, multiple cell types expressing both latent and lytic genes are readily detected in the tonsil (4; Laichalk and Thorley-Lawson, submitted). Thus, the CTL response does not seem competent to restrict infection to a specific subset, at least in that particular organ.

One limitation of our study is that patients are only tested when they present to the clinic. It is possible that during the very early stages of the infection, before the onset of symptoms, multiple cell types are infected in the blood. Subsequently, the infection could become restricted to memory cells by the time of onset of symptoms, when we first study the patients. A scenario such as this is seen with MHV68, where initially all cell types are infected but after 6 months the virus becomes restricted to memory B cells (12, 50). The caveat to direct extrapolation of those studies to EBV is that they were done with the spleen. In the case of EBV, infected cells are found in the spleen, but even in persistent infection, the virus remains in multiple cell types (29). Only in the blood is specificity for memory cells observed.

A more analogous situation to that seen with the spleen and MHV68 might be the tonsil. Recent immunohistochemical studies have identified directly infected memory and germinal center cells which express EBNA2 and appear to have undergone EBV-driven clonal expansion (27, 28) in the tonsils of AIM patients. The infection of all B-cell subsets in the tonsils of healthy carriers has also been seen (4); however, in this case, each subset uses a discrete transcription program and EBNA2 was not detected in the memory or germinal center cells. Rather, these cells express a limited subset of latent genes referred to as the default program (4, 5). It is conceivable that, during acute infection, all subsets are directly infected, in the tonsil, and that this resolves into the more regulated pattern seen in persistent infection.

The work on AIM tonsils has led to the suggestion that EBV may establish persistent infection in the memory compartment by direct infection (26, 28). Our model does not preclude this possibility, although no evidence was found for it in healthy carriers (3–5). One possibility is that the virus gains access to

the memory compartment by direct infection during acute infection but not during persistent infection. Another possibility is that the presence of proliferating clones of directly infected memory and germinal center cells in AIM tonsils has occurred because of the large number of virions and tissue damage present during AIM, allowing direct infection of cells that would not otherwise be exposed to the virus. It was striking that EBV-driven clonal expansion of naive B cells was not seen in the studies of Kurth et al. (28). This would be predicted by our model, which states that only infected naive B cells in the tonsil can differentiate out of the cell cycle by becoming memory cells. If other cell types, such as memory or germinal center cells, were infected directly they would be stuck as proliferating clones. This would mean that the predominant infected cell types seen in AIM tonsils by immunohistochemistry would be these expanding clones that cannot exit the cell cycle and will eventually be destroyed by cytotoxic T cells.

One major caveat with immunohistochemical approaches to identify infected cells *in vivo* is that the lower threshold for detection is not known. Thus, it is impossible to know how many infected cells have been missed and how representative the identified cells are. Unfortunately, tonsils from AIM patients are not generally available for study, making it impossible for us to apply our techniques that use quantitative analysis of whole-cell populations. Thus, we cannot assess whether the reported direct infection of memory and germinal center cells in AIM tonsils represents (i) a mechanism for establishing persistent infection or (ii) a deregulated state of infection or (iii) whether these observations are a consequence of technical artifacts.

In conclusion, we have shown that EBV in the peripheral blood remains restricted to the resting memory B-cell subset, even when the compartment is massively overwhelmed by virus-infected cells.

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