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Identification of the Site of Epstein-Barr Virus Persistence In Vivo as a Resting B Cell

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Epstein-Barr (EBV) is a powerful immortalizing virus for human B lymphocytes in vitro and is associated with several human neoplasias in vivo. Previously, we have shown that the majority of EBV-infected cells in the peripheral blood of healthy, persistently infected individuals do not express the activated phenotype, e.g., high levels of cell surface CD23 and CD80 (B7), characteristically expressed on in vitro-immortalized cells. Here, we show that ≥90% of the CD23$^-$, virus-infected cells in the peripheral blood are in G_0 and therefore resting. The remaining cells may be G_1 arrested, but we were unable to detect a significant number of cells traversing the S-G_2-M stages of the cell cycle. The mRNA for LMP2A, but not EBNA1 originating from Qp, was readily detected in this population, and these cells appear competent in the processing and presentation of antigen by class I major histocompatibility complex. We propose that these resting B cells are the site of long-term latent persistence for EBV. We further propose that the persistence of the virus in a resting B7$^-$ B cell provides an important mechanism to escape immunosurveillance. The demonstration that EBV can persist latently in a resting B cell means that the immortalizing functions of EBV can be down regulated in a normal B cell. This conclusion has important implications for understanding and controlling EBV-associated neoplasia.

Epstein-Barr virus (EBV) has the unique ability to transform human B cells in culture into continuously proliferating lymphoblastoid cell lines (LCLs) (reviewed in reference 13). The immortalizing functions of the virus are believed to be predisposing factors for the development of a number of different EBV-associated human malignancies that occur both in and out of the lymphoid system (reviewed in reference 22). This ability to immortalize cells distinguishes EBV from other human herpesviruses; however, it shares with these viruses the ability to persist for the lifetime of the infected host. The virus persists in a latent state but is also actively replicating, since infectious virus is shed, probably continuously, into the saliva.

These observations set up a number of interesting and important biological questions centering around how EBV interacts with the normal biology of its target cell, the B lymphocyte. Specifically, why does the virus need to relentlessly drive the proliferation of B cells when it establishes latency, whereas other herpesviruses can establish latency without this function, and how does the virus maintain both lifetime latency and viral replication in the face of a strong cellular immune response to immortalized B cells and a humoral response to the virus?

Recently, information about the mechanism of EBV persistence in vivo has begun to accumulate. It has long been speculated that EBV persistence in vivo requires both B cells and epithelial cells (14, 17, 22, 23, 29); however, the role of epithelial cells has been questioned recently. By employing highly sensitive in situ techniques, it has not been possible to detect EBV in either tonsillar epithelium (1, 27, 28) or exfoliated epithelial cells in the saliva (11) of acutely infected individuals, even though virus-infected B cells were readily detected in both locations. Thus, infection of epithelial cells is probably an epiphenomenon and is not likely to play a significant role in normal EBV biology in healthy hosts (31). Nevertheless, the ability of EBV to infect epithelial and other non-B-cell tissues is critically important in the development of neoplastic diseases such as nasopharyngeal and gastric carcinomas and T and NK lymphomas (reviewed in reference 22).

Since the entire virus life cycle can be accomplished in B lymphocytes (31), we recently performed studies to identify the cell surface phenotype of B cells infected with EBV in the periphery of healthy persistently infected individuals. Specifically, we questioned whether the cells were phenotypically related to the EBV-immortalized B lymphoblasts seen in tissue culture. Through the application of a quantitative DNA PCR assay that can detect a single viral genome (12), we could definitively conclude that all of the cells were latently infected B cells (4), but they lacked expression of activation markers, such as CD23, which are classically expressed at high levels on immortalized B lymphoblasts in vitro (19, 20). Furthermore, the frequency of the infected cells is individual specific and remarkably stable over the course of several years in healthy donors (12). This led us to propose that a previously unknown form of latency exists in vivo.

In order to understand how this new form of latency is maintained and what role it plays in long-term persistence of the virus, it is essential to known whether the latently infected cells are proliferating. If they are resting, then they would need to proliferate occasionally in order to replace cells that die, through attrition or when they enter the lytic cycle, and thereby maintain a stable frequency. If, on the other hand, the EBV-infected cells are proliferating, then the cells would automatically be self-replacing and expansion would have to be contained, presumably by the cytotoxic T-lymphocyte (CTL) response. Although the available evidence is predominantly consistent with the idea that the cells are resting, direct or formal proof is completely lacking. For example, expression of an activated phenotype is usually predictive that a cell is proliferating; however, the converse is not true. Thus, it is well established that activated, proliferating normal B cells may lack the expression of activation markers such as CD23 (7). In particular, germinal center centroblasts are cycling, CD23$^-$ B cells that have been proposed as a potential site of EBV persistence (6).

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Analysis of viral gene expression in peripheral blood by reverse transcription-PCR (RT-PCR) techniques has been unable to detect the growth-promoting latent EBNA2 and LMP1 genes (21), consistent with the cells being in a resting state. This result is inconclusive, however, because the RT-PCR techniques used are not quantitative, and another latent gene associated with the proliferating lymphoblastoid state, LMP2a, was readily detected. This raises the possibility that failure to detect EBNA2 and LMP1 may simply reflect the lack of sensitivity of the RT-PCR. More-recent reports have confirmed these observations (3, 32). In addition, one of these studies claimed uniform detection of the EBNA1 transcript (3) but could not identify promoter usage, and one further study claimed that EBNA1 is expressed from the "EBNA1-only," or latency 1, promoter Qp (32). Since it has been shown recently that Qp contains Rb-dependent elements (25a), it is expected that the promoter would function only in cycling cells. This raises the possibility that latency in the peripheral blood involves a proliferating cell that expresses only EBNA1. These cells are presumably driven to proliferate, not under the direction of viral latent genes, but through the action of signals that normally drive B cells, such as CD40 ligand. Exactly where LMP2A expression fits into this is unclear. It is likely that the nonquantitative nature of the RT-PCR and its inability to distinguish what fraction of the latently infected cells are expressing a specific transcript are generating the confusion.

Since EBV has classically been characterized as a lymphoproliferative virus, the demonstration that it can reside in resting B cells would represent a radical departure in our perception of how viral latency functions in vivo. Thus, it is essential to demonstrate directly whether EBV truly can reside in resting B cells. To resolve these issues, we have used a DNA PCR technique that allows us to make definitive and quantitative conclusions about the presence or absence of infected cells in different populations. We have purified $CD23^-$ B cells from the peripheral blood of healthy donors and directly separated them into resting and cycling populations on the basis of DNA content alone or in combination with the expression of the marker of cell proliferation Ki67 (10). We have then used limiting-dilution analysis to measure the frequency of EBV-infected cells in each population. We show that most, if not all (>90%), of the cells are resting in G_0; a significant number of infected cells were not detected in the cell cycle. This population of cells expresses LMP2A, but EBNA1 was not consistently detected. We propose that (i) resting $CD23^-$, $LMP2A^+$ B cells are the site of long-term persistence for latent EBV; (ii) these cells avoid immunosurveillance through lack of expression of the costimulatory molecule B7 (CD80); and (iii) the term "latency program" be adopted to describe this type of latency to distinguish it from forms of latency where the growth-promoting latent genes are expressed, for which we suggest the general term "growth program."

MATERIALS AND METHODS

Cells and cell lines. ER (this laboratory) is an EBV-positive LCL. Namalwa (American Type Culture Collection) and Rael (gift of S. Speck) are EBV-positive Burkitt's lymphoma cell lines. BJAB (gift of E. Kieff) is an EBV-negative B-cell lymphoma line. All these cell lines were maintained in 5% CO_2 in 10% fetal bovine serum–RPMI 1640 with penicillin and streptomycin.

Peripheral blood from healthy individuals was drawn in heparinized syringes and layered onto Ficoll-Hypaque (Pharmacia). The buffy coat was removed and washed once in RPMI 1640 containing 1% fetal bovine serum and Heplock (5 U) and once in PBSA wash (phosphate-buffered saline [PBS; pH 7.4] with 0.5% bovine serum albumin fraction IV [Sigma], 5 mM EDTA, 50 μM alpha-thioglycerol, 20 nM bathocuproinedisulfonic acid, and 1 mM sodium pyruvate).

Antibodies. The murine monoclonal antibodies used in this study were biotinylated anti-CD19 (Dako), fluorescein isothiocyanate (FITC)-coupled anti-CD20 (Dako), biotinylated anti-CD23 (Dako), and FITC-coupled anti-major histocompatibility complex (anti-MHC) class I (Ancell) for cell surface markers. FITC-coupled Ki67 antibody (Immunotech) was used to distinguish G_0 from G_1-S-G_2-M cells. Where necessary, biotinylated antibodies were detected with phycoerythrin-coupled streptavidin (Dako).

Magnetic cell sorting (MACS) separations. Peripheral blood cells were resuspended at 2×10^7/ml in 1.5-ml microcentrifuge tubes. The cells were separated according to the instructions of the manufacturer, with some modifications. Biotinylated antibody against CD23 and FITC-conjugated antibody against CD20 were added to each tube, and the tubes were rotated at 4°C for 30 min. The cells were washed twice with 1 ml of PBSA. The cells were then incubated on ice for 15 min with 1.5 μl of MACS beads (Miltenyi Biotec) conjugated to streptavidin. An aliquot (0.5×10^6 cells) was set aside for fluorescence-activated cell sorter (FACS) analysis. All separations were performed at 4°C to prevent capping of the antibodies. If approximately 5×10^7 cells were expected to bind the magnetized column, then B1 columns (Miltenyi Biotec) were used. They were prepared by a washing with 70% ethanol followed by 10 column volumes of PBSA to remove the ethanol. The column was placed in the magnetic stand, and cells were added to the top. The flow rate was controlled with a 23.5-gauge needle. The cells were passed through the column twice to ensure the efficient removal of $CD23^+$ cells. The columns were then washed with 5 to 7 ml of PBSA and removed from the stand to elute the positively selected cells. The negative cells, the flowthrough, were resuspended to a concentration of 2×10^7/ml.

Biotinylated antibody against CD19 was added to each tube, and the tubes were incubated at 4°C on a rotator. The cells were washed twice and resuspended in PBSA. Depending on the number of $CD19^+$ cells, either another B1 column or a mini column (fewer than 10^7 positive cells) was used to select the $CD19^+$ cells. If mini columns were used, the flow rate was not regulated. Positive and negative cells were collected. Cell purity was monitored throughout the magnetic bead purification. FITC anti-CD20 and phycoerythrin-coupled streptavidin were added to the cells before and after separation on the columns to monitor the percentages of $CD23^+$ $CD20^+$ and $CD20^+$ $CD23^-$ cells, respectively. The cells were subsequently analyzed on a FACSCAN (Becton Dickinson) with Lysys II software. The $CD23^-$ population was always >90% depleted of $CD23^+$ cells. For an example of reanalysis after magnetic bead depletion, see reference 20.

Magnetic bead separation with Dynal beads. Isolation of total peripheral blood was performed as described above. The buffy coat was incubated with neuraminidase-treated sheep erythrocytes to remove the T cells (E^+ cells). The E^- cells were separated into $CD23^-$ and $CD23^+$ cells by using magnetic beads (Dynal) coated with antibody against CD23 as previously described (20).

Cell cycle analysis. To measure DNA content, cells were permeabilized and stained with propidium iodide (PI) by incubation overnight in the dark at 4°C in a solution of 50 μg of PI per ml in 0.1% Triton X-100–0.1% sodium citrate. Ki67 expression was analyzed by using the protocol described by Jacob et al. (10), with some modifications. $CD23^-$ $CD19^+$ cells were washed with RPMI 1640–2% human serum and permeabilized with 3% saponin (Sigma) in PBS by incubation on ice for 20 min. FITC-Ki67 or FITC-immunoglobulin G was diluted in 0.1% saponin–PBS and incubated with the cells for 15 min on ice. PI solution was added to the permeabilized cells, and they were kept in the dark at 4°C overnight. The cells were sorted with a FACStar Plus into 96-well V-bottom plates containing either 10^4 or 10^5 EBV negative BJAB cells/well to serve as a carrier. The cells were pelleted by centrifugation for 5 min at $500 \times g$. The supernatants were carefully removed, and the pellets were resuspended in proteinase K buffer and digested overnight as previously described (20). DNA was extracted by standard methods, as previously described (20), for DNA PCR. PCR was performed in a 50-μl volume by using Microamp reaction tubes in a GeneAmp PCR System 9600 (Perkin-Elmer) thermocycler according to an established method outlined previously (20). Oligonucleotide primers against the W repeat region of the EBV genome were used as previously described (20). For double staining of the $CD23^-$ cells for surface markers and DNA content, the cells were first labelled with either anti-CD20 or anti-CD23 and then permeabilized by alcohol fixation prior to staining with PI as described previously (30).

Calculation of expected versus observed frequencies of EBV-infected cells in the proliferating $CD23^-$ B-cell populations. We used the measured frequency of EBV-infected B cells in the G_0-G_1 population of the $CD23^-$ B cells to estimate the expected frequency of EBV-infected cells in the S-G_2-M population if all of the infected cells were proliferating and therefore all of the positive cells in the G_0-G_1 fraction were in G_1 (see Table 1). This number was then compared to the number of signals obtained experimentally. To make the estimate, the following calculation was performed, taking experiment 2 in Table 1 as an example. A total of 3.1% of the $CD23^-$ B-cell population was in S-G_2-M. Therefore, for every 10^6 G_0-G_1 $CD23^-$ B cells there are $3.1/97 \times 10^6 = 3.2 \times 10^4$ cells in S-G_2-M. The frequency of EBV-infected cells was $83/10^6$ in the G_0-G_1 population. In a typical cycling EBV-positive LCL, the percentage of cells in S-G_2-M is (45 ± 4)%. Therefore, if we assume that the $CD23^-$ EBV-infected cells have a similar cell cycle distribution, then for every 83 EBV-positive G_0-G_1 cells there should be $45/55 \times 83 = 68$ EBV-positive cells in S-G_2-M. Therefore, we should find 68 EBV-positive cells in 3.2×10^4 S-G_2-M cells.

In this experiment, we analyzed a total of 1.3×10^4 S-G_2-M cells. Therefore, we expect $68 \times 1.3/3.2 = 28$ infected cells in the S-G_2-M population we tested. We actually obtained one signal.

Later (see Table 2), we used the measured frequency of EBV-infected B cells in the unfractionated $CD23^-$ B-cell population to estimate the expected fre-

quency of EBV-infected cells in the G_1-S-G_2-M population, assuming that all of the cells were proliferating, and then compared this number to the number of signals actually obtained. To make the estimate, the following calculation was performed, taking experiment 1 in Table 1 as an example. Cell cycle analysis showed that 10.2% of the $CD23^-$ cells were in G_1-S-G_2-M. Therefore, 10^6 unfractionated $CD23^-$ cells contain 10.2×10^4 cells in G_1-S-G_2-M. The frequency of EBV-positive cells was $14/10^6$ unfractionated $CD23^-$ cells. If the 14 EBV-positive cells were in G_1-S-G_2-M, they should be at a frequency of 14 in 10.2×10^4 G_1-S-G_2-M cells. In this experiment, we analyzed 12.5×10^4 G_1-S-G_2-M cells; therefore, we expected $14 \times 12.5/10.2 = 17$ EBV-infected cells. We actually found two.

RT-PCR for LMP2A and EBNA1. A total of 1×10^6 to 5×10^6 cells/tube were pelleted in microcentrifuge tubes at $1,000 \times g$ in an International Equipment Company centrifuge and immediately processed or snap frozen in liquid nitrogen. RNA was extracted by using Trizol according to the manufacturer's instructions. The RNA was dissolved in 10 μl of diethylpyrocarbonate H_2O. Samples were heated for 10 min at 55°C to ensure that the RNA was completely solubilized, and 1 to 5 μg of RNA was used to synthesize cDNA after being primed with random hexamers (50 ng/μl). Primer and RNA were denatured for 10 min at 70°C and then placed on ice. The following reagents were premixed and heated at 42°C for each reverse transcriptase reaction: 2 μl of 0.1 M dithiothreitol, 2 μl of 10× PCR buffer (500 mM KCl, 25 mM $MgCl_2$, 200 mM Tris [pH 8.4]), and 1 μl of 10 mM deoxynucleoside triphosphates (Gibco-BRL). This mixture was added to the primer-RNA, and the combination was mixed very gently by being pipetted a few times. Then 1 to 3 μl of Superscript II reverse transcriptase (200 U/μl) (Gibco-BRL) was added, and the contents of the tube were collected by a 10-s centrifugation. The reaction mixtures were incubated at 42 to 45°C for 50 min and then at 70°C for 15 min to deactivate the enzyme. Subsequently, 1 μl of *Escherichia coli* RNase H (2.5 U/ml) (Gibco-BRL) was added, and the reaction mixtures were incubated for 20 min at 37°C. The volume of the reaction mixtures was increased by the addition of 300 μl of high-pressure liquid chromatography H_2O, and 0.5 μg of poly(dI-dC) (Pharmacia) was added as a carrier to minimize the loss of cDNA. cDNA was precipitated at −20°C with a 1/5 volume of 10 M ammonium acetate and 2.5 volumes of 100% ethanol for at least 1 h. Tubes containing cDNA were subjected to centrifugation at $13,000 \times g$ for 20 min at 4°C. The resulting pellets were washed with 1 ml of 70% ethanol, air dried for 15 min at room temperature, and resuspended in 20 or 50 μl of 10 mM Tris-HCl (pH 8.4).

For cDNA PCR, 5 to 10 μl of cDNA was mixed with 10× PCR buffer (500 mM KCl, 200 mM Tris-HCl [pH 8.5], 2.0 mM $MgCl_2$), 200 μM deoxynucleoside triphosphates (Pharmacia), 20 pmol of each primer, and 1.0 U of *Taq* polymerase (Perkin-Elmer Cetus) by the hot-start method. The total reaction volume was 50 μl in Microamp tubes (Perkin-Elmer Cetus), and a Perkin-Elmer 9600 thermocycler was used. For LMP2A, 40 cycles of 95°C for 15 s, 49°C for 15 s, and 72°C for 30 s were used. For the EBNA1 transcript originating from Qp, 40 cycles of 95°C for 15 s, 64°C for 30 s, and 72°C for 30 s were used. The primers used for LMP2A were as described by Tierney et al. (32), and the primers used for EBNA1 were TCGCCCCTCGTCAGACATGATT (Q exon) and AGCGTGCG CTACCGGAT (K exon). The expected size of the EBNA1 product is approximately 200 bp. RT-PCR products were analyzed in the same manner as the DNA PCR products, except the PCR products were detected by probing Southern blots with ^{32}P-random-primed LMP2A or EBNA1 PCR product. The EBV-positive LCL IB4 was used as a positive control for LMP2A, and the Rael cell line was used as a control for the Qp EBNA1 transcript. These protocols detect the mRNA from 10 EBV-positive cells in the presence of 10^6 EBV-negative BJAB cells.

RESULTS

The EBV-infected cells in the periphery are not traversing the cell cycle. We have used a FACS to sort $CD23^-$ B cells, from the peripheral blood of healthy donors, after being stained for cell cycle stage with the DNA intercalating dye PI. Staining with PI alone allows clear distinction of cells in G_0 and G_1 from those in S-G_2-M, which consists entirely of proliferating cells. Typical staining of an established EBV-positive LCL is shown in Fig. 1A. A large peak is seen due to cells that have a DNA content of 2 N, which includes resting cells (G_0) and cells in the G_1 phase of the cell cycle. As the cells enter S phase and begin to synthesize DNA, the amount of staining increases until the cells reach G_2 and M, when they have a 4 N DNA content and therefore stain with double the intensity of G_0 or G_1 cells. In a typical EBV-immortalized cell line, 50 to 60% of the cells are in G_1 and the rest are distributed throughout S, G_2, and M. This staining technique was then applied to purified $CD23^-$ B cells from the peripheral blood of healthy donors. The cells were usually more than 90% pure (Fig. 1B). The PI staining profile (Fig. 1C, left panel) reveals that most of the cells (>90%) are in the G_0-G_1 peak. However, a small but significant population of cells was always found to be cycling. Typically, we have detected ~2 to 5% of the cells in S-G_2-M with this type of analysis. Because this percentage is so small, we could not exclude the possibility that they represent a very small contaminating population. However, in double-staining analysis (not shown), it was possible to confirm that this cycling population was representative of the bulk $CD23^-$ B-cell population. This means that ~5 to 10% of the cells are in the cell cycle, directly confirming that the $CD23^-$ phenotype does not necessarily imply that B cells must be resting. We have used the FACS to purify the G_0-G_1 and the S-G_2-M populations. Reanalysis of the sorted populations demonstrating their purity is shown in the middle and right-hand panels of Fig. 1C. We then measured the frequency of EBV-infected cells in each population, using a DNA PCR-based assay to detect EBV in a limiting-dilution analysis. This assay can detect a single viral genome in the presence of 10^6 uninfected cells, and its reproducibility and sensitivity have been established in detail elsewhere (12, 20). Examples of the results obtained in the PCR analysis are shown in Fig. 1D, and a summary of the results obtained in three separate experiments is shown in Table 1. In all three experiments, sufficient numbers of infected cells were detected in the G_0-G_1 population to allow frequencies to be estimated, and these are shown in Table 1. We never obtained enough positive samples in the S-G_2-M population to estimate a frequency. Therefore, these experiments demonstrate that essentially all of the detectable virus-infected cells are in the G_0-G_1 population.

These experiments exclude the possibility that all of the virus-infected cells are proliferating; otherwise, we would have seen a strong enrichment into the S-G_2-M population. We have attempted to estimate the level of confidence with which we could exclude the possibility that any EBV-infected cells were in the cell cycle, using the data from the experiments in Table 1. We used the measured frequencies of EBV-positive cells in the G_0-G_1 population to estimate the number of positive cells we would expect to find in the S-G_2-M population if all of the positive cells in the G_0-G_1 population were from proliferating cells in G_1 (see Materials and Methods). The results are presented in Table 1, and, combining the data from three independent experiments, it is apparent that, of an expected 54 positive cells, we obtained only 1 positive sample. This demonstrates that essentially all of the EBV-positive cells in the peripheral blood are in the G_0-G_1 population, with only 2% in the S-G_2-M population (95% confidence interval, 0 to 5.3%), which would correspond to 4% of the EBV-infected $CD23^-$ cells being in the cell cycle. This result is consistent with all of the cells being out of the cell cycle or the EBV-positive cells behaving like the bulk $CD23^-$ population, with only a small fraction traversing the cell cycle at any one time.

The majority of EBV-infected cells in the periphery are resting in G_0. The analysis described above demonstrates that the latently infected cells in peripheral blood are not actively cycling. However, this analysis cannot distinguish genuinely resting cells from cells that are arrested in G_1. Therefore, we have used two-color fluorescence and the FACS to sort $CD23^-$ B cells from the peripheral blood of healthy donors into resting (G_0) and proliferating (G_1-S-G_2-M) populations and then analyzed these populations for the presence of EBV-infected cells. The two-color FACS analysis involves the permeabilization of the cells followed by staining with PI for DNA content in conjunction with staining by an FITC-coupled monoclonal antibody to the nuclear antigen Ki67 (10). The Ki67 antigen is expressed in proliferating, but not in resting, cells (5). Figure 2

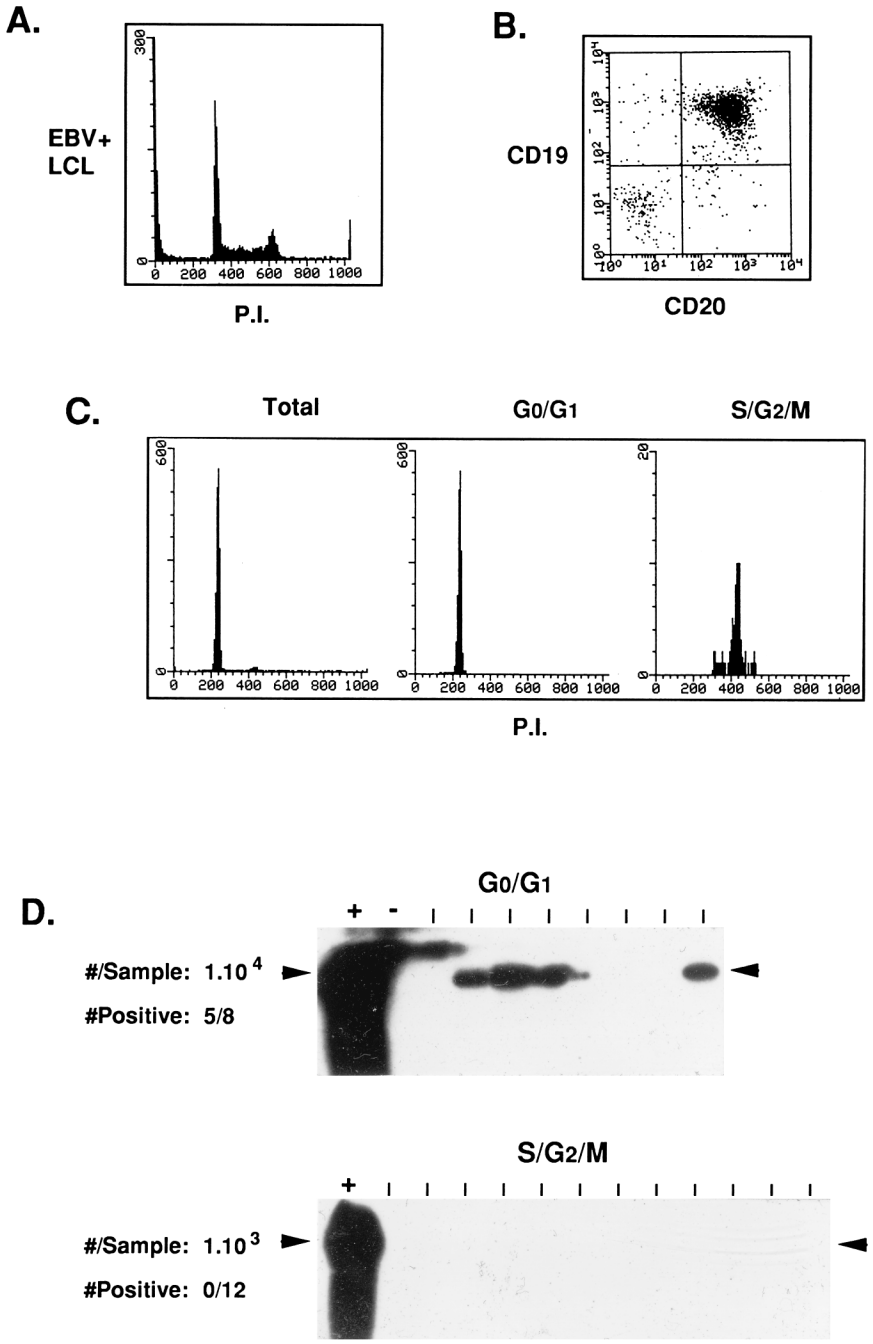

FIG. 1. Detection of EBV-infected cells in separated G_0-G_1 and S-G_2-M populations of CD23$^-$ B cells. CD23$^-$, CD19$^+$ B cells were isolated by using MACS beads. The cells were permeabilized in 0.1% Triton X-100 and stained with PI. The cells were then sorted with a FACStar cell sorter into the G_0-G_1 and S-G_2-M populations. DNA PCR for EBV was performed with replicate samples of various numbers of cells. (A) FACS analysis of an EBV-positive LCL stained with PI, demonstrating the typical distribution of proliferating cells through the cell cycle. (B) FACS analysis of the purified population of cells used in the experiments. The samples were typically greater than 90% pure B cells. (C) FACS analysis of the purified CD23$^-$ B cells stained for DNA content before and after fractionation into G_0-G_1 and S-G_2-M populations. (D) Southern blot of the DNA PCR products obtained from single dilution points from each of the two fractionated populations. The blot was hybridized with a probe from the *Bam*HI W repeat sequence of EBV labelled by random priming.

demonstrates staining with Ki67 alone (Fig. 2A) and in combination with PI (Fig. 2B) on control populations of resting and proliferating cells and mixtures of the two. This figure demonstrates that the staining technique allows the separation of resting G_0 cells from the proliferating G_1-S-G_2-M cells, although there is approximately a 10% overlap between the G_0 and G_1 populations (lower panels).

Analysis of purified CD23$^-$ B cells by the two-color staining method is shown in Fig. 3A. The left-hand panel shows the staining with PI alone, and the middle panel shows double staining with PI and Ki67. In this panel, the different populations of cells are identified. During the course of these experiments, it was again apparent that 5 to 10% of the CD23$^-$ B cells were in the cell cycle. We then proceeded to isolate the

TABLE 1. Frequency of EBV-containing cells in G_0-G_1 and S-G_2-M populations of $CD23^-$ B cells[a]

Expt	Frequency/10^6 cells in G_0-G_1[b]	No. observed[c] in S-G_2-M	No. expected in S-G_2-M[d]
1	22 ± 7	0	7 ± 2
2	83 ± 28	1	28 ± 9
3	44 ± 15	0	19 ± 6
Total		1	54 ± 17

[a] The percentages of cells in S-G_2-M were 4.7, 3.1, and 1.6% for experiments 1 to 3, respectively.
[b] The error of the frequency measurements has previously been shown to be ±30% (12).
[c] The total numbers of cells tested in the limiting-dilution analysis were 1.7×10^4, 1.3×10^4, and 0.86×10^4 for experiments 1 to 3, respectively.
[d] The expected values for the S-G_2-M population were calculated as described in the text.

resting population. The reanalysis of the purified G_0 cells is shown in the right-hand panel of Fig. 3A, confirming the purity of the population to be greater than 99%. The frequency of EBV-infected cells in this population was then estimated by using the DNA PCR-based limiting-dilution analysis for EBV. Examples of Southern blots of the PCR products are shown in Fig. 3. Positive samples were readily detected in the G_0 population, and the frequency of virus-infected cells could be estimated and compared to that of the unfractionated $CD23^-$ cells. In two independent experiments, there was no significant difference between the frequencies of virus-infected cells per 10^6 $CD23^-$ B cells in the unfractionated population (14 ± 5 and 47 ± 16) and in the G_0 fractions (16 ± 5 and 32 ± 11) (means ± standard deviations; error of ±30% [12]), indicating that the majority of the infected cells are resting.

We performed a control experiment to eliminate the possibility that the results we obtained were affected by the method involved in permeabilizing and staining the cells. The processing could have selectively enriched for or destroyed EBV-positive cells. Therefore, the frequencies of EBV-infected cells in a population of 10^6 $CD23^-$ B cells before processing (9.5 ± 3), after permeabilization with 3% saponin for Ki67 staining (9 ± 3), and after permeabilization and staining with PI containing 0.1% Triton X-100 (12 ± 4) were estimated as described previously (12, 20) (values are means ± standard de-

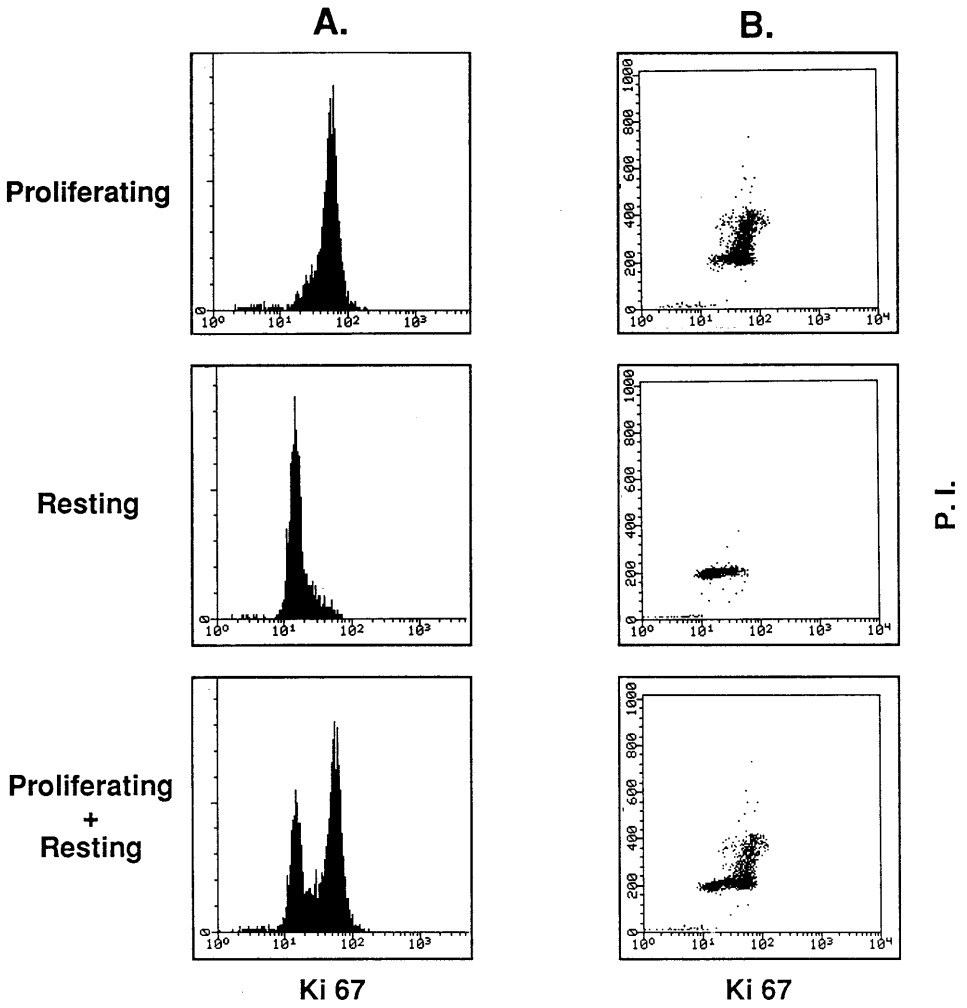

FIG. 2. Double staining with the Ki67 antibody and PI allows the resolution of proliferating cells from cells in G_0. Cells were costained with PI for DNA content and Ki67 to distinguish proliferating from resting cells. The proliferating population is an established EBV-positive LCL, the resting population is whole PBMC, and the third population is an admixture of the two. Columns A and B represent the same analysis, with column A showing only the Ki67 staining and column B showing double staining with both Ki67 and PI.

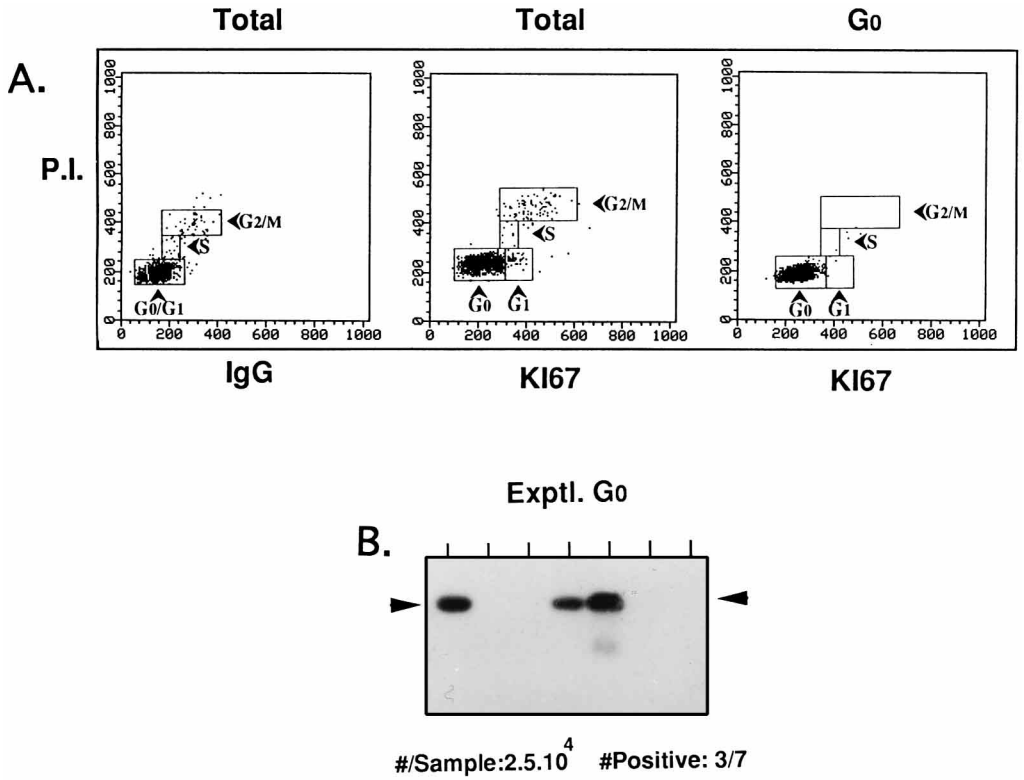

FIG. 3. Detection of EBV-infected cells in resting CD23$^-$ B cells. CD23$^-$ B cells were costained with PI for DNA content and Ki67 to distinguish resting from proliferating cells. The cells were then sorted for the G_0 populations, and DNA PCR for EBV was performed with replicate samples of various numbers of cells. A summary of the quantitation of these experiments is shown in Table 2. (A) FACS analysis of the CD23$^-$ CD19$^+$ B cells. The left-hand panel shows staining of the CD23$^-$ B cells with PI and an isotype-matched control for Ki67. The middle panel shows staining with PI and Ki67. The locations of cells in the different stages of the cell cycle are shown. The right-hand panel shows PI and Ki67 staining of the sorted G_0 population that was used for DNA PCR. IgG, immunoglobulin G. (B) Southern blot of the DNA PCR products obtained from the sorted G_0 population hybridized with a probe from the *Bam*HI W repeat sequence of EBV labelled by random priming. Exptl., experimental.

viations, with an error of ±30% [12]). The frequency of EBV-positive cells was not detectably altered by either process.

We performed a second series of analyses to see how rigorously we could exclude the possibility that there were proliferating cells. The G_1-S-G_2-M populations from two donors were separated and analyzed for the presence of EBV with the limiting-dilution assay (Fig. 4A and Table 2, experiments 1 and 2). In both experiments, we analyzed all of the cells isolated. Nevertheless, we never obtained enough positive reactions from this population to estimate a frequency of EBV-infected cells. This rules out the possibility that there were large numbers of proliferating infected cells.

One explanation for this result is that the processing involved in the staining and sorting of the cells preferentially depletes or destroys only the EBV-infected cells that are proliferating. To try to control for this possibility, we repeated the experiment described above, except in this case the peripheral blood mononuclear cells (PBMC) were "spiked" with cells from an EBV-positive LCL. The positive cells were added at a frequency of 1 in 10^5, comparable to the frequency of endogenously infected cells in the donor used for experiment 2. As a precaution, we spiked PBMC from a donor with a very low frequency (~4 EBV-positive cells in 10^7 PBMC). The cell mixture was processed as before for Ki67 and PI staining, and the cycling population was isolated and analyzed for the presence of EBV-positive cells by DNA PCR. A Southern blot of the PCRs for one cell dilution is shown in Fig. 4B, and the results are summarized in experiment 3 in Table 2. Even though the number of cells used for the PCRs in the example shown in Fig. 4B was 10-fold less than the number of cells from the proliferating populations in the experiment whose results are shown in Fig. 4A, EBV-positive cells were now readily detected in the G_1-S-G_2-M population.

On the basis of the percentage of B cells (10.25%) in the PBMC sample that was spiked, the purity of the B cells when isolated (62%), and the fraction of the B cells in the cell cycle (14.2%), we expected to find approximately 13 EBV-positive cells in the 1.2×10^4 G_1-S-G_2-M cells analyzed due to the spiked LCL cells, if they survived the process. Overall, we observed 10. This control experiment demonstrates directly that, had the latently infected CD23$^-$ B cells been proliferating, we would have readily detected them at the cell numbers we tested.

We have repeated the calculation used for experiment 3 with the data from experiments 1 and 2 in Table 2, assuming that all of the EBV-positive cells were proliferating (see Materials and Methods). These numbers, compared to what was actually found, are also shown in Table 2. Combining the data from the two experiments, we may conclude that we should have detected 29 EBV-infected cells in G_1-S-G_2-M if all of the cells were proliferating; however, we detected only 3. Therefore, no more than 10% of the EBV-infected cells are proliferating. There are two explanations for this finding. The first is that these signals represent contamination of the G_1-S-G_2-M population with G_0 cells. As demonstrated above and in Fig. 2, there is sufficient overlap between the two populations to ac-

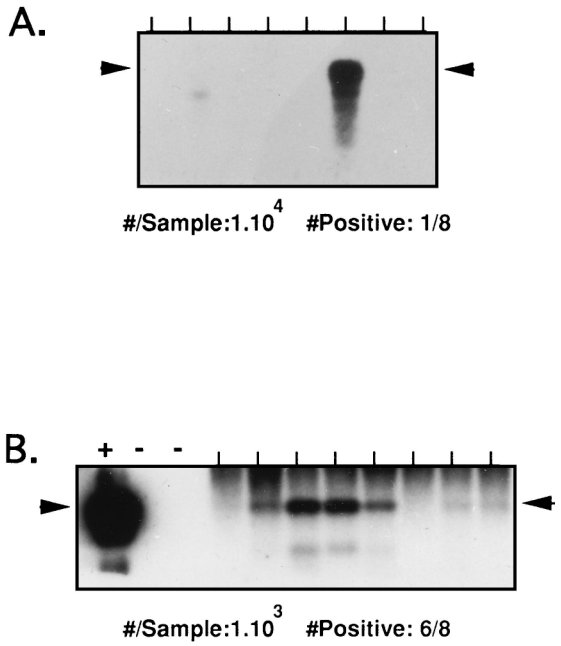

FIG. 4. Detection of EBV-infected cells in proliferating CD23$^-$ B cells. The experiment was performed as described for Fig. 3 except that in this case the G_1-G_2-M population was analyzed. A summary of the quantitation of these experiments is shown in Table 2. (A) Experimental G_1-S-G_2-M population. Southern blotting of the DNA PCR products obtained with a sorted G_1-S-G_2-M population hybridized with a probe from the *Bam*HI W repeat sequence of EBV labelled by random priming. (B) Spiked population. The experiment was done as for panel A but with PBMC of a very low frequency donor that had been spiked through the addition of proliferating EBV-positive LCL cells to give a frequency of EBV-infected cells (1 in 10^5) similar to that of the donor cells used in panel A.

count for such a level of contamination. Alternatively, it is conceivable that the EBV-infected CD23$^-$ B cells behave like the bulk CD23$^-$ population; that is, at any one time, approximately 10% are proliferating.

LMP2A is expressed in the CD23$^-$ population. RT-PCR analysis by several groups has established that the transcript for the EBV-encoded LMP2A protein is expressed in peripheral blood B cells (3, 21, 32) and that the growth-promoting LMP1 and EBNA2 genes are not detectable. One group has also reported the detection of EBNA1 transcripts originating from the Q promoter (32), characteristic of the EBNA1-only type of latency found in Burkitt's lymphoma tumor cells and long hypothesized to be the form of long-term latency in resting B cells in vivo (14, 17). LMP2A is expressed in immortalized LCLs and is a known CTL target; therefore, its expression makes the infected cell susceptible to T-cell surveillance. It seemed unlikely that such a product would be expressed in a site of long-term latency, and we reasoned that the presence of the LMP2A transcript represented the presence of very rare immortalized lymphoblasts in vivo and that LMP2A mRNA was simply easier to detect than the other latent genes associated with immortalization. If correct, then it would be predicted that the LMP2A transcripts should be detected in the CD23$^+$ population. On the other hand, if EBNA1 only is truly the form of latency in resting B cells, then the EBNA1 transcripts originating from Qp should be localized in the CD23$^-$ resting B cells. To test this, we employed an RT-PCR for LMP2A and EBNA1 mRNAs that was able to detect the transcript from 1 EBV-positive cell in the presence of 10^5 EBV-negative cells. mRNAs were prepared from CD23$^-$ and CD23$^+$ B cells from two donors whose frequency of EBV-positive cells was approximately 1 in 10 peripheral B cells. RT-PCR analysis was performed for LMP2A, and the results are shown in Fig. 5. The opposite result to that expected was obtained; namely, mRNA for LMP2A was readily detected in the CD23$^-$ population but was undetectable in multiple samples of CD23$^+$ B cells. By comparison, we were also to detect the EBNA1 transcript initiating from the EBNA1-only promoter, Qp, but this detection was sporadic. Thus, of multiple samples of 10^6 CD23$^-$ B cells, all of which should contain several latently infected cells, only occasional samples were positive (not shown). Furthermore, occasional positive samples were found in the CD23$^+$ population as well; therefore, there was no obvious segregation of the signal with cellular phenotype. The reasons for this result are unclear, but the RT-PCR results are clearly not consistent with the idea that the form of latency in resting peripheral blood B cells is EBNA1 only or that the LMP2A mRNA expression is due to the presence of

TABLE 2. Frequency of EBV-containing cells in proliferating, G_1-S-G_2-M, CD23$^-$ B cells[a]

Expt	Frequency/10^6 CD23$^-$ B cells[b]	Cells tested (10^4)	No. positive	
			Expected[c]	Observed
1	14 ± 5	12.5	17 ± 6	2
2	50 ± 17	2.5	12 ± 4	1
3[d]	62	1.2	13	10

[a] The percentages of cells in G_1-S-G_2-M were 10.2, 10.5, and 14.2% in experiments 1 to 3, respectively.

[b] The error of the frequency measurements has previously been shown to be ± 30% (12).

[c] The expected values were calculated as described in Materials and Methods.

[d] In experiment 3, EBV-immortalized LCL cells were used to spike the peripheral blood cells, to give an estimated frequency of 1 in 10^5, prior to isolation of the CD19$^+$ lymphocytes.

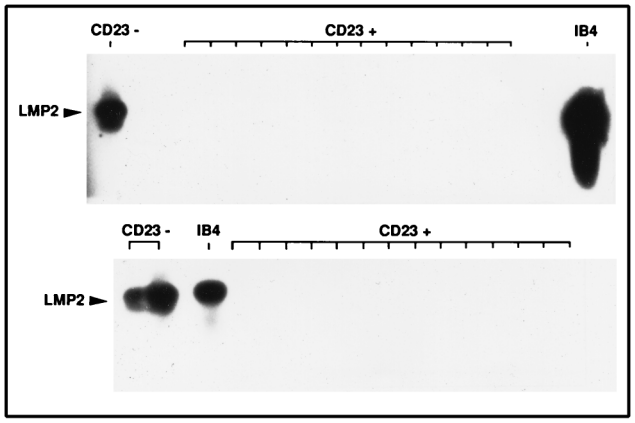

FIG. 5. Detection of the mRNA for LMP2A in the CD23$^-$ B-cell fraction. CD23$^-$ and CD23$^+$ B-cell fractions were isolated from E-rosette-negative cells using the Dynal bead system. IB4 EBV-positive LCL cDNA was used as the positive control. RT-PCR for LMP2A was performed as described in Materials and Methods. The PCR products were fractionated on a 3% Nusieve–0.6% agarose gel and detected by Southern blotting with a probe from the LMP2A PCR product labelled by random priming. The LMP2A PCR product (arrowhead) is approximately 277 bp. The lanes contain amplified LMP2A cDNA from 3×10^6 CD23$^-$ or CD23$^+$ cells (top) or from 5×10^6 CD23$^-$ cells or 2×10^6 CD23$^+$ cells. Reanalysis showed that in the experiment whose results are shown at the top the CD23$^-$ cells were 96.4% depleted of CD23$^+$ B cells and were 16.2% B cells, and in the other experiment the CD23$^-$ cells were 99.6% depleted of CD23$^+$ B cells and were 16.9% B cells. The CD23$^+$ cells could not be reanalyzed due to the magnetic beads but are assumed to be essentially pure CD23$^+$ B cells.

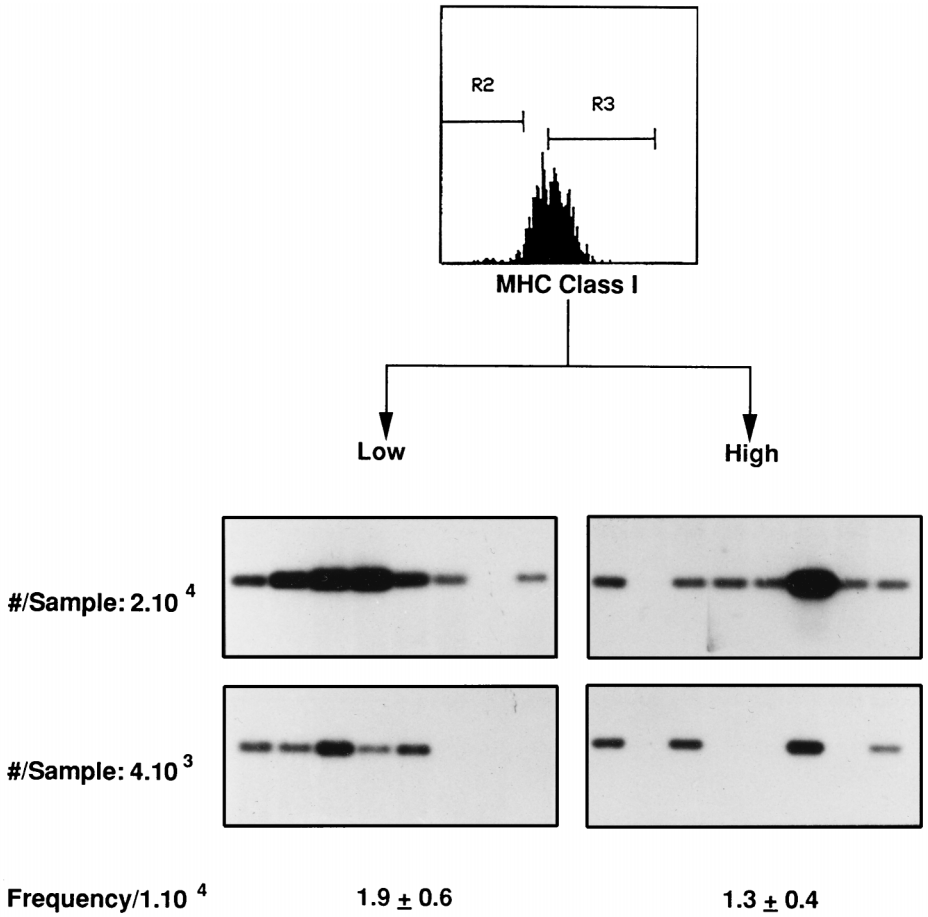

FIG. 6. The presence of EBV-infected cells does not correlate with HLA expression. $CD23^-$ $CD19^+$ B cells were stained for the expression of MHC class I molecules, and the brightest and dimmest 25% of the population was separated with a FACStar cell sorter. The frequency of EBV-infected cells in each population was then assessed by DNA PCR.

small numbers of EBV-immortalized B lymphoblasts. Rather, it seems consistent with the notion there may be an LMP2A-only form of latency, as originally proposed by Qu and Rowe (21).

Antigen processing and presentation in latently infected, resting $CD23^-$ B cells. By expressing LMP2A, the latently infected, resting $CD23^-$ B cells in the peripheral blood could become targets for CTLs. However, the cells are resting and therefore metabolically quiescent, so it was formally possible that they did not process and present antigen efficiently. We have tested this by performing pulse-chase experiments to study the maturation of MHC class I complexes. These studies demonstrate that the $CD23^-$ B cell population is as efficient as established EBV cell lines in forming mature-peptide-containing complexes at the cell surface (not shown). However, one mechanism that viruses, including herpesviruses, have evolved to avoid T-cell immunosurveillance is to encode proteins that directly or indirectly result in diminished expression of MHC class I molecules so that insufficient target peptides are presented at the surface of the infected cells. If this were the case for the latently infected, resting $CD23^-$ B cells in the peripheral blood, then it would be expected that they would have diminished levels of MHC class I molecules on their surface compared to that of the bulk population of uninfected cells. To test this, we used the FACS to separate the cells into the highest 25% and lowest 25% for expression of MHC class I molecules and then measured the frequencies of EBV-positive cells in the two populations. This experiment was performed with cells from two individuals, and the results for one are shown in Fig. 6. It was found that the frequencies of EBV-positive cells in the two populations were identical, demonstrating that the EBV is not selectively latent in B cells with down-regulated levels of MHC class I molecules. Therefore, LMP2A peptides should be efficiently processed and presented by these cells.

DISCUSSION

In this study, we have provided the first direct quantitative evidence that the major site of EBV persistence in the peripheral blood is a resting B cell. We propose that this resting, $CD23^-$, $B7^-$ B cell is the site of long-term persistence for EBV in vivo for healthy individuals chronically infected with EBV.

We have used two different experimental approaches to reach the conclusion that >90% of the latently infected cells are out of the cell cycle. In the first analysis, we used PI staining to isolate the G_0-G_1 cells and estimate the frequency of EBV-infected cells. We then used this estimate to calculate how many EBV-infected cells we should detect in the S-G_2-M population if all of the infected cells were proliferating. On the basis of this analysis, we could conclude that no detectable cells (<5%) were actively proliferating. The drawback to this anal-

ysis is that it depends on the unverifiable assumption that cycling cells in vivo would have a cell cycle distribution similar to that of an established LCL (see Materials and Methods). In the second analysis, we used the Ki67 marker to separate resting and proliferating cells. From the frequency of EBV-infected cells in the unfractionated $CD23^-$ population, we could estimate how many positive cells we should find in the proliferating population if the cells were cycling. This analysis indicates that ≥90% of the EBV-infected cells in the periphery are resting in G_0. Although this estimate does not depend on unverifiable assumptions, it is limited by the partial overlap between the G_0 and G_1 populations. Therefore, we cannot rule out the possibility that ~10% of the EBV-infected cells are arrested in G_1 or in a prolonged G_1 (26). A potentially more interesting scenario which is also consistent with our data is the possibility that the EBV-infected cells behave just like the bulk of $CD23^-$ B cells, with a very small fraction (~5%) in the cell cycle at any one time.

The observation that a strong immortalizing virus such as EBV persists latently in resting cells confirms the absence of the growth-promoting latent genes and implies that these genes can be down regulated in normal cells. This raises the possibility that expression of these genes could be manipulated to control the growth of EBV-associated neoplasias. It also opens a series of new questions that can now be addressed about EBV infection in vivo. The two central questions are: how does a virally infected, resting B cell avoid immunosurveillance and how does the virus gain entry to and exit from a resting B cell?

The first question may be moot. The simplest model of EBV persistence is for no viral proteins to be expressed. This would be analogous to the neurotropic herpesviruses. To date, RT-PCR techniques are not sensitive or quantitative enough to definitively analyze latent-gene expression for EBV in vivo. So far, the LMP2A gene is the only viral latent gene consistently found by RT-PCR to be expressed in the peripheral blood of healthy donors (3, 21, 32). Our experiments show that expression of this gene is localized to the $CD23^-$ population and is not due to a few residual lymphoblastoid cells. Still, we cannot know if the LMP2A is expressed in all of the latently infected cells or only a small subpopulation that does not play a critical role in viral persistence. On the basis of the work of Miller et al., there would be an advantage to expressing LMP2A (18). LMP2A acts as a dominant negative signalling molecule that prevents fortuitous reactivation of the virus. LMP2A expression in all of the latently infected cells would prevent viral replication in the peripheral blood (4), perhaps limiting viral reactivation to the mucosal surfaces, where it is shed into saliva.

How could the latently infected cells avoid the immune response if they are expressing LMP2A, which is a known target for CTLs? The explanation comes in part from the demonstration that the cells are resting and from our previous observation that they do not express the costimulatory molecule B7 (20). It has been shown previously that B7 expression is required for a resting B cell to reactivate a memory T-cell response (2). Thus, the $LMP2A^+$ latently infected cells in vivo, being $B7^-$ and resting, will be shielded from the large numbers of memory CTLs carried by healthy donors. Nevertheless, $B7^-$ cells are able to activate CTLs indirectly through a mechanism that is believed to involve the release of antigenic peptides to be picked up and presented by $B7^+$ professional antigen-presenting cells (8, 33). We have shown that resting B cells in general process and present antigen efficiently, and the presence of normal levels of MHC class I molecules on the surface of the EBV-positive cells indicates that this is also true of the latently infected resting B cells. Therefore, if there is a significant amount of LMP2A in the cells, it should be processed, presented, and shed. However, only LMP2A mRNA has been detected, and we know nothing about the levels of protein expression. It is likely that, since the cell is resting, only small amounts of the protein are made. Furthermore, the frequency of infected cells is very low, and shed peptides have a short half-life. Taken together, these three effects may result in insufficient processed peptide being produced to elicit a response. If correct, then the consistent failure to detect expression of the growth-promoting genes in the peripheral blood by RT-PCR (3, 21, 32) would not reflect a need for the cells to avoid immunosurveillance or a lack of sensitivity of the PCR but would simply be a consequence of the expression of these genes being incompatible with the majority of EBV-infected cells being in a resting state.

There are two likely mechanisms that could explain how EBV establishes latency in a resting B cell. Either the virus is able to directly enter resting cells and establish latency, or B cells induced to proliferate by viral infection are capable of subsequently entering into a resting state. In general, we favor the second mechanism, for three reasons. First, the viral genome in the infected cells is in the form of a covalently closed episome. It has been shown previously that covalent circularization of the viral genome in vitro requires activation of the infected resting B cells into the cell cycle (9). Therefore, the latently infected cells in vivo must have been activated at some point in time to allow circularization of the genome. Second, all of the evidence from in vitro studies of freshly isolated B cells suggests that infection with EBV invariably leads to activation. Third, if the virus is capable of establishing latency directly in a resting B cell, it is unclear why it would need the growth-promoting latency program associated with immortalization. The most satisfying explanation would be that the virus uses the growth-promoting latency program to expand and spread during acute infection. This would explain why this type of latency has been detected only during acute infection (24, 32). These activated proliferating B cells would then either be killed by CTLs or enter into a dormant resting state of long-term latency. This model explains why EBV has a transcriptional program that drives the activation and proliferation of B cells, since this would be essential for the establishment of latency.

In situ immunohistology suggests that B cells replicating the virus are found in the epithelium of mucosal lymphoid tissue, at least in acutely infected individuals (1). This explains how EBV is shed into saliva. It also raises questions as to how the resting, latently infected B cells in the circulation home to the mucosal epithelium and what signals cause the virus to reactivate. Presumably, these events are occurring in the secondary lymphoid tissue.

On the basis of these observations, we have suggested a new nomenclature to describe the different types of EBV latency in normal B cells (31). The form of latency we have described in resting B cells is referred to as the latency program. This distinguishes it from the program found in in vitro-immortalized B cells, usually referred to as latency 3 (22), for which we suggest the term growth program. These terms are more flexible because they are not rigidly defined by specific expression of viral latent genes; rather, they are distinguished by a resting versus proliferating cell type and are defined by the absence or presence of growth-promoting genes such as the LMP1 and EBNA2 genes. A third form of latency, previously known as latency 1, is when only EBNA1 is expressed (25), and we suggest that the term EBNA1-only latency is more appropriate. This form of latency is found in Burkitt's lymphoma

but has yet to be demonstrated in normal B cells. Nevertheless, there is strong circumstantial evidence for its existence. EBNA1 is essential for the replication of the latent, episomal viral genome in proliferating cells (35). It is also the only latent protein with a domain that prevents it from being processed and presented to CTLs (15). Therefore, EBNA1 may be expressed in an activated, proliferating B cell without being detected by the immune response. Such a form of latency likely plays a role in allowing the virus to persist in proliferating B cells without evoking a CTL response.

The possibility that EBV persists latently in a resting cell has been raised previously on the basis of the finding that EBV could be found in high-density B cells, as assessed by spontaneous-outgrowth assays (16, 34). The interpretation of those studies is compromised, however, because it is unknown what fraction of latently infected cells are detected in the outgrowth assay; infected cells were equally readily detected in the low-density fractions, and the assumption that the cells were resting was based solely on cell density. In contrast, the fractionation used in our study depends directly on whether the cells are proliferating and demonstrates that the majority of latently infected cells in vivo are resting.

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