Germinal Center B Cells Latently Infected with Epstein-Barr Virus Proliferate Extensively but Do Not Increase in Number\textsuperscript{\textdagger}

Jill E. Roughan,† Charles Torgbor,‡ and David A. Thorley-Lawson*

Department of Pathology, Tufts University School of Medicine, Jaharis Building, Boston, Massachusetts 02111

Received 22 August 2009/Accepted 22 October 2009

In this study we show that in long-term persistent infection, Epstein-Barr virus (EBV)-infected cells undergoing a germinal center (GC) reaction in the tonsils are limited to the follicles and proliferate extensively. Despite this, the absolute number of infected cells per GC remains small (average of 3 to 4 cells per germinal center; range, 1 to 9 cells), and only about 38 to 55% (average, 45%) of all GCs carry infected cells. The data fit a model where, on average, cells in the GC divide approximately three times; however, only one progeny cell survives to undergo a further three divisions. Thus, a fraction of cells undergo multiple rounds of division without increasing in numbers; i.e., they die at the same rate that they are dividing. We conclude that EBV-infected cells in the GC undergo the extensive proliferation characteristic of GC cells but that the absolute number is limited either by the immune response or by the availability of an essential survival factor. We suggest that this behavior is a relic of the mechanism by which EBV establishes persistence during acute infection. Lastly, the expression of the viral latent protein LMP1 in GC B cells, unlike in vitro, does not correlate directly with the expression of bcl-2 or bcl-6. This emphasizes our claim that observations made regarding the functions of EBV proteins in cell lines or in transgenic mice should be treated with skepticism unless verified in vivo.

Epstein-Barr virus (EBV) is a human herpesvirus that establishes a lifetime persistent infection in >90% of all adults (reviewed in references 21 and 51). The virus persists in resting, latently infected memory B cells that circulate in the periphery and lymph nodes (5; reviewed in references 53 and 55). In these cells the virus is quiescent, at least at the level of viral protein expression (23). This lack of viral proteins is presumably a major reason why these cells are able to persist in the face of a healthy immune response. The other property for which EBV is well known is its ability to infect resting B cells and drive them to become activated proliferating lymphoblasts through the expression of nine latent proteins and several untranslated RNAs (reviewed in reference 45). The latter include a large number of microRNAs (miRNAs) (7, 12, 17, 44).

The current model of Epstein-Barr virus persistence holds that the virus drives resting B cells to become activated lymphoblasts so that they can differentiate through a germinal center (GC) reaction to become resting memory B cells (55–57), where the virus persists. The GC is the structure in the follicles of secondary lymphoid organs where antigen-activated B cells undergo a T-cell-dependent immune response (1, 35, 37). The production and maintenance of GCs are absolutely dependent on the expression of the transcription factor bcl-6 (13, 58) and are initiated by the rapid proliferation of antigen-specific B cells. During this expansion phase, B cells divide approximately every 6 to 12 h (2, 37) so that 3 ± 2 founder cells can create a GC of approximately 10^5 cells in a few days (29, 36, 37). In the classical model of GC development (37), it was proposed that in the proliferating state, the B cells (centroblasts) reside in the dark zone of the follicle and do not express their antigen receptor while actively undergoing the processes of somatic hypermutation and immunoglobulin class switching. These processes are governed by the enzyme AID (activation-induced cytidine deaminase), whose expression is also a defining feature of GC B cells (39). After several rounds of division, the cells move to the light zone, rest, reexpress their surface immunoglobulin (centrocytes), and compete for antigen binding and T-cell help. Survival requires that the B cell successfully competes for both signals. It should be noted, though, that the clear-cut distinction between centroblasts and centrocytes has been called into question (1).

While in the GC, B cells are highly apoptosis prone, and a failure to receive the requisite survival signals leads rapidly to death. If the survival signals are received, then there are three possible fates for the GC cell. It can recapitulate the process by once more becoming a centroblast, or it can upregulate pro-survival molecules such as bcl-2 (29, 36) and leave the GC as a plasma or memory B cell. Which fate the B cell follows depends on the cytokine environment and the relative strength and endurance of its interaction with antigen and T-helper cells.

In a previous study we presented direct evidence that EBV-infected cells transiting from activated lymphoblast to memory B cells are found to physically reside in tonsil GCs and participate in the GC reaction (46). This included the expression of bcl-6 and AID, defining makers of the GC, and GC-specific chemokine receptors that would cause them to be retained in the GC. Moreover, the cells express markers associated with...
centrocytes and centroblasts, suggesting that EBV-infected GC cells also cycle through rounds of proliferation and resting. Thus, in all aspects that we analyzed, EBV-infected cells were indistinguishable from normal B cells undergoing a GC reaction. The exception was that small numbers of EBV-infected cells were detected in the GCs, and they did not show signs of undergoing the typical massive proliferation and expansion characteristic of GC cells.

EBV latent protein expression in tonsil GC cells is limited to three proteins, EBNA1, LMP1, and LMP2 (6, 46). The major function of EBNA1 is to tether and direct the replication of the viral genome, which is episomal in the latent state (34). LMP1 and LMP2, on the other hand, were previously shown in experimental systems to have all the signaling functions necessary to potentially drive an EBV-infected cell into and through the GC reaction to become a memory B cell (8, 10, 16, 19, 42, 53). This led us to propose originally that EBV-infected cells undergoing a GC reaction may receive all of the necessary signals from LMP1 and LMP2 without the necessity of interacting with antigen or T-cell help or even physically residing in the follicle (23, 55, 56). However, a phenotypic and functional analysis of latently infected GC cells in the tonsils showed no obvious impact of LMP1 and LMP2 on the behavior of the cells (46). Furthermore, analyses of expressed immunoglobulin genes in latently infected memory B cells found in the periphery suggested that they had undergone selection by antigen (50, 51). This led to the conclusion that the potent signaling effects of LMP1 and LMP2 observed with experimental systems were artifacts, produced by constitutive, unregulated expression, that may be more relevant to EBV-associated diseases. The normal roles of these two proteins in vivo must be more modest, i.e., not so much to drive the GC process but to provide EBV-infected cells a survival advantage in the highly competitive environment of the GC. Specifically, we previously suggested (46) that LMP2 could augment B-cell receptor signaling so that the EBV-infected cells would not have to effectively compete for cognate antigen and that extended CD40 signaling, provided by LMP1, could ensure that the latently infected cells were steered toward the memory compartment (4) for long-term persistence.

In the current study we demonstrate that EBV-infected cells in the GCs of healthy carriers undergo extensive proliferation but without the concomitant massive expansion. The data support a simple model whereby the virus is maintained via a balance between proliferation and cell death. These results provide a direct link through the GC between new EBV infection of B cells in the tonsils and the memory B cells in which the virus persists. In addition, we demonstrate a differential expression of viral latent proteins that calls into question previously published results from in vitro studies.

MATERIALS AND METHODS

Cells and cell lines. The EBV-positive (EBV + ) lymphoblastoid cell line B95-8 (gift of Eliot Kieff) was performed on the samples as described above, and the signal was compared supplemented with 10% fetal bovine serum, 2 mM glutamine, 2 mM sodium pyruvate, and 100 IU of penicillin-streptomycin (culture medium).

This study was approved by the Tufts Medical Center Institutional Review Board. Heparinized blood was collected from healthy volunteers by venipuncture. Human palatine tonsils were obtained from patients 18 years of age or younger receiving a tonsillectomy performed at the Tufts Medical Center, Boston, MA. Tonsil lymphocyte suspensions were derived by mincing the tissue in phosphate-buffered saline (PBS) plus 1% bovine serum albumin (BSA). Mononuclear cells were isolated from blood or tonsil suspensions by using Ficoll-Paque Plus (Fisher Scientific) centrifugation as described previously (6). B cells were isolated by using StemSep according to the manufacturer’s instructions. B-cell subpopulations were purified by fluorescence-activated cell sorting (FACS) as described previously (46).

Histology staining and dissection of tonsil tissues. Tonsil tissue sections were prepared for dissection as described previously (46). Stained sections were examined under a microscope, and either the GC, mantle zone, or interfollicular region was dissected by scraping out the cells using a sterile scalpel and placing them into a RNase-free Eppendorf tube filled with PBS plus 5% BSA on ice. For whole-single-GC dissections, individual GCs were identified from sequential slices, and the sequential, dissected tissue regions were pooled separately for each GC. Mantle zones and interfollicular regions were pooled from multiple sections from up to 30 slides.

Limiting-dilution analysis, EBER1 RT-PCR, and W-repeat DNA PCR. Limiting-dilution analysis, EBER1 RT-PCR, and W-repeat DNA PCR were performed as described previously (46).

Calculation of the total number of cells and the number of EBV-infected cells in GC dissections. For each tonsil to be analyzed by scraping/dissection, a piece was set aside for the purification of GC cells (CD19+ CD3−) by StemSep. To estimate the number of cells in dissection specimens, a serial dilution of flow-purified GC cells was made and aliquoted into sterile Eppendorf tubes, usually with five replicates for each dilution. Each of the replicates was further diluted 1/2, 1/4, and 1/8. cDNA was synthesized, and quantitative RT-PCR (qRT-PCR) for CD10 or β-actin was then performed for each dilution as described previously (46). A calibration curve of cell number versus CD10 or β-actin qRT-PCR cycle number was plotted and used to convert the CD10 or β-actin qRT-PCR signal obtained from the individual dissected tissues from the same tonsil to an estimate of cell number.

To estimate the number of infected GC cells, limiting-dilution analysis was performed by using isolated CD19− CD19+ cells. The cycle number for single cells was then estimated from the quantitative PCR readout for either EBER1 RT-PCR or W-repeat DNA PCR at the limit dilution. For EBER1, this was 40 cycles (standard deviation of 1), and for the W-repeat, this was 38 cycles (standard deviation, 2.2). Note that the larger standard deviation for the W-repeat sequence is consistent with and presumably due to the range of episome copy numbers (1 to 20 copies) present in single cells, as shown in Fig. 5. These values were then used to estimate the number of EBV-infected cells in the pooled dissection specimens by using real-time PCR for EBER1 RNA or the W-repeat DNA sequence.

Estimation of episomal copy number. The viral genome copy number was calculated for tonsil GC B cells or newly infected B cells in culture. Prior to analysis, tonsil GC B cells were isolated by FACS as described above, and the frequency of infected cells was estimated by limiting-dilution W-repeat DNA PCR as described above and as described previously (46). GC B cells were subsequently sorted into a 96-well V-bottom plate at numbers predicted to give one infected cell per sample. In all, three separate tonsils were analyzed.

For newly infected cells, the following protocol was performed. StemSep-purified B cells were infected at a density of 105 cells/ml by resuspending cells into undiluted supernatant from the B95-8 cell line. The supernatant was prepared as described previously (28). After 2 to 3 h at 37°C, an equal volume of fresh medium was added, and the cells were incubated overnight at 37°C. The cells were then spun down and resuspended at 106 cells/ml in prewarmed (37°C) PBS plus 0.1% BSA (PBSA), to which 2 μl of a 5 mM stock of CFSE (carboxyfluorescein diacetate succinimydyl ester) in dimethyl sulfoxide (DMSO) per ml of cells (final concentration, 10 μM CFSE) was added. The cells were then incubated for 10 min at 37°C, followed by the addition of 2 volumes of culture medium and incubation on ice for 5 min. The cells were then washed five times, once with prewarmed medium and four times with ice-cold medium. The cells were then harvested at various times postinfection and analyzed for the number of cell divisions based on CFSE staining using FACS. Cells that had undergone 0, 1, 2, 3, and 4 divisions were sorted singly into the wells of a 96-well V-bottom plate that contained 106 EBV-negative filler CB59 cells.

To estimate genome copy number, W-repeat quantitative DNA (qDNA) PCR was performed on the samples as described above, and the signal was compared.
to that obtained from a standard curve of cells with a known genome copy number. To generate this curve, dilutions of IB4 (4 genomes/cell [26]) and Namalwa (2 genomes/cell [27]) cells were made by FACS that contained five replicates each of 10,000, 100, 10, 5, 3, 2, and 1 cells, and the cell number was brought to 10⁴, when necessary, through the addition of CB59 cells. W-repeat qDNA PCR was then performed as described above. An analysis was accepted if the standard curve yielded a straight line over the entire range of cell numbers tested when the log of the genome copy number was plotted versus the threshold cycle (C_{T}) value from the qDNA PCR.

RESULTS

Distribution of EBV-infected cells in tonsil GCs. In a previous study we developed a novel technique to dissect and pool sections of GCs from human tonsils (46). We have modified this technique to study whole individual GCs. Sections of snap-frozen tonsils were placed onto slides, stained with hematoxylin and eosin (H&E), and examined under a microscope to identify sequential slices through individual GCs (Fig. 1a). Areas corresponding to sections of the same GC were then scraped off the slide, pooled, and subjected to analysis. To estimate the number of EBV-infected cells in each GC, we took two completely independent approaches, one based on RT-PCR and the other based on DNA PCR. We reasoned that if the two approaches produced comparable results, we could have confidence that the values were not produced by an artifact. For the first method, we measured the total number of GC cells in the pooled GC scraping samples by performing RT-PCR for the GC-specific marker CD10. We then used a CD10 RT-PCR calibration curve, generated from CD10-positive cells from the same tonsil purified by FACS, to estimate the number of whole-cell equivalents in the GC dissection pools. To estimate the number of EBV-infected cells, we used the same approach except that we performed RT-PCR for the EBV-specific RNA EBER1. For the DNA-based method, we estimated total cell numbers by performing DNA PCR for a genomic sequence (β-actin) and the numbers of EBV-infected cells by DNA PCR for the W-repeat sequence of the viral genome. Detailed results for the EBV-positive GCs from three separate tonsils are summarized in Table 1, and the results for all of the GCs analyzed, both EBV positive and negative, are...
summarized in Table 2. From a total of 96 GCs analyzed, the average number of cells per GC ranged from 1.6 × 10⁴ to 2.6 × 10⁵ cells. The estimates based on the RNA approach (average, 1.87 × 10⁵ cells) consistently gave higher values than did estimates based on the DNA approach (average, 6.0 × 10⁴ cells). However, the overall difference was only about 3-fold, and all the estimates are within the range of previously reported estimates that GCs contain 10⁴ to 1.5 × 10⁵ GC cells (33). This provided confidence that our techniques were working correctly. When we analyzed the presence of EBV, the results from both techniques were again in good agreement with the average number of EBV-infected cells, varying from 1.5 to 4.6 cells per GC depending on the tonsil and method of measurement. Again, the RNA approach gave slightly but consistently higher estimates than the DNA-based technique (average of 3.8 versus 2.6 cells). Nevertheless, given the completely independent methods of measurement, the results were in remarkable concordance, indicating that 38 to 55% (average, 45%) of GCs contained EBV-infected cells and that there were, on average, approximately 3.5 infected cells (range, 1 to 9 cells) per EBV-positive GC.

The distribution of EBV-infected GC cells is consistent with a simple proliferation model.

<table>
<thead>
<tr>
<th>Table 1. Estimated numbers of EBV-infected cells in whole-GC dissection specimens based on RNA or DNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonsil and parameter</td>
</tr>
<tr>
<td>1. No. of positive GCs/no. of GCs dissected (%)</td>
</tr>
<tr>
<td>Avg. no. of cells (10⁵)/GC (SEM)a</td>
</tr>
<tr>
<td>Avg. no. of EBV⁺ cells/GC</td>
</tr>
<tr>
<td>2. No. of positive GCs/no. of GCs dissected (%)</td>
</tr>
<tr>
<td>Avg. no. of cells (10⁵)/GC (SEM)a</td>
</tr>
<tr>
<td>Avg. no. of EBV⁺ cells/GC</td>
</tr>
<tr>
<td>3. No. of positive GCs/no. of GCs dissected (%)</td>
</tr>
<tr>
<td>Avg. no. of cells (10⁵)/GC (SEM)a</td>
</tr>
<tr>
<td>Avg. no. of EBV⁺ cells/GC</td>
</tr>
<tr>
<td>All. No. of positive GCs/no. of GCs dissected (%)</td>
</tr>
<tr>
<td>Avg. no. of cells (10⁵)/GC (SEM)a</td>
</tr>
<tr>
<td>Avg. no. of EBV⁺ cells/GC</td>
</tr>
</tbody>
</table>

a Estimated using a CD10 (RT-PCR) or β-actin (DNA PCR) standard curve from CD19⁺ CD10⁺ cells sorted by FACS obtained from the same donor.

b Estimated based on EBER1 (RNA) RT-PCR of CD19⁺ CD10⁺ cells sorted by FACS obtained from the same donor.

c Estimated based on W-repeat (DNA) RT-PCR of CD19⁺ CD10⁺ cells sorted by FACS obtained from the same donor.

<table>
<thead>
<tr>
<th>Table 2. Estimated number of EBV-infected GCs and number of EBV-infected cells per GC from whole-GC dissection specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonsil and parameter</td>
</tr>
<tr>
<td>1. No. of positive GCs/no. of GCs dissected (%)</td>
</tr>
<tr>
<td>Avg. no. of cells (10⁵)/GC (SEM)a</td>
</tr>
<tr>
<td>Avg. no. of EBV⁺ cells/GC</td>
</tr>
<tr>
<td>2. No. of positive GCs/no. of GCs dissected (%)</td>
</tr>
<tr>
<td>Avg. no. of cells (10⁵)/GC (SEM)a</td>
</tr>
<tr>
<td>Avg. no. of EBV⁺ cells/GC</td>
</tr>
<tr>
<td>3. No. of positive GCs/no. of GCs dissected (%)</td>
</tr>
<tr>
<td>Avg. no. of cells (10⁵)/GC (SEM)a</td>
</tr>
<tr>
<td>Avg. no. of EBV⁺ cells/GC</td>
</tr>
<tr>
<td>All. No. of positive GCs/no. of GCs dissected (%)</td>
</tr>
<tr>
<td>Avg. no. of cells (10⁵)/GC (SEM)a</td>
</tr>
<tr>
<td>Avg. no. of EBV⁺ cells/GC</td>
</tr>
</tbody>
</table>

a Estimated using a CD10 (RT-PCR) or β-actin (DNA PCR) standard curve from CD19⁺ CD10⁺ cells sorted by FACS obtained from the same donor.

b Data from Table 1.
EBV-infected cells in a large number of independent GCs, we were in a position to describe and analyze the distribution of these cells between GCs. To do this, we used only the data obtained based on EBER1 expression. We did not use the data based on W-repeat DNA PCR because we found a wide variation in the DNA PCR signal between single GC cells. This was, as shown later, because the genome copy number in any given infected cell in the tonsil ranges from 1 to 20. Since the number of infected cells in any given GC is small, the variation in per-cell genome numbers makes estimations of cell numbers from genome copy numbers unreliable for any given GC. However, the RT-PCR signal for EBER1 was remarkably constant between single GC cells (see Materials and Methods for a detailed discussion of this issue). Therefore, we used only the EBER1 data to estimate the distribution. This is displayed in Fig. 2 and demonstrates a simple distribution where most of the GCs had only one EBV-infected cell, with 9 cells being the most that were detected. We have compared this observed distribution with two simple models (Fig. 2A and B). The first model assumes that infected B cells are randomly entering and leaving GCs, so the resulting distribution should follow a Poisson distribution. We calculated the expected distribution predicted from the Poisson distribution using either the measured average number of EBV-infected cells in all GCs or the average number computed from the Poisson equation using the fraction of GCs that lack EBV. Both methods yielded similar results. In Fig. 2A, we show the distribution derived by the latter method compared to the actual values. Chi-square analysis of these two data sets yielded a P value of <0.001, indicating that the data do not fit a Poisson distribution. The second model assumes that the cells enter GCs randomly but then proliferate. Given that the largest number of infected cells per GC that we detected was 9, we tested a model limited to 3 divisions (“three and out”). The expected results from this model are shown in Fig. 2B. Chi-square analysis of these data versus what was observed gave a P value of 0.48, whereas 4 divisions gave a P value of 0.03. Thus, the data approximate a simple model whereby EBV-infected cells randomly enter the GC and undergo ~3 divisions.

**EBV-infected GC cells undergo proliferation and maturation.** The conclusions described above predict that, despite their small numbers, EBV-infected GC cells are proliferating. To test this, we have isolated proliferating GC cells from tonsils by FACS after staining with the GC-specific marker CD10 and the proliferation-associated marker Ki67 (15). The frequency of EBV-infected cells in each population was assessed by performing a limiting-dilution analysis for cells expressing the ubiquitous EBV-encoded small RNA EBER1. In addition, the cells were also tested for the expression of the latent genes LMP1 and LMP2. The results from the analysis of three separate tonsils are shown in Fig. 3. It is apparent that the latently infected GC cells are equally distributed between the Ki67-positive and -negative fractions. Interestingly, although the cells in both populations were LMP1 positive, there was a trend toward a significantly smaller fraction of the proliferating population expressing LMP2.

When GC cells receive appropriate rescue signals, they up-regulate the antiapoptotic gene bcl-2 (29, 36). To test if EBV-infected GC cells undergo a similar process, we repeated the analysis described above but this time stained for bcl-2 expression instead of Ki67. The results for three tonsils are shown in Fig. 4. It is apparent that EBV-positive cells were contained in both populations, with a slight trend toward a higher frequency in the bcl-2-positive (bcl-2+) population, suggesting that EBV-infected GC cells undergo normal maturation. Both bcl-2+ and bcl-2-negative (bcl-2−) EBV+ GC cells expressed LMP1, demonstrating that unlike in vitro studies, LMP1 expression in vivo does not always correlate with bcl-2 expression. However, the bcl-2+ population did not express detectable levels of LMP2 or EBNA1, suggesting that the expression of these two genes may be extinguished prior to the cells leaving the GC. This is the first example where we have seen a discordant expression of LMP1 and LMP2 in any non-tumor-infected cell type in vivo.

**EBV-infected GC cells undergo extensive proliferation but do not expand.** From the data described above, it is apparent that the EBV-infected cells in the GC are proliferating but never achieve numbers greater than 8 to 9 cells per GC. Thus, it appears that they do not undergo the massive expansion characteristic of normal GC B cells. However, we have shown
previously that the resulting latently infected memory B cells have accumulated normal levels of somatic hypermutations (6/100 bp [50, 51]) in their immunoglobulin genes, which would require many cell divisions to accumulate given the previously reported rate of somatic hypermutation in the GC of 1/10³ per division (3, 38, 47). To investigate this contradiction, we exploited the fact that the average EBV genome copy number increases in a newly infected population as it proliferates. We have shown previously that upon infection in vitro, only a single viral genome forms an episome (28, 30). The genome copy number then amplifies as the cells proliferate until a heterogeneous population of cells is produced in long-term culture, with an average number of episomes of around 7 to 10 per cell but with a highly skewed distribution, with small numbers of cells having 15 or more copies (40). These two conditions are exemplified in Fig. 5A to C, which show the distribution of episomes in newly infected peripheral B cells that had undergone 1 (Fig. 5A), 3 (Fig. 5B), or 20 to 25 (Fig. 5C) divisions in culture. As expected, the population that had undergone only one division consisted almost entirely (92%) of cells with a single viral genome, and no cell had more than two genomes. Even after 3 divisions, 40% of the cells contained only one genome, and two-thirds had 2 genomes or fewer, with no cell having more than 8 genomes. In comparison, cells that had undergone 20 to 25 divisions had reached equilibrium, with a mean of 10.1 episomes per cell (standard deviation, 8.7). Only ~10% had two genomes or fewer. Figure 5D shows the result when we analyzed the viral genome copy numbers in latently infected tonsil GC B cells from three separate tonsils. The resulting distribution was not uniform, with about one-third of the cells containing only one genome and the remainder containing a wide range of copy numbers (mean, 5.5; standard deviation, 5.0). From this analysis, it appears that latently infected tonsil GC cells are a mixture of cells that have undergone very few divisions and cells that have undergone many divisions. To demonstrate this, we show the results of two simple modeling experiments in Fig. 6. In the first case, we assumed that the cells followed the simple “three-and-out” model described above and that the tonsil would therefore be a mixture of cells that had undergone 0, 1, 2, or 3 divisions. Given these assumptions, we derived a predicted distribution by combining the observed episome distribution curves for cells that had undergone 0, 1, 2, or 3 divisions, giving equal weight to each group. This distribution was then normalized to the total number of cells, 32 cells, in the observed tonsil distribution. The predicted and observed distributions are shown in Fig. 6A. From the data shown in Fig. 6A, it is apparent that the simple “three-and-out” model does not explain the epi-
some distribution that we found in the tonsil GC cells (P value from chi-square analysis of <0.001). In the second model, we combined the distribution from the “three-and-out” hypothesis (Fig. 6A) with the distribution for cells that had undergone many divisions (Fig. 5C), giving equal weight to both distributions. Again, this distribution was normalized to the total number of cells, 32, in the observed tonsil distribution, and the predicted and observed distributions are shown in Fig. 6B. As shown in Fig. 6B, this result approximates what was actually seen for tonsil GC cells (P value from chi-square analysis of 0.28). We conclude that the tonsil GC consists of a mixture of cells, most of which undergo only approximately three divisions before they die or leave but a subset of which continues to proliferate without expanding; i.e., they must be dying/leaving at the same rate at which they are proliferating.

**EBV-positive cells are not undergoing a GC reaction outside of the follicle.** The studies described above and previously (46) demonstrate that EBV-infected cells reside in GCs and are participating in a GC reaction. We wished to address the question of whether such cells were restricted to GCs in the follicle or whether EBV-infected cells could also undergo the GC reaction outside of the follicle. To test this, we extended our dissection technique to include the mantle and interfollicular regions. Figure 1d to g demonstrates the sequential dissection of these regions in and around a GC. Multiple dissection specimens were then pooled and assessed for total cell numbers based on β-actin expression and for the presence of GC cells based on the expression of the GC-specific markers CD10 (43) and bcl-6 (58). Lastly, the presence of EBV in the pooled dissection specimens was assessed based on EBER1 expression. The data for the three tonsils are summarized in Table 3. As expected, cells expressing the two GC-specific markers and EBV were routinely detected in the GC dissection pools. The actual estimated cell number was always significantly smaller based on the two GC markers compared to β-actin. This is not surprising since the GC dissection specimen would also include stromal and other non-B cells that physically reside in the GC. However, we were unable to detect any cells expressing the GC markers in either the mantle or interfollicular region, although infected cells were detected readily in the latter region. The interfollicular region is believed to contain latently infected memory B cells, plasma cells replicating the virus, and newly infected naïve B cells (6, 32). This would explain our detection of infected cells in dissection specimens from this region. To confirm this supposition, we tested the interfollicular tonsil tissue fraction for the expression of marker genes for new infection (EBNA2) and virus replication (BZLF1). As expected, we found EBNA2 expressed in one-third (2/6 samples) and BZLF1 in one-half (3/6 samples) of the EBER-positive interfollicular dissection specimens that we tested. Interestingly, we have never found evidence for the presence of EBV-infected cells in the mantle zone with any of the PCR methods that we tried. These results demonstrate that there are no detectable EBV-infected cells in the mantle zone and that the EBV-infected cells in the interfollicular region are not undergoing a GC reaction.

**DISCUSSION**

The current model of EBV persistence holds that the virus infects naïve B cells in Waldeyer’s ring and activates them using the growth transcription program (latency 3) so that they can then differentiate through a GC process to become resting memory B cells, the site where the virus persists. We have previously shown that EBV-infected cells in the GCs of tonsils from healthy carriers of the virus are undergoing a GC reaction.
In the model of EBV persistence, the virus expresses the growth program (latency 3) in newly infected naïve B cells in order to drive them to become activated lymphoblasts, thereby allowing them to subsequently differentiate into memory B cells via the GC. Naïve B cells emerge from the peripheral circulation into the tonsil via high endothelial venules (HEV) and then migrate through the interfollicular region to take up residence in the follicular mantle zone. The observation that we detected no infected cells in the mantle zone implies, therefore, that naïve B cells become infected as they transit from the HEV to the follicle and directly enter the GC without residing in the mantle zone. This scenario is completely consistent with our suggestion, based on the expression of BZLF1 and EBNA2, that virus production and new infection occur in the interfollicular region. Furthermore, we observed that about one-third of the latently infected GC cells have only one episome. This raises the possibility that newly infected naïve B lymphoblasts undergo very limited proliferation prior to entering the GC, which is in contrast to the behavior of newly infected lymphoblasts in vitro, which proliferate indefinitely. This behavior in vivo is consistent with an idea that we proposed previously, that the growth program in vivo is self-limiting. In this scenario, the purpose of the growth program is to activate newly infected B cells so that they can enter the GC and not to drive proliferation per se (54, 56). Consequently, the continuous proliferation of in vitro-infected lymphoblasts expressing the growth program may be a consequence of the failure of the cells to have access to a GC environment.

We have shown that, on average, there are 3 to 4 infected cells per EBV-positive GC, with a range of 1 to 9 cells. This is consistent with data from our previous qPCR studies (46) and immunohistochemical studies reported previously by others (25, 41) suggesting that there are small numbers of EBV-infected cells in the GC. We are confident in our results for

![Graph](image-url)

**FIG. 6.** Comparison of the observed distribution of viral genomes in tonsil B cells with that predicted by two models. (A) The observed distribution (black bars) is compared to the expected outcome if the cells divide three times and then either leave or die (white bars). The expected outcome was derived by combining equal portions of the genome distributions obtained from cells that had divided 0, 1, 2, and 3 times and then normalizing the data to 32 cells (the total number of cells in the observed tonsil distribution). (B) The observed distribution (black bars) is compared to the expected outcome if half the cells divide three times and then either leave or die and half undergo a large number of divisions (white bars). The expected outcome was derived by combining the results from Fig. 5C and 6A and then normalizing the results to 32 cells (the total number of cells in the observed tonsil distribution).

but do not undergo the massive expansion characteristic of GC cells (46). However, the memory B cells, in which the virus persists, show clear evidence, based on the extent of somatic hypermutation in their immunoglobulin genes (6/100 bp) (50, 51), of having proliferated extensively while in the GC. In this paper we have now resolved this issue. It is apparent that EBV-infected cells in the GC, expressing the viral default transcription program, are indeed proliferating and maturing and that some cells proliferate extensively yet fail to expand.

The only logical conclusion to draw is that an equilibrium is achieved where the proliferating cells are dying at a rate equal to their rate of proliferation. We hypothesize that the cells are dying either because of predation by cytotoxic T lymphocytes (CTLs) that recognize LMP1 and/or LMP2 or because there is some limitation on survival signals or factors in the GC that the EBV-infected cells require.

<table>
<thead>
<tr>
<th>Tonsil and region</th>
<th>Total no. of cells detected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All cells&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>2.5 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>MZ</td>
<td>9 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>IF</td>
<td>8 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>8 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>MZ</td>
<td>5 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>IF</td>
<td>1.6 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>1.3 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td>MZ</td>
<td>2 × 10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>IF</td>
<td>9.6 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Estimated using a β-actin RT-PCR standard curve generated from CD19<sup>+</sup> CD10<sup>+</sup> tonsil cells sorted by FACS.

<sup>b</sup> Estimated using a CD10 RT-PCR standard curve generated from CD19<sup>+</sup> CD10<sup>+</sup> tonsil cells sorted by FACS.

<sup>c</sup> Estimated using a bcl-6 RT-PCR standard curve generated from CD19<sup>+</sup> CD10<sup>+</sup> Bcl-6<sup>+</sup> tonsil cells sorted by FACS.

<sup>d</sup> Estimated based on EBER RT-PCR cell cycle analysis from a limiting dilution of CD10<sup>+</sup> cells sorted by FACS.

<sup>e</sup> IF, interfollicular zone; MZ, mantle zone.
There are approximately $1.2\times10^5$ mature B cells in the average adult (estimated from (14)). There are $\approx10^5$ mature B cells in Waldeyer’s ring (based on our own measurements). Overall 30% of B cells are memory cells i.e.:

- $4\times10^7$ total memory B cells in the average adult and
- $3\times10^8$ memory B cells in the average adult Waldeyer’s ring.

The median frequency of EBV infected memory B cells is $1.8\times10^5$ in the tonsil and $1\times10^6$ (15 fold lower(31)) in the rest of the lymphoid tissue. Therefore, the median level of infection is $5.4\times10^4$ infected memory B cells in the tonsil and $4\times10^4$ in the rest of the lymphoid tissue.

Therefore the total number of infected memory B cells is $9.4\times10^8$.

The half-life of a latently infected memory B cells is about 7 days(18).

Therefore $4.7\times10^4$ memory B cells die every 7 days or $6.7\times10^3$ per day.

There are approximately $10^{10}$ B cells in Waldeyer’s ring of which $\approx30\%$ are GC cells. From the current study and previous estimates we can assume that there are approximately $10^5$ GC cells per GC or about $3\times10^3$ GCs in Waldeyer’s ring. From the current study about half of these are infected or $1.5\times10^6$.

The simple proliferation model suggests that the cells undergo about 3 divisions and then leave or die. The average division time for GC cells has been estimated to be 6-12 hours (2).

Therefore it would take about 1 day for the 3 divisions that would produce 8 cells.

If these cells all leave then this would produce $1.2\times10^5$ memory cells per day.

Comparing lines A and B it is apparent that $>90\%$ of the GC production must die before entering the circulation.

FIG. 7. Most of the EBV-infected GC B cells must die.

several reasons. First, we arrived at the same conclusion using two completely different techniques, one based on detecting the viral small RNA EBER1 and the other based on the detection of viral DNA. Second, we have shown that only EBV-infected cells located within the GC express the GC-specific markers CD10 and bcl-6. Thus, we may conclude that all EBV-infected cells undergoing a GC reaction reside in the GC. Conversely, we can conclude that EBV does not detectably drive a GC reaction independently of residence in the follicle.

We can exclude other models that could account for small numbers of infected B cells in the GC. For example, based on recent studies of mice, employing two-photon vital microscopy, we know that GCs are dynamic open structures where non-GC cells may occasionally pass through (48). Therefore, one possibility is that we are detecting EBV-infected cells that are randomly passing through the GC. However, this suggestion implies that the distribution of the cells should follow a Poisson distribution, which it does not, and we have shown that the cells are proliferating within the GC and not just passing through. In addition, we have shown previously that infected GC cells express chemokine receptors that would tend to attract them to and retain them in the GC (46).

We have previously estimated the half-life of EBV-infected memory B cells (18) and the median number and range (14, 31) of these cells present in healthy carriers. As detailed in Fig. 7, this leads to the prediction that a median number of $6.7\times10^3$ memory cells will die each day and need to be replaced by either homeostatic cell division or new infection to maintain the stable levels that we observed over time. Based on the data in this paper, we might assume that the infected GC cells undergo, on average, only three divisions. Given the number of GC cells in Waldeyer’s ring, the average number of cells per GC, and the expected output of EBV-infected cells per GC, we can then estimate that Waldeyer’s ring would produce, on average, about $10^5$ memory cells per day (Fig. 7). This would mean that $>95\%$ of the GC output must die before they enter the periphery, which fits well with our conclusion that the infected GC cells are dividing but that the vast majority of them die. Thus, the simplest model is that, on average, after every three rounds of cell division, only one cell survives to undergo another three rounds of division. Therefore, we favor a model whereby the time needed for three divisions is about the time available for an infected cell in the GC to proliferate before most of its progeny is either destroyed by CTLs or dies due to some limiting survival signal or factor.

If this model is correct for persistent infection, what happens in the first days of an acute infection? One possibility is that early in infection, EBV-infected cells undergo large-scale expansion in the GC. Indeed, if Waldeyer’s ring is the main source of latently infected memory cells, it is difficult to conceive how the extremely large numbers of such cells present during acute infection (24) could be produced without such a massive involvement of the GCs. The CTL response would then arise and effectively shut down this process. Thus, when patients with infectious mononucleosis (acute EBV infection) arrive at the clinic, they already have a very highly active CTL response (9) and a rapidly declining level of infection in the blood (18). Subsequently, the latently infected memory B cells would be maintained by normal homeostasis (23, 50). The alternate hypothesis is that the number of EBV-infected cells in the GC is small due to some limiting survival factor. In this case, the numbers per GC may remain low even during the
acute phase of infection. Current evidence would seem to favor the latter hypothesis because only a few infected cells have been found in the GCs of tonsils from patients with infectious mononucleosis patients (41). However, it is unclear if these small numbers are representative of acute infection or reflect the fact that the infection is already resolving by the time clinical symptoms occur (18). For example, at day 14 after infection with mouse gammaherpesvirus 68 (MVH68), up to 15% of all splenic GC cells contain the virus, but this number rapidly declines to 5% by day 18 (11), and it is generally accepted that the symptoms of infectious mononucleosis do not arise for at least 5 weeks postinfection (22).

One crucial issue is whether any of the infected GC cells produced during persistent infection actually enter the memory compartment or if they all die. Specifically, is a continuous supply of new latently infected memory B cells required to maintain stable levels of persistently infected B cells in the blood, or is homeostasis sufficient? The latter scenario would predict that all of the latently infected memory cells present during persistent infection originated during the acute phase of infection. This distinction is nontrivial since if continual new infection is required, then extremely potent drugs that block viral replication, or a highly effective vaccine that prevented reinfection, should result in a progressive and relentless loss of the virus, i.e., the infection could, in principle, be cleared. If, however, homeostasis mechanisms are sufficient, then such treatments will be wholly ineffective. Unfortunately, no drugs or vaccines potent enough to test this hypothesis currently exist (49).

The observations presented in this paper reinforce our previously reported conclusions about the necessity of confirming in vivo any observations made in vitro or with transgenic mice. First, our data raise the possibility that cells expressing the LMP1 to turn off bcl-6 and turn on bcl-2.

In conclusion, we have presented quantitative measurement to show that EBV-infected cells undergoing a GC reaction in persistently infected tonsils are restricted to the GC. There, they undergo extensive proliferation and maturation but do not increase in number, indicating that an equilibrium is in place between cell growth and death.

Acknowledgments

We thank Michael Shapiro for help with statistical analysis and modeling and Steve Kwok and Allen Parmelec for FACS.

Dynamics of EbV in the Germinat Center

This work was supported by Public Health Service grants RO1 CA65883, RO1 AI18757, and RO1 AI062989 to D.T.-L.

References


