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The Expression Pattern of Epstein-Barr Virus Latent Genes In Vivo Is Dependent upon the Differentiation Stage of the Infected B Cell

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Summary

Epstein-Barr virus–infected B cells in vivo demonstrate three distinct patterns of latent gene expression, depending on the differentiation stage of the cell. Tonsillar naive B cells express the EBNA2-dependent lymphoblastoid phenotype, characteristic of direct infection. Germinal center centroblasts and centrocytes as well as tonsillar memory B cells express a more restricted pattern of latent genes (EBNA1(Q-K)$^+$, LMP1$^+$, LMP2$^+$, EBNA2$^-$) that has only been seen previously in EBV-positive tumors. Peripheral memory cells express an even more restricted pattern where no latent genes are expressed, with the possible exception of LMP2. These results are consistent with a model where EBV uses the normal biology of B lymphocytes to gain access to and persist within the long-lived memory B cell compartment.

Introduction

Epstein-Barr virus is a B lymphotropic human herpesvirus. The virus persists in the peripheral blood in a very specific subset, the resting memory B cell (Miyashita et al., 1997; Babcock et al., 1998). These cells express little or no viral genetic information (Qu and Rowe, 1992; Babcock et al., 1999)—a condition we have referred to as the "latency program" (Thorley-Lawson and Miyashita, 1996). As such, they constitute no pathologic threat and may be invisible to the host's immune system. This behavior contrasts with the events seen in newly infected B cells in vitro. In this case, the virus has no subset preference but indiscriminately infects any B cell and drives it to become an activated lymphoblast through the concerted expression of a set of latent genes under the regulation of the transcription factor EBNA2 (reviewed in Kieff, 1996). The latent genes encode six nuclear antigens (EBNAs) and three membrane proteins (LMPs). We have referred to this as the "growth program" (Thorley-Lawson and Miyashita, 1996) because the latently infected blasts are highly proliferative. As such, they are potentially dangerous for the host, but they are also strongly immunogenic and are readily recognized and controlled by cytotoxic T cells (reviewed in Khanna et al., 1995).

These observations lead to two critical questions. First, how does a potent growth-promoting virus achieve the quiescent state observed in the resting peripheral memory cells? Second, if the virus can persist in this quiescent state, why does it possess a latency mechanism, the growth program, that places the host at risk of developing life-threatening neoplasias? The simplest explanation is that EBV infects resting B cells directly in vivo and remains quiescent. Any infected cells that express the growth program would be eliminated by CTL, explaining why the virus is only detected in resting B cells. This explanation is unsatisfactory for a number of reasons. First, there is no evidence that an EBV-infected resting B cell can stay resting. All of the in vitro studies suggest that EBV is poised to initiate the growth program when it infects a resting B cell. Second, it does not explain why the virus is restricted to memory cells. Third, this model offers no explanation for the existence of the growth program.

We assume that the growth program is in some way essential for establishing and/or maintaining the persistent infection. If it was not essential, evolutionary pressure would have eliminated it because it is potentially detrimental to the host. In what way could the growth program be essential? One possibility is that it provides a mechanism to replicate and spread the virus. Replication is unlikely, since viraemia will produce much more virus more quickly than dividing cells. Spreading the virus is more likely because latently infected B cells may be able to distribute the virus to all of the lymphoid tissues to establish a disseminated persistent infection. The difficulty is that a mechanism is required that allows the latently infected proliferating lymphoblasts, expressing the growth program, to exit the cell cycle and become resting, and this mechanism must only apply to infected memory cells to account for the restriction of EBV to resting memory cells in the periphery. To resolve this issue, we have turned to normal B cell biology. We have proposed (Thorley-Lawson and Babcock, 1999) that the growth program is essential for the establishment of latency because it allows the latently infected B cells to differentiate into resting memory B cells. In this model, the EBV-activated lymphoblast, driven by the growth program, is equivalent to a normal antigen-activated B cell blast, which can differentiate into a resting memory cell through the process of germinal center formation (reviewed in MacLennan, 1994; Liu and Arpin, 1997). In this process, antigen activates B cells to become blasts that enter primary follicles, proliferate, and alter their Ig genes through isotype switching and hypermutation. At intervals, these centroblasts stop dividing and become centrocytes. If cognate antigen and T cell help are available, the cells will again proliferate, if not, they die. Eventually, the cells exit the cell cycle either by becoming antibody-secreting plasma cells or, as antigen begins to wane, memory cells.

We have proposed that EBV infects normal resting B cells in the lymphoid tissue and drives them to become proliferating blasts through the EBNA2-dependent growth program. It does this to activate them so they can then differentiate through a germinal center. Latently infected cells could survive germinal center selection by expressing the latent proteins LMP1 and LMP2. LMP1 can, in a ligand-independent fashion, mimic the

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signal generated by CD40 (Gires et al., 1997) on the B cell when it interacts with an antigen-specific T helper (Th) cell. Similarly, LMP2 can, in a ligand-independent fashion, mimic the rescue signal delivered by the antigen receptor (Caldwell et al., 1998). Thus, LMP1 and LMP2 have the potential to provide the necessary signals, independent of real antigen and T cell help, to allow latently infected cells, which have entered the germinal center, to survive and differentiate into memory cells. Evidence to support this idea comes from studies suggesting that the EBV-associated tumor Hodgkin's disease (HD) originates from a germinal center or memory B cell (Kuppers and Rajewsky, 1998) and only expresses EBNA1, LMP1, and LMP2 (Pallesen et al., 1991; Oudejans et al., 1996; Niedobitek et al., 1997) . These genes are expressed in an EBNA2-independent way that, in the case of EBNA1, involves the use of a unique promoter, Qp (Schaefer et al., 1995; Tsai et al., 1995; Nonkwelo et al., 1996).

If EBV is using the normal pathways of B cell activation and differentiation to gain access to the memory compartment, then the important intermediate steps in persistence should, as with antigen-driven activation/differentiation, be occurring in the secondary lymphoid tissue not in the peripheral circulation. The tonsil is a known site of viral persistence with both latently infected cells and viral replication (Babcock et al., 1998) and is readily available for study. Therefore, to test the ideas discussed above, we have analyzed the status of EBV infection in specific subsets of B cells from the tonsil. To do this, we have taken advantage of a series of elegant studies (reviewed in Liu and Arpin, 1997) establishing that specific cell surface markers can be used to identify and isolate functionally distinct subsets of human B cells from the tonsil.

Results

EBV-Infected Cells Are Present in All Tonsillar B Cell Subsets

If EBV is using the germinal center to gain access to the memory compartment, then there should be latently infected germinal center cells in the tonsil. We have shown previously that tonsils contain infected naive (IgD^+) and IgD^- B cells (Babcock et al., 1998) and that the cells are latently infected (Decker et al., 1996). However, IgD^- cells in the tonsil are a mixture of both germinal center cells and memory cells. To test if both are infected, we separated the IgD^- B cell fraction into germinal center and memory populations, based on the expression of the germinal center–specific marker CD10 (Pascual et al., 1994). The frequency of virus-infected cells in each population was then estimated using a modified version of the limiting dilution DNA PCR method described previously (Babcock et al., 1998). In this technique, cell populations are serially diluted, and then multiple samples of each dilution are tested for the presence of EBV with a DNA PCR assay that can detect a single copy of the viral genome. By applying Poisson statistics, the absolute frequency of virus-infected cells can then be calculated. The results of one such analysis are shown in Figure 1A. This experiment was repeated on eight tonsils, and representative results from three tonsils (numbers one through three) are summarized in Table 1. In all cases, significant numbers of infected cells were found in all three populations of cells tested. FACS analysis of the purified populations from one experiment is also shown in Figure 1A. The populations were always ≥90% pure and contaminated with ≤5% of other B cell subsets. The remaining contamination consisted of T cells, which lack EBV and are therefore irrelevant to the analysis. Based on these results, we conclude that the naive, germinal center, and memory B cell subsets all contain cells latently infected with EBV and that the presence of these cells cannot be accounted for by cross-contamination with other B cell subsets.

A similar study was performed for the germinal center subsets. In this case, the IgD^- B cells were stained for

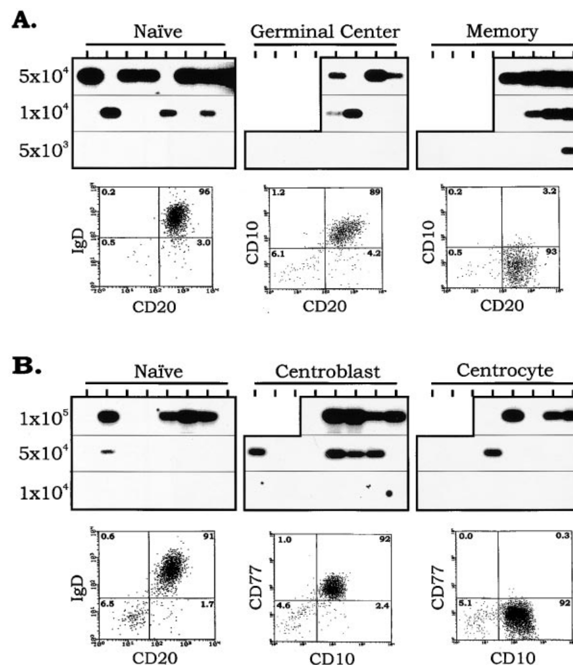

Figure 1. EBV Is Present in All the Major B Cell Subsets Found in the Tonsil

(A) Germinal center, memory and naive B cells contain EBV. Naive (IgD^+), germinal center ($CD10^+$), and memory B cells were purified as detailed in Experimental Procedures. The purified populations were costained for the pan-B cell marker CD20 for reanalysis to check purity. The cells were subjected to limiting dilution DNA PCR analysis. In this technique, cell populations are serially diluted and then multiple samples of each dilution tested for the presence of EBV with a DNA PCR assay that can detect a single copy of the viral genome. By applying Poisson statistics, the absolute frequency of virus-infected cells can then be calculated. Southern blot analysis of the PCR products is shown. Note that the sample in any given lane is not a direct dilution of the sample directly above it but a replicate of samples with the same cell number. N.B., We have shown previously that all $CD19^+$ B cells in the tonsil, that carry EBV, are latently infected (Babcock et al., 1998).

(B) Germinal center, centroblasts and centrocytes contain EBV. Naive B cells and germinal center centroblasts ($CD77^+$, $CD10^+$) and centrocytes ($CD77^-$, $CD10^+$) were isolated as described in Experimental Procedures. PCR and FACS analysis were as described in (A).

Table 1. Frequency of EBV-Infected Cells in the Tonsillar Memory B Cell Compartment

Number of Infected Cells/10^7 Total Cells					
Tonsil	Naive	Memory	Germinal Center	Centroblasts	Centrocytes
1	50	25	70	–	–
2[a]	270	1000	300	415	500
3[a]	75	100	40	200	70
4	70	–	–	35	140
5	<20	140	<20	–	–
6	<20	800	<20	–	–
7	<20	>800	<20	<20	<20

N.B., Tonsils five to seven were the only three analyzed that lacked infected naive cells, whereas tonsils one to four are representative of a larger panel of 13 tonsils that demonstrated similar results.
[a] Fractionations into memory versus germinal center and memory versus centroblasts and centrocytes were performed on separate occasions.

CD10 and CD77 and fractionated into the centroblasts ($CD10^+$, $CD77^+$) and centrocytes ($CD10^+$, $CD77^-$) (Pascual et al., 1994; Liu and Arpin, 1997). An example of one such experiment is shown in Figure 1B. A total of seven tonsils were tested altogether with similar results, and the quantitative results from three representative tonsils (numbers two through four) are shown in Table 1. Both germinal center subsets had significant levels of virus-infected cells that could not be accounted for by cross-contamination.

We conclude, therefore, that tonsils with infected naive cells also contain infected memory cells and germinal center cells, including both centroblasts and centrocytes.

Viral Gene Expression in Tonsillar B Cell Subsets

We have shown previously that viral replication is ongoing in the tonsil (Babcock et al., 1998). Therefore, the simplest interpretation of our DNA PCR analysis is that all B cell subsets are being directly infected in the tonsil and should express the EBNA2-dependent growth program. However, our model predicts that latently infected germinal center and memory cells are not derived through direct infection but by differentiation of directly infected B lymphoblasts. In this scenario, the infected germinal center population should not express the EBNA2-dependent form of latency but a restricted latency such as is found in EBV-positive HD. To distinguish these two possibilities, we have performed RT-PCR analysis on the isolated tonsillar B cell subsets. Since we wanted to analyze multiple genes on the same samples, we selected diagnostic genes to test. Specifically, we chose EBNA2 as a marker for the growth program that drives the lymphoblastoid form of latency characteristic of directly infected cells. We choose EBNA1(Q-K) to represent the restricted forms of latency and LMP1 plus LMP2 to distinguish the BL form (EBNA1(Q-K) only) from the HD/NPC form (EBNA1(Q-K) plus LMP1 and LMP2a). We have analyzed naive (IgD^+), germinal center (IgD^-, $CD10^+$), centroblast (IgD^-, $CD10^+$, $CD77^+$), centrocyte (IgD^-, $CD10^+$, $CD77^-$), and memory populations (IgD^-, $CD10^-$) from 17 tonsils, although not every subset was always tested for every tonsil. Representative examples are shown in Figure 2, and a summary of all the results is shown in Table 2. It is apparent that, unlike the peripheral blood, latent gene expression was readily and reproducibly detected in tonsillar subsets and in patterns previously defined in vitro (lymphoblastoid growth program) or in tumor models (HD/NPC). Figure 2A shows the analysis from one tonsil. Both the germinal center (IgD^-, $CD10^+$) and memory (IgD^-, $CD10^-$) populations expressed EBNA1(Q-K), but it was not detected in the naive (IgD^+) population. Conversely, EBNA2 was only expressed in the naive population. All three subsets expressed LMP1 and LMP2. Figure 2B shows the results for two tonsils that were fractionated in parallel into either naive or memory populations, and the same results were observed: EBNA2 was only found in the naive subset and EBNA1(Q-K) only in the memory subset. Most of our experiments were performed on memory cells defined as $CD3^-$, IgD^-, $CD10^-$. It was conceivable that the cells were not truly memory cells but were aberrant sIg^- B cells. Therefore, we performed a limiting dilution DNA PCR and an RT-PCR analysis on memory cells that were positively selected for sIg as the last step in the purification. All of the virus-infected cells in the $CD3^-$, IgD^-, $CD10^-$ population fractionated into the sIg^+ subset, and the viral gene expression pattern was found to be identical to the unselected $CD3^-$, IgD^-, $CD10^-$ cells (data not shown).

Lastly, germinal center cells were isolated from six tonsils and separated into centroblasts ($CD77^+$) and centrocytes ($CD77^-$). The RT-PCR analysis for these samples is shown in Figure 2C. EBNA2 was never detected in either population from any of the six tonsils, whereas EBNA1(Q-K) was found in centroblasts from 6/6 tonsils and in centrocytes from 3/6 tonsils. LMP1 was found in both populations from all of the tonsils studied and LMP2a in centroblasts and centrocytes from 5/6 tonsils. These results are in agreement with those obtained for whole unfractionated germinal center cells shown in Figure 2A.

When we last studied peripheral blood, the only latent gene we found to be expressed was LMP2. However, since that time, we have increased the sensitivity of our RT-PCR assays to the point where we routinely detect all four transcripts upon RT-PCR of a single infected cell in the presence of 2×10^6 EBV-negative tonsil cells. This is demonstrated in Figure 3B, which shows an example of the sensitivity titration and multiple negative controls that were performed with each experiment. It was conceivable that the more complex patterns of gene expression we now report in the tonsil, compared to our previous peripheral blood studies, simply reflected this increased sensitivity. To check this, we performed RT-

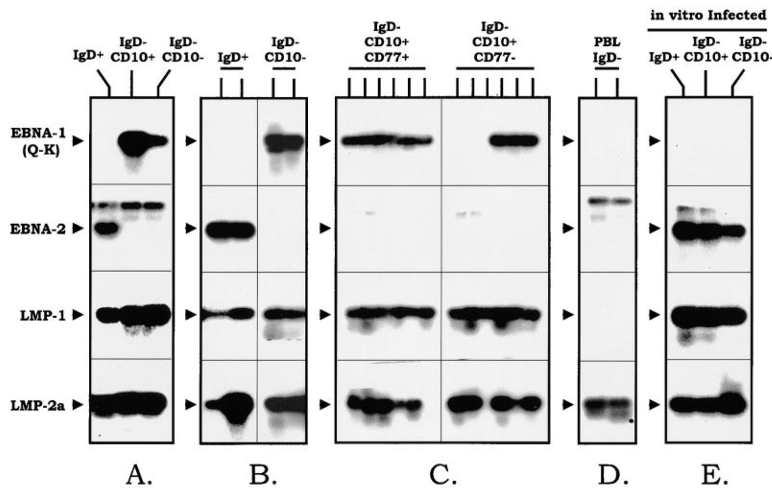

Figure 2. RT-PCR Analysis for Latent Gene Expression in Tonsillar B Cell Subsets

(A) B cells from a single tonsil were fractionated into naive (IgD^{+}), germinal center (CD10^{+} IgD^{-}), and memory (CD10^{-}, IgD^{-}) subsets as described in Experimental Procedures. The cells were assayed for expression of the latent transcripts EBNA1(Q-K), EBNA2, LMP1, and LMP2a.

(B) As (A), except only naive and memory cells from two different tonsils are shown.

(C) As (A), except only germinal center centroblasts and centrocytes from six different tonsils are shown.

(D) As (A), except only memory cells from two peripheral blood samples are shown.

(E) As (A), except the subsets were first purified from an EBV-negative tonsil and then infected in vitro for 48 hr with the B95-8 strain of EBV.

Southern blot analysis of the PCR products is shown. The expected size of the PCR product is indicated by an arrow to the left. Note that a band larger than the expected PCR product was sometimes seen in the EBNA2 RT-PCR. This represents the unspliced sequence derived from contaminating genomic DNA.

PCR analysis on peripheral memory cells from two donors (Figure 2D) and confirmed again that even with the more sensitive assays we only detected LMP2a.

Of the 11 tonsils (Table 2) and two peripheral blood samples studied, only the tonsil naive populations expressed the latent gene pattern (EBNA2^{+}, LMP1^{+}, LMP2a^{+}, EBNA(Q-K)$^{-}$) characteristic of the lymphoblastoid latency that occurs after direct infection. EBNA1(Q-K) was never detected in the tonsil naive population, indicating that it does not contain a mixture of cells expressing the restricted and lymphoblastoid forms of latency. Whole germinal center cells, germinal center cells separated into centroblasts and centrocytes, and memory cells never expressed detectable EBNA2. They all expressed the limited form of latent gene expression previously only found in tumors such as HD and NPC (EBNA2^{-}, EBNA1(Q-K)$^{+}$, LMP1^{+}, LMP2^{+}). EBNA1(Q-K) expression (characteristic of BL) was never detected in the absence of LMP1 and LMP2. Therefore, we were unable to confirm the existence of an "EBNA1(Q-K) only" form of persistent infection.

Germinal Center and Tonsillar Memory Cells Express the Lymphoblastoid Transcription Pattern, Not Restricted Latency, upon Direct Infection In Vitro

It is our interpretation that the EBNA1(Q-K)$^{+}$, EBNA2^{-} phenotype of the IgD^{-} subsets had to arise through the differentiation of a directly infected cell because all cells directly infected with EBV express the EBNA2^{+} lymphoblastoid form of latency. However, it was conceivable that the EBNA1(Q-K)$^{+}$, EBNA2^{-} phenotype was the natural phenotype of directly infected tonsillar germinal center and memory B cells. To prove that this was not the case, we have isolated all three B cell subsets from an EBV-negative tonsil and infected them in vitro with the B95-8 laboratory strain of virus. The cells were analyzed by RT-PCR at 48 hr postinfection. This was before proliferation had started and eliminates the possibility that lymphoblastoid cells could have overgrown cells expressing the restricted phenotype. These experiments revealed (Figure 2E) that naive, germinal center, and memory populations all expressed the EBNA2^{+}, LMP1^{+}, LMP2^{+} phenotype expected of the lymphoblastoid growth program form of latency found in newly infected cells. No hint of EBNA1(Q-K)$^{-}$ activity was detected. It is conceivable that direct infection of B cell subsets, such as germinal center B cells, in special microenvironments in vivo could cause them to go directly to a EBNA1(Q-K)$^{+}$, EBNA2^{-} type of latency; however, the simplest interpretation of our results is that the restricted latency seen in the IgD^{-} subsets does not arise from direct infection and must, therefore, arise through the differentiation of newly infected cells.

The Viral Latent Gene Expression Patterns Are Not Due to Cross-Contamination of Purified Subsets

One issue that arises from the RT-PCR analysis is whether the signals seen are representative of a subset or are a consequence of contamination with other cell types. This is not of concern for the naive population. The presence of EBNA2 and the absence of EBNA1(Q-K) indicates no significant cross-contamination, because all other subsets express EBNA1(Q-K) and lack EBNA2. Similarly, we can be confident about the memory subset since, for rare tonsils, this was the only subset infected (see below). Analysis of gene expression in one such tonsil revealed the same pattern of gene expression (EBNA2^{-}, EBNA1(Q-K)$^{+}$, LMP1^{+}, LMP2^{+}) as memory cells from other tonsils (data not shown). The major contamination issue concerns the germinal center populations. Specifically, are the cells truly expressing EBNA1(Q-K), LMP1, and LMP2, or is this the result of cross-contamination with memory cells? To attempt to address this question, memory, centroblast, and centrocyte populations were isolated from a single tonsil. FACS analysis of the populations are shown in Figure 3C. For the centroblasts and centrocytes, contaminating memory cells would reside in the bottom left-hand quadrant of the FACS dot plot. Since contaminating T cells also reside in this quadrant, we can conclude that there

Table 2. EBV Latent Gene Expression in Tonsillar B Cell Subsets of All Tonsils Tested[a]

Population	EBNA2	EBNA1(Q-K)	LMP1	LMP2a
IgD^+ naive	8/11	0/10	8/8	9/11
IgD^- germinal center and memory	0/2	2/2	2/2	2/2
IgD^- $CD10^+$ germinal center (GC)	0/1	1/1	1/1	1/1
IgD^- $CD10^+$ $CD77^+$ GC centroblasts	0/6	6/6	6/6	5/6
IgD^- $CD10^+$ $CD77^-$ GC centrocytes	0/6	3/6	6/6	5/6
IgD^- $CD10^-$ memory-tonsil	0/6	5/6	6/6	5/6
IgD^- memory-peripheral blood	0/2	0/2	0/2	2/2

[a] In all, 17 tonsils were analyzed. Not all genes were tested for all tonsils.

was 5% contamination with memory B cells in the centroblast and centrocyte populations. Serial dilutions of the same cells were then performed (Figure 3A) on all three subsets to determine the endpoint of detection by RT-PCR of the latent genes. As expected, EBNA2 was not detected in any of the samples. The signals for the other three genes titrated to points where it was possible to conclude that the centroblasts and centrocytes would have to have been massively contaminated, around 50%, with memory cells to account for the RT-PCR results seen. For example, the lowest dilution where LMP1 was detected was 5×10^3 memory cells and 10^4 centroblasts. Therefore, the centroblasts would have to be 50% contaminated with memory cells to account for the signal seen. We conclude, therefore, that both germinal center populations express the EBNA1(Q-K)$^+$, LMP1$^+$, LMP2a$^+$, EBNA2$^-$ phenotype.

EBV-Infected Cells Are Absent from the Naive and Germinal Center Populations in Tonsils that Lack Infectious Virus

We have shown previously that viral replication is ongoing in the tonsils. However, in rare tonsils, we found no evidence of viral replication, and, in these cases, we also found no infected naive (IgD^+) B cells (Babcock et al., 1998). This was not because the virus replicates in the IgD^+ cells. Viral replication in the tonsil is restricted to cells with an IgD^-, $CD19^-$ phenotype (Babcock et al., 1998). This led to the suggestion that naive B cells are being directly infected, a conclusion supported by the RT-PCR analysis discussed above. Our model predicts that these infected naive cells should differentiate into germinal center cells. It follows that in a tonsil that lacks viral replication and infected naive cells there should be no infected germinal center cells either. We have been able to analyze three such tonsils. The results from one are detailed in Figure 4. The lack of viral replication in the unfractionated cells is documented in Figure 4A, which shows the result of a DNA PCR Gardella (Decker et al., 1996) analysis. This gel technique allows the linear form of the virus, indicative of replication, to be resolved from the episomal form, found in latent infection. The viral DNA is detected by DNA PCR. As can be seen in Figure 4A, only the episomal form of the viral genome was detected. Since the DNA PCR can detect a single copy of the genome (Miyashita et al., 1995), we conclude

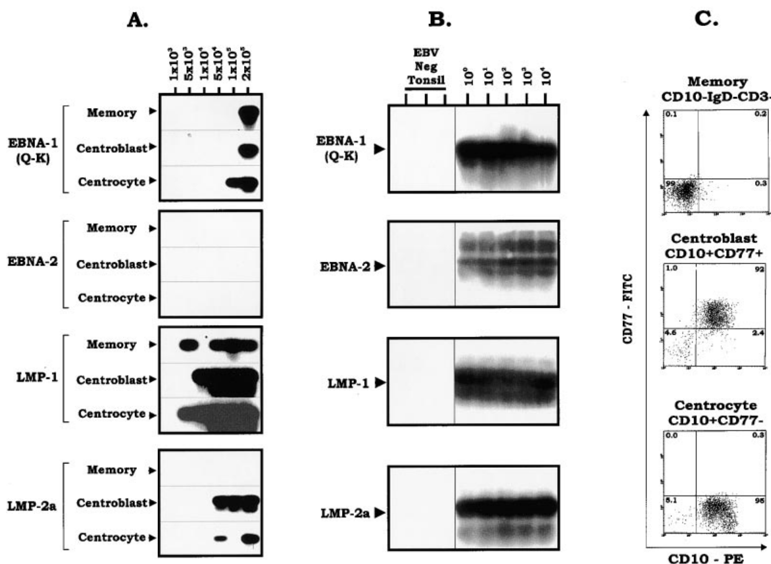

Figure 3. EBNA1(Q-K), LMP1, and LMP2a in the Germinal Center Centroblasts and Germinal Center Centrocytes Are Not Due to Contamination from Memory Cells

(A) Memory cells, germinal center centrocytes, and centroblast were purified as described in Experimental Procedures and the legend to Figure 1. RT-PCR analysis was then performed for EBNA1(Q-K), EBNA2, LMP1, and LMP2a on serial dilutions of cells for all four populations. Note, EBNA2 was not detected in any of the populations tested, and LMP2a was not detected at the highest number of memory cells tested. For EBNA1(Q-K), LMP1, and LMP2a, contamination of the order of 50% would be needed of memory into centroblast or centrocytes to account for the signals seen. A similar level of contamination would also be needed of centrocytes into centroblasts or vice versa to account for the signals seen.

(B) An example of the positive and negative controls that were performed for every experiment. Cells from a tonsil that lacked EBV, based on limiting dilution DNA PCR, were used as a negative control. The positive control was cells from the Rael cell line for EBNA1(Q-K) and from an EBV lymphoblastoid cell line for EBNA2, LMP1, and LMP2. The EBV-positive cells were serially diluted into EBV-negative tonsillar cells and used as both a positive control and a sensitivity control. The cells were analyzed by RT-PCR as described in (A).

(C) FACS reanalysis of the populations used. Cells were isolated as described above. Memory cells and non-B cells would be located in the bottom left, centroblasts in the top right, and centrocytes in the bottom right quadrants of the dot plots. Therefore, there is $\leq$5% contamination of memory cells in either centroblast or centrocytes, $\leq$2.4% contamination of centrocytes in centroblasts, and $\leq$1% of centroblasts in centrocytes.

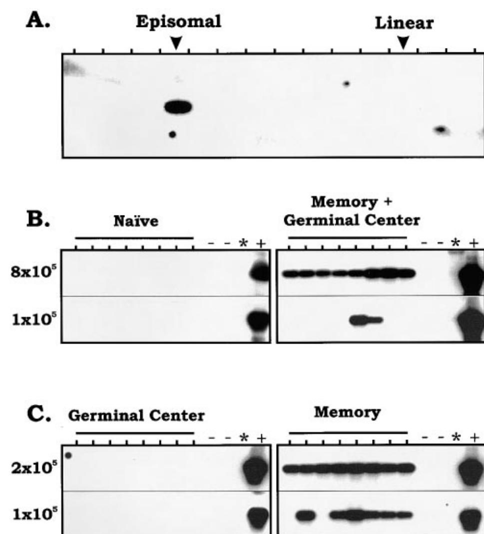

Figure 4. Tonsils that Lack Viral Replication Also Lack Infected Naive and Germinal Center Cells

(A) PCR-modified Gardella gel analysis was performed on purified tonsillar B cells. The Gardella gel allows the episomal and linear forms of the viral DNA to be fractionated. The episomal DNA migrates more slowly than the linear DNA. The position of the viral DNA is determined by slicing the gel into a series of equal-size pieces and performing DNA PCR on DNA extracted from each slice. Southern blot analysis of the PCR products is shown. The migration point of episomal and linear DNA is denoted at the top.

(B and C) DNA PCR limiting dilution analysis was performed on naive versus memory and germinal center B cells (B) and on germinal center versus memory cells (C) purified from the tonsil shown in (A) above. For details, see Experimental Procedures. The cells were subjected to limiting dilution DNA PCR to estimate the frequency of virus-infected cells. Southern blot analysis of the PCR products is shown. The absolute number of cells per sample are given to the left. Lanes with experimental samples are labeled with a vertical line, positive controls with a plus, negative controls with a minus, and the molecular weight marker lane with an asterisk. The calculated frequencies of infected cells for each population are shown in Table 1, experiment #1.

that no viral replication is ongoing. Figure 4B shows the limiting dilution DNA PCR analysis on the IgD^+ (naive) and IgD^- (mixture of germinal center and memory) cells. As shown previously, no infected naive cells are detected when linear viral DNA is absent. Figure 4C shows the same analysis carried out after the IgD^- population was fractionated into the germinal center and memory subfractions. No infected germinal center cells were detected; all of the virus-infected cells were located in the memory compartment. The results for all three such tonsils are summarized in Table 1 (tonsils five through seven). These results can be compared to those in Figure 1A, which shows that the virus is detected in the germinal center population of tonsils that have infected naive B cells. We conclude, therefore, that in tonsils that lack viral replication, naive and germinal center cells are not latently infected.

Discussion

The studies presented here demonstrate that persistent infection by EBV is characterized by viral gene expression patterns that vary according to the differentiation stage of the infected B cell. Naive B cells in the tonsil express the EBNA2-dependent lymphoblastoid growth program, whereas germinal center and tonsillar memory cells express a pattern of genes restricted to EBNA1, LMP1, and LMP2, and peripheral memory cells express no detectable latent genes, with the exception of LMP2. The pattern of gene usage agrees well with our model that EBV needs the lymphoblastoid growth program to activate the latently infected cells so they can gain access, through the normal pathways of germinal center differentiation, to the memory compartment, the site of long-term persistence. The ability of the growth program to drive proliferation, the most striking property of the virus in vitro, may be of secondary importance to its ability to activate the cells so they can differentiate.

There are several notes of caution that need to be taken into account when interpreting these experiments. Our purification procedures involve multiple fractionation steps and take several hours to complete. This could affect the integrity of the mRNA within the cells. However, the tissue where we found the least number of genes expressed, peripheral memory B cells, was the one that had the shortest and simplest purification procedure. Therefore, the complexity of gene expression detected was not simply a function of how long the procedure took. The subset where this was of greatest concern was the germinal center centrocytes, which are known to undergo rapid apoptosis in culture (MacLennan, 1994). Instability of these cells may account for the inconsistent results shown in Figure 2, where EBNA1(Q-K) was only found in 50% of the samples tested. By testing multiple samples, we could distinguish whether negative results are real or a result of occasional technical difficulties in the assay. Another possible source of false negatives is that the absolute sensitivity of the RT-PCR assays is not known. We can detect transcripts in a single infected tissue culture cell, but the transcript copy numbers in those cells are not known, and the copy number per cell in vivo may be lower. RT-PCR results also have to be interpreted with caution because the assay only detects steady-state levels of mRNA; long-lived mRNA or proteins such as EBNA1 may persist in cells that no longer transcribe the gene.

We do not believe these issues compromise our results. LMP1 and LMP2 were detected in all of the tonsil subsets; therefore, false negatives are not an issue for these mRNAs. Particularly convincing is the inverse relationship between the detection of EBNA2 and EBNA1(Q-K). Every tonsil subset tested expressed one or the other but never both. It is difficult to account for this result based on false negatives within a given subset. We have also performed some RT-PCR assays, when the sensitivity of all four assays was optimal, on samples that contained as few as two infected cells, based on limiting dilution DNA PCR frequency estimates. Under these circumstances, we have never seen expression of latent genes inconsistent with our results, for example, EBNA2 in $EBNA1(Q-K)^+$ populations or vice versa. Despite the concerns, therefore, it is apparent that, at the level of analysis presented here, the patterns of gene expression seen were not garbled or random. Defined patterns of gene expression, consistent with those seen previously in cell lines and tumors, are seen to be associated with specific subsets of B cells.

Our experiments are limited to the analysis of stable

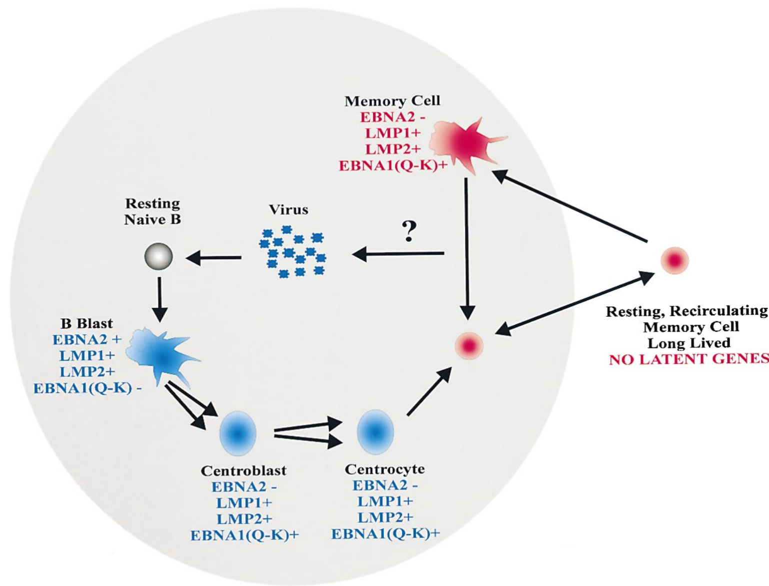

Figure 5. A Diagrammatic Representation of the Infected Populations Present in the Tonsil and Peripheral Blood and Their Possible Relationship

Free virus infects naive B cells and drives them to become proliferating lymphoblasts expressing the EBNA2-dependent transcription program. These cells can differentiate through a germinal center as centroblasts and centrocytes that express the HD/NPC form of latency EBNA1(Q-K)$^+$, LMP1$^+$, LMP2a$^+$, and EBNA2$^-$. These cells exit into the peripheral circulation as resting memory cells that are transcriptionally quiescent for viral latent genes. These memory cells recirculate through lymph nodes where they undergo reactivation that leads to the reexpression of the HD/NPC transcription program. These cells can return to the recirculating resting state or proceed to replicate the virus. Differentiation to a plasma cell is a likely candidate mechanism for the step marked with a question mark in the diagram. The key to the model is that LMP1 and LMP2a are expressed at the crucial stage where T cell help and antigen receptor signaling are required for survival of the B cell; namely, the germinal center and the memory cell. The pathway shown in blue only occurs when infectious virus is around and likely represents steps involved in the establishment of the initial infection. The pathways marked in red occur independently of infectious virus and likely represent the key steps in viral maintenance. The gray area demarcates events that occur in the tonsillar lymph node.

infection phenotypes found in the tonsil and peripheral blood; we have not directly addressed the issue of dynamic changes in the state of latency of infected cells nor the possibility that other latency states could exist in other lymphoid tissues. However, certain relationships can be inferred, based on our experiments and through analogy to normal B cell biology. A diagram summarizing our results within the context of our proposed model is presented in Figure 5. Only naive B cells express the growth program, suggesting that they are the only cells undergoing direct infection with the virus. We suggest that these cells then turn off EBNA2 so they can differentiate through a germinal center reaction. Consistent with this, infected germinal center cells do not express the growth program, as they do when directly infected in vitro, but a limited transcription program, suggesting that they have arisen from another infected cell type. Since infected germinal center cells are only detected in the presence of infected naive B cells, we infer that the germinal center cells are derived from the infected naive cells. The program used by the infected germinal center cells consists of EBNA1, LMP1, and LMP2. EBNA1 allows retention of the viral genome, whereas LMP1 can provide a surrogate T help signal (Gires et al., 1997) and LMP2 a surrogate antigen receptor signal (Caldwell et al., 1998). Therefore, this program is all that is required to rescue the latently infected germinal center cells into the memory compartment. We speculate that all the latent genes may be shut down when germinal center cells exit the cell cycle to become centrocytes so that they can enter the recirculating memory pool, just as in a normal waning immune response. The only transcripts found in the periphery would be residues from transcription in the lymph nodes.

The long-term survival of memory B cells depends on T cell help (Gray et al., 1996) and antigen receptor signals received in lymph nodes (Gray and Skarvall, 1988; Lam et al., 1997). LMP2a and LMP1 can provide these signals; therefore, the latency program discovered in tonsillar memory B cells, LMP1$^+$, LMP2a$^+$, EBNA1(Q-K)$^+$, could allow the long-term survival of virus-infected cells within the recirculating memory compartment.

We find no evidence that memory B cells are being directly infected with EBV. Based on limiting dilution analysis, we estimate that $<$1% of infected memory cells expresses EBNA2. It is unlikely that memory cells are directly infected and then killed immediately by CTL. Our assay can detect a single infected cell expressing EBNA2, and it is not apparent why memory cells would be killed much more rapidly than naive B cells. In a recent study (Kurth et al., 2000 [this issue of *Immunity*]), the immunoglobulin genes have been examined in single infected cells microdissected from the tonsils of acute IM patients. Occasional infected naive B cells were found, but the expanding EBNA2-positive clones were all memory cells. This is consistent with our idea that the infected naive B cells would differentiate rather than undergo virus-driven expansion. The infected memory cells, on the other hand, being unable to differentiate, would proliferate until the CTL response arose to destroy them. Since we have never seen evidence of EBNA2 expression in the memory population of healthy carriers, we interpret the presence of such cells in IM to indicate that IM is an atypical state where the virus is infecting cells other than the intended target, the naive B cell. This means that the previous assumption that IM is simply an amplified version of asymptomatic infection (Rickinson and Kieff, 1996) may be incorrect.

EBV can infect any B cell; therefore, the restriction of direct infection to the naive subset implies that viral replication and reinfection must be restricted to regions of the tonsil where naive B cells predominate, such as

the mantle zone (Perry and Whyte, 1998). An alternative origin for the EBNA2^{+} naive cells is that they arose during the acute stage of infection and have persisted ever since. This is unlikely because infected naive cells are not present in tonsils that lack viral replication (Babcock et al., 1998). Therefore, the lymphoblastoid naive cells are not self sustaining. The naive subset showed no evidence, based on EBNA1(Q-K) expression, that some of the cells were expressing a more restricted form of latency. It is possible that there is a population of naive cells with very limited gene expression, like peripheral memory cells. To achieve this, however, the virus would have to directly infect some naive cells and keep latent gene transcription silent. It is unclear how this could be achieved.

Several investigators have proposed that long-term persistent infection with EBV is associated with expression of EBNA1 alone from the Q promoter (Masucci and Ernberg, 1994; Rickinson and Kieff, 1996). However, we have never found EBNA1(Q-K) expressed alone. Although it is possible that more sophisticated fractionations will dissect out an EBNA1-only latency, we believe it is more likely that EBNA1 will always be expressed along with LMP1 and LMP2a. Germinal center centroblasts, germinal center centrocytes, and memory cells all express EBNA1(Q-K) with LMP1 and LMP2. We believe this is because they provide the signaling functions necessary to rescue both germinal center B cells into memory and to maintain latently infected memory cells within the memory compartment. It now seems likely that this program is expressed in tumors because it is the default transcription program that the virus uses to promote the survival of latently infected cells. For this reason, we propose that this form of latency be referred to as the default or survival program to contrast with the growth program of the proliferating lymphoblasts and the latency program of the quiescent recirculating memory cells.

Recent claims that transgenic expression of LMP1 blocks germinal center formation are contradictory to our model (Uchida et al., 1999). However, the failure to develop germinal centers may be an artifact of constitutive expression of LMP1 from the transgene, since the mice had other abnormalities, as evidenced by the development of lymphomas. Interestingly, the B cells were able to undergo isotype switching, raising the possibility that EBV-driven lymphoblasts might differentiate into memory without developing identifiable germinal centers.

If our model is correct, then EBV latency consists of continuous infection and proliferation followed by germinal center differentiation into memory cells that in turn undergo periodic activation and expansion. The countervailing force is the immune response. Potentially, every step in the pathway is a target for regulation by the immune response (Thorley-Lawson and Poodry, 1982; Khanna et al., 1992; Bogedain et al., 1995), with the possible exception of the peripheral memory cells. Why doesn't the immune system either clear the infection or the memory compartment steadily fill up with latently infected cells? Since the memory cells are quiescent and express minimal if any viral genes, their numbers may be regulated by the same counting mechanisms that regulate homeostasis in the memory compartment. Exactly how all these competing forces are balanced to produce stable persistent infection is a central question that remains to be answered.

Experimental Procedures

Primary Cells and Cell Lines

Namalwa (ATCC), an EBV-positive BL line, was used as a positive control and BJAB, an EBV-negative B cell line, as a negative control for DNA PCR studies. An EBV-positive lymphoblastoid cell line was used as a positive control for RT-PCR of EBNA2, LMP1, and LMP2a. Rael (gift of Dr. S. Speck), an EBV-positive BL line, was used as a positive control for EBNA1 (Q-K) RT-PCR. Cells from tonsils that were EBV-negative, by limiting dilution DNA PCR, were used as negative controls for all RT-PCR experiments and as carrier cells when the cell number tested was less than 2×10^{6} cells.

Tonsils were obtained from patients undergoing routine tonsillectomies for obstructed breathing disorders at the Massachusetts General Hospital. Tonsillar lymphocytes were prepared as described previously (Babcock et al., 1998).

Magnetic Bead Separations

The details of the MACS-based protocol have been described previously (Babcock et al., 1998), except the type of MACS column used depended on the amount of cells expected in the positive-bound fraction. For an expected number less than 3×10^{7} total positive cells, the AS column (Miltenyi Biotec) was used, and for expected numbers greater than 3×10^{7} but less than 2×10^{8} total positive cells, the CS column was used. All antibodies were titrated for optimal recovery and purity of the desired population. The amounts of biotinylated antibody added were 0.060 mg of αIgD antibody (Southern Biotechnology), 0.072 μg of αCD19 (our lab), and 0.03 mg of αCD3 (Pharmingen). A typical MACS separation takes about 3 hr; all operations were performed at 40°C.

FACSort Separations

Due to the a lack of biotinylated antibodies for markers of germinal center B cells, the FACSort, in conjunction with fluorochrome-labeled antibodies, was employed. First, IgD^{-} B cells were purified using the MACS system, as described above, by negative selection for CD3 and IgD. Given that tonsils contain $<$1% monocytes, this typically yielded $>$80% purity in the negative fraction for IgD^{-} B cells. For separation of CD10^{+} B cells in tonsil, cells were stained with PE-coupled αCD10 (1:50) (Pharmingen) and FITC-coupled αIgD (1:500), and the CD10^{+}, IgD^{-} B cells were sorted. For purification of centroblasts and centrocytes, CD77 was used as a marker. CD77 is expressed on centroblasts but not centrocytes. Cells were first stained with unconjugated αCD77 antibody (Immunotech) at a 1:100 dilution and washed. The cells were then stained with FITC-coupled αrat IgM (Jackson Labs) at a 1:20,000 dilution and PE-coupled αCD10. The CD77^{+}, CD10^{+} centroblasts and CD77^{-}, CD10^{+} centrocytes were then sorted. A typical FACS separation could take up to 6 hr. All operations were performed at 40°C.

FACS Analysis and Antibodies

Flow cytometric analysis was used to assay the purity of all isolated populations. In all experiments, the purified population was $\geq$90% pure and frequently $>$95% pure for the desired marker, with $\leq$5% contamination by any undesired B cell subset. The remaining contamination consisted of T cells, which lack EBV and are therefore irrelevant to the analysis. Based on these results, we made the conservative assumption that we cannot be sure of the status of virus infection in any subset where the estimated frequency of virus-infected cells could be accounted for by $\leq$10% contamination with another B cell subset.

All fractionated populations were analyzed using a Becton Dickinson FACScan with Lysis II software as described previously (Babcock et al., 1998).

DNA PCR and Limiting Dilution DNA PCR

Limiting dilution DNA PCR analysis was performed on isolated populations exactly as described previously (Babcock et al., 1998). Cell populations were serially diluted, and multiple samples of each dilu-

tion were tested by DNA PCR. Serial dilutions were never performed on cell extracts or isolated DNA.

RT-PCR

RNA was purified from 5×10^6 cells with Trizol (GIBCO), as described by the manufacturer. If necessary, lymphocytes from an EBV-negative tonsil were added to samples to bring the cell number up to 5×10^6. cDNA was prepared as described previously (Babcock et al., 1998), except the 20 μl cDNA mixture was not ethanol precipitated but brought up to 100 μl with HPLC H_2O and used directly. The cDNA suspension (20 μl) was aliquoted to 200 μl Microamp reaction tubes, and PCR was carried out in a final volume of 50 μl consisting of 50 mM KCl, 20 mM Tris (pH 8.4), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, and 20 pM each of the amplimers (3.0 mM $MgCl_2$ was used for LMP1). This allowed RT-PCR to be performed for all four latent genes from one cDNA pot. The amplimers were EBNA1(Q-K), TGGCCCCTCGTCAGACATGATT and AGCGTGCGCTACGGAT; EBNA2, CATAGAAGAAGAAGAGGATGAAGA and GTAGGGATTCG AGGGAATTACTGA (Qu and Rowe, 1992); LMP1, TTGGTGTACTC CTACTGATGATCACC and AGTAGATCCAGATACCTAAGACAAGT (Qu and Rowe, 1992); and LMP2a, ATGACTCATCTCAACACATA and CATGTTAGGCAAATTGCAAA (Qu and Rowe, 1992). Reactions were incubated at 95°C for 5 min, and 1 U of Taq DNA Polymerase (Perkin Elmer) was added to each tube. The tubes were loaded in a Geneamp 9600 thermocycler, and the following conditions were run: for EBNA1 (Q-K) and EBNA2, 95°C for 15 s, 62°C for 30 s, and 72°C for 30 s, repeated for 40 cycles; LMP1, 95°C for 15 s, 65°C for 30 s, and 72°C for 30 s, repeated for 40 cycles; and LMP2a, 95°C for 15 s, 55°C for 30 s, and 72°C for 1 min, repeated for 40 cycles. All PCRs were concluded with a 5 min incubation at 72°C to complete the extension of all synthesized products. PCR products were visualized by Southern blotting as described above. Blots were probed using PCR product derived from the IB4 or Rael cell lines.

Gardella Gel Analysis

PCR-modified Gardella gel analysis was performed as previously described (Decker et al., 1996).

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