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EBV Persistence in Memory B Cells In Vivo

Gregory J. Babcock,* Lisa L. Decker,*
Mark Volk,† and David A. Thorley-Lawson*‡
*Department of Pathology
Tufts University School of Medicine
Boston, Massachusetts 02111
†Department of Otolaryngology
New England Medical Center Hospital
Boston, Massachusetts 02111

Summary

Epstein-Barr virus establishes latency in vitro by activating human B cells to become proliferating blasts, but in vivo it is benign. In the peripheral blood, the virus resides latently in resting B cells that we now show are restricted to the sIgD$^-$ memory subset. However, in tonsils the virus shows no such restriction. We propose that EBV indiscriminately infects B cells in mucosal lymphoid tissue and that these cells differentiate to become resting memory B cells that then enter the circulation. Activation to the blastoid stage of latency is an essential intermediate step in this process. Thus, EBV may persist by exploiting the mechanisms that produce and maintain long-term B cell memory.

Introduction

Infection by the human herpesvirus Epstein-Barr virus (EBV) is a predisposing factor for several human neoplasias (reviewed in Rickinson and Kieff, 1996). The 175 kb genome carries all of the information necessary to infect any resting, mature B cell in vitro and activate it to become a proliferating B lymphoblast. To achieve this, the virus expresses five essential latent genes (reviewed in Kieff, 1996). A complex and tightly regulated transcriptional process precisely controls the timing and levels of expression of these proteins during the establishment and maintenance of the proliferating, latently infected state. Thus, the virus has evolved a sophisticated mechanism to guarantee that whenever it comes into contact with a target B cell it will efficiently establish latency and drive the cell to proliferate.

Nevertheless, EBV is usually a benign, persistent virus. No viral replication is detectable in the peripheral blood (Decker et al., 1996) of healthy individuals (>95% of the adult population). The infected B cells are all latent, but they are resting cells, not activated blasts (Miyashita et al., 1997). The periphery is a reservoir of persistent virus, since the absolute number of infected cells remains constant over several years (Khan et al., 1996) and infectious virus can be recovered from the cells when they are cultured (Rickinson et al., 1977; Miyashita et al., 1995). The only latent gene consistently found to be expressed in the periphery is LMP2a (Qu and Rowe, 1992), which is dispensable for immortalization but may play a critical role in the maintenance of latency in resting cells.

The presence of latent EBV in resting B cells creates a paradox—how does EBV, which drives infected cells to proliferate in vitro, manage to persist in a resting cell in vivo? There are two possibilities. Either the virus is able to establish latency directly in resting B cells in vivo, or the newly infected cells become activated and proliferate but are able to exit from the cell cycle. Several arguments favor the latter explanation. First, the viral DNA in the resting B cells is a covalently closed circular episome (Decker et al., 1996) that forms in vitro when an infected cell becomes activated and enters the cell cycle (Hurley and Thorley-Lawson, 1988). Therefore, the resting cells must have been proliferating at some point in time. Second, the transcriptional program of EBV is complex and appears to be specifically designed to efficiently activate newly infected B cells and drive them into the cell cycle. It is unclear how EBV could enter a resting B cell and quiescently establish latency without initiating this program, but if it could the activation mechanism would be unnecessary. In fact, the ability to activate B cells has been retained even though it poses a threat, predisposition to neoplasia, to the very host organism that the virus depends on for long-term persistence and survival. Therefore, EBV must have evolved a mechanism for B cell activation because it is an essential step in the establishment of long-term persistence. In this case, acute EBV infection in vivo would initially result in the indiscriminate infection, activation, and proliferation of B cells. Subsequently, these cells would enter into a quiescent state, associated with down-regulation of the latent genes. An attractive aspect of this proposal is that it precisely mimics the known behavior of B cells during a response to an antigen (reviewed in MacLennan, 1994; Liu et al., 1996).

When a circulating naive (surface IgD$^+$) or memory (sIg$^+$ but sIgD$^-$) B cell percolates through lymphoid tissue, it becomes activated by specific antigen in combination with T cell help, in the form of CD40 cross-linking and lymphokines. The B cells become proliferating blasts phenotypically indistinguishable from B cells activated by EBV infection. These activated B cell blasts then loose their sIgD, express a switched isotype (IgM, G, E, or A) on their surface, and terminally differentiate into antibody-secreting plasma cells until antigen becomes limiting. In this case, the cells either die or exit the cell cycle and become memory B cells. The important point is that activated naive or memory B cell blasts can exit the cell cycle, without terminally differentiating, only by becoming sIg$^+$, IgD$^-$ memory B cells. The B cell blasts can never dedifferentiate back into a naive state.

If EBV-infected B blasts in vivo become resting B cells through the same pathway as antigen-activated B blasts, then a number of experimentally testable predictions can be made. First, in sites of EBV replication, any mature B cell, naive (sIgD$^+$) or memory (sIgD$^-$), could be infected. However, to reach the resting state, the B blasts would have to become memory cells. Therefore,

‡To whom correspondence should be addressed (e-mail: DThorley-Lawson@Infonet.Tufts.Edu).

Table 1. Cells Latently Infected with EBV Are Restricted to the IgD^- B Cell Subset in the Periphery Blood but Not Tonsils of Persistently Infected Healthy Donors

		Number of Infected Cells/10^7 Total Cells		
Experiment[a]		Total B	IgD^+	IgD^-
PBL	1	5	0[b]	25
	2	25	1[c]	100
	3	40	1[c]	230
	4	95	6	375
	5	380	7	830
Tonsil	1		40	75
	2		3[d]	70
	3		70	35
	4		15	30
	5		25	200

[a] Each experiment represents a different donor.
[b] No positive cells detected in 1.2×10^7 IgD^+ B cells tested (95% confidence limit 0, 3).
[c] One positive cell detected in 1.2×10^7 IgD^+ B cells tested (95% confidence limit 0, 5).
[d] Two positive cells detected in 0.6×10^7 IgD^+ B cells tested (95% confidence limit 1, 7).

at sites lacking viral replication, such as the peripheral blood, resting, latently infected B cells should all be in the memory subset.

In this study, we show that the EBV-positive cells in the peripheral blood have the characteristics of memory B cells (sIg^+ $sIgD^-$). Significant numbers of infected naive B cells ($sIgD^+$) were not found. We propose therefore that memory B cells are the site of long-term EBV persistence. By comparison, both IgD^+- and IgD^--infected cells were detected in tonsillar lymphoid tissue, a site of active viral replication and infection. We conclude that the activated, proliferating state of latent infection is required to allow the virus to gain access to resting memory B cells. Access to these cells occurs not through direct infection but through the differentiation of latently infected B cells using the normal pathways by which B cell blasts become memory cells.

Results

EBV-Infected B Cells in the Peripheral Blood Are Restricted to the IgD^- B Cell Subset

Since there is no viral replication in the peripheral blood, our model predicts that infection occurs elsewhere and that latently infected cells become resting by differentiating into memory cells prior to entering the peripheral circulation. Therefore, only latently infected, memory B cells should be found in the periphery. To test this prediction, we have separated B cells on the basis of surface IgD expression. IgD is expressed on naive B cells

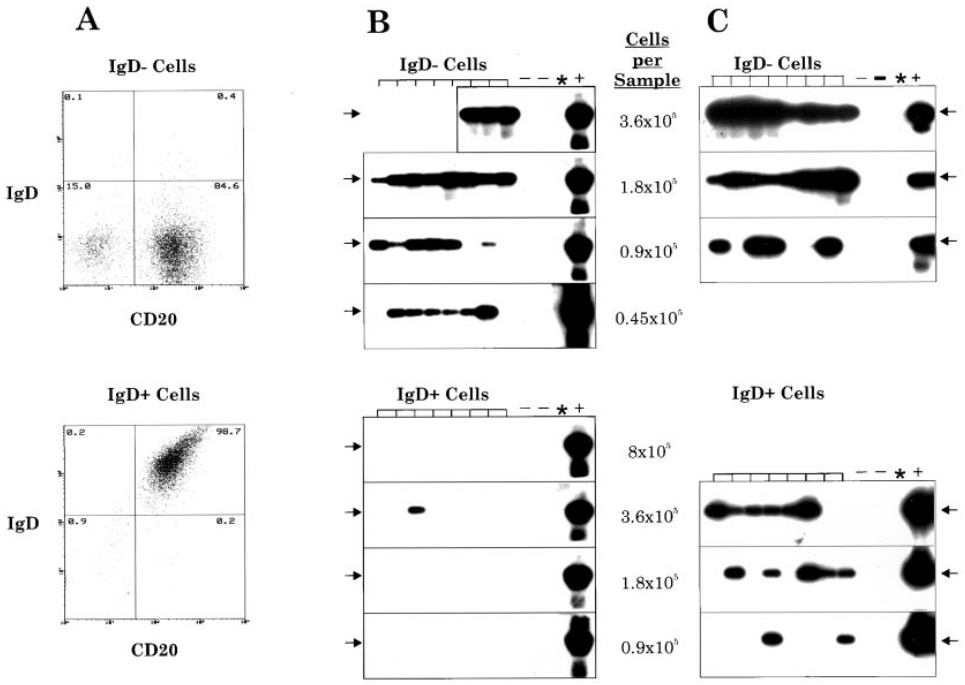


Figure 1. EBV-Infected Cells Are Restricted to the IgD^- B Cell Subset in the Peripheral Blood but Not Tonsils of Healthy Individuals

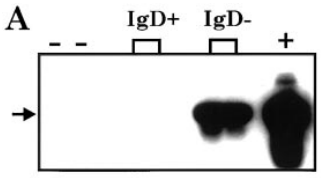
IgD^- and IgD^+ B cells were fractionated from either the peripheral blood or tonsils using the MACS system as described in Experimental Procedures. PCR products from IgD^- and IgD^+ B cells from healthy donors were analyzed by Southern blot as described in Experimental Procedures. Limiting dilution analysis was employed and absolute frequencies of EBV-infected cells in each population were determined using Poisson statistics. The expected size of the PCR product is indicated by an arrow to the left and the absolute number of cells per sample are given in the middle. 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 are shown in Table 1. (A) FACS analysis of IgD^+ and IgD^- B cell subsets isolated from whole PBMC by sequential MACS fractionation as described in Experimental Procedures. The populations were costained for the pan-B cell marker CD20 (FITC) and for IgD expression (PE). (B) B cells from the peripheral blood of donor #3 (Table 1). (C) Tonsillar mononuclear cells from donor #1 (Table 1). Note that in contrast to peripheral blood, it is apparent that both the IgD^+ and IgD^- B cell subsets from tonsils harbor EBV.

but is lost during germinal center maturation that yields memory B cells expressing the switched isotypes (M, G, E, and A) of hypermutated immunoglobulin genes (Liu et al., 1996).

IgD$^+$ B cells were positively selected from PBMC using a biotinylated αIgD monoclonal antibody and the MACS cell fractionation system. The remaining, predominantly IgD$^-$, B cells were then isolated using an αCD19 antibody (a pan-B cell marker) again in conjunction with MACS beads. The absolute numbers of virus-infected cells in each population were then assayed by performing limiting dilution analysis. The presence or absence of viral genomes in each sample was established by performing a virus-specific DNA PCR reaction (Miyashita et al., 1995). The products of the PCR reaction were detected by Southern blotting with a virus-specific probe. We have shown previously that this assay can detect a single viral genome in the presence of 10^6 uninfected cells (Khan et al., 1996). Southern blots from one representative donor (#3 in Table 1) are shown in Figure 1B. Only one signal was detected in all of the dilutions of IgD$^+$ B cells tested (a total of 1.2×10^7 cells). By comparison, signals were readily detected in the IgD$^-$ population. The frequencies of virus-infected cells were calculated to be 230 per 10^7 IgD$^-$ B cells. This experiment was repeated on several donors with a wide range in frequencies of infected cells, from the lowest with 5 per 10^7 B cells to the highest with 380 per 10^7. The results are summarized in Table 1. It is apparent that less than 1% (95% confidence limits 0.3 and 1.3) of the EBV-infected cells reside in the naive (IgD$^+$) B cell population. Only in donors with the highest numbers of EBV-infected cells did we observe sufficient signals in the IgD$^+$ population to estimate a frequency. These numbers, however, were still so low compared to the IgD$^-$ population that they could be accounted for by $<$1.5% contamination of the IgD$^+$ population by IgD$^-$ B cells. Therefore, we cannot tell if the infected cells in the IgD$^+$ population represent a very small fraction, $\leq$5%, of virus-infected cells that are truly IgD$^+$ or are simply due to very low levels of contaminating IgD$^-$ cells.

For every experiment, we monitored the purity of the populations before and after fractionation by staining for the specific marker selected, in this case IgD, and the pan-B cell marker CD20. An example of such an analysis is shown in Figure 1A. The purified cells were always $<$5% contaminated with the unwanted B cell population. In some preparations, there was significant contamination with non-B cells (e.g, 15% in the IgD$^-$ B cells in Figure 1). However, these cells do not contain virus (Miyashita et al., 1995) and do not affect the interpretation of the results. We also measured the frequencies of virus-infected cells in the unfractionated B cell population (total CD19$^+$ cells). Knowing this and the purity of each population, we could estimate the expected yield of virus-infected cells after fractionation and compare this to the observed yield. By this means, we could determine that essentially all of the virus-infected cells were recovered in the IgD$^-$ population and that there had been no gross loss of cells during the isolation procedures.

We conclude therefore that there is not a significant number of infected naive B cells in the peripheral blood of healthy, persistently infected individuals and that essentially all of the EBV-infected cells reside in the IgD$^-$ subset.



B

	No. Infected cells/10^6 Total Cells	
Expt.	IgD+	IgD-
DNA PCR*	0.6	38
Outgrowth	0#	7.5

* - This is Expt. PBL#4 in Table 1

- No outgrowth from $3x10^6$IgD+ B cells tested (95% confidence: 0,1)

Figure 2. EBV Is Only Found in the IgD$^-$ B Cell Subset When Assayed by RTPCR or Spontaneous Outgowth

(A) RT-PCR for LMP2a was performed as described in Experimental Procedures on the IgD$^-$ and IgD$^+$ B cell subsets, and the Southern blot analysis of the PCR products is shown. Sample lanes for IgD$^+$ and IgD$^-$ cells are labeled and contained the PCR products from 10^6 of the appropriate cell population. The positive control is denoted as a plus and negative controls labeled with a minus. Only in the IgD$^-$ B cell subset were we able to detect the transcript for LMP2a.
(B) IgD$^-$ and IgD$^+$ B cells from the peripheral blood were assayed, by limiting dilution, for the frequency with which EBV transformed cells spontaneously arose in culture. For details see Experimental Procedures. The same cells were also assayed for the frequency of infected cells by limiting dilution and DNA PCR.

EBV Is Restricted to the IgD$^-$ B Cell Population as Judged by RTPCR

LMP2a is the only viral transcript that has been consistently detected in all of the published RT-PCR studies on the peripheral blood of healthy, persistently infected individuals (Qu and Rowe, 1992; Tierney et al., 1994; Chen et al., 1995; Miyashita et al., 1997). Thus, LMP2a expression is a second molecular marker for the presence of EBV in the periphery. To confirm the results obtained by DNA PCR, we performed RT-PCR analysis for expression of LMP2a in IgD$^-$ and IgD$^+$ B cell populations. As shown in Figure 2A, LMP2A transcripts were readily detected in the IgD$^-$ memory B cell subset only. Although it is not possible to quantitate the absolute numbers of cells containing the transcript, these results confirm, qualitatively, that viral gene expression, like EBV DNA, is limited to the IgD$^-$ B cell subset.

Only the IgD$^-$ B Cell Population Undergoes Spontaneous Outgrowth

When placed into culture, infected peripheral blood B cells release infectious virus that will immortalize bystander B cells—so called spontaneous outgrowth (Rickinson et al., 1977). It has been reported previously that the virus is released almost exclusively from IgD$^+$ B cells (Lam et al., 1994). This result would appear to be in

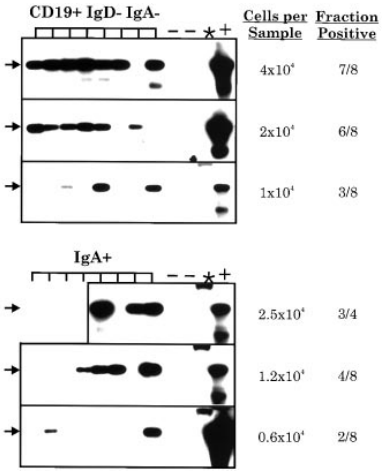


Figure 3. EBV Is Evenly Distributed throughout the Memory B Cell Subset

DNA-PCR limiting dilution analysis was performed on IgD^- B cells depleted ($IgD^-IgA^-CD19^+$, upper panel) or enriched (IgA^+, lower panel) for the expression of IgA. The expected size of the PCR product is indicated by an arrow to the left, and the absolute number of cells per sample and the fraction of samples positive for each cell number tested are given to the right. 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 2, experiment #1.

contradiction to our observation that essentially all of the EBV-infected cells in the peripheral blood are IgD^-. To check this apparent contradiction, we have repeated the spontaneous outgrowth experiments. Virus release was assayed on both the IgD^+ and IgD^- B cell subsets from the peripheral blood. In parallel, the same cell fractions were assayed for the presence of viral DNA by PCR. Limiting dilution analysis was performed to measure the frequency of cells spontaneously releasing virus or containing viral DNA. The results are shown in Figure 2B. Only in the IgD^- population did we observe significant numbers of virus-infected cells by either assay. By DNA PCR, the absolute frequency of EBV-infected cells was 38 per 10^6 IgD^- and 0.6 per 10^6 IgD^+ B cells. By outgrowth, we estimated a frequency of 7.5 virus-releasing cells per 10^6 IgD^- B cells. For the IgD^+ cells, we observed no outgrowth in 30 wells each containing 10^5 IgD^+ B cells per well (0 per 3.10^6). Why the estimated frequency of infected cells measured by detecting the release of infectious virus is 5-fold lower than by DNA PCR is not clear. One possibility is that outgrowth is an inefficient process and is not as sensitive as PCR for detection of virally infected cells. Another possibility is that only a subset of the cells is able to release infectious virus. We also have no explanation for the discrepancy between our results and those published previously. However, in our hands the outgrowth assay is in concordance with the DNA and RT PCR studies—all of the virus-infected cells detected in the peripheral blood reside in the IgD^- population.

EBV Resides in and Is Distributed throughout the Memory B Cell Subset

In the previous section, we demonstrated that latently infected cells reside exclusively in the IgD^- subset of B cells ($CD19^+$, $CD20^+$). Although sIgD expression is the only reliable surface marker for naive B cells in the peripheral blood, lack of sIgD does not prove that a cell is a memory B cell. It was conceivable that the infected B cells lacked sIg expression completely and were unrelated to memory cells. We therefore performed experiments to test if the virus-infected cells expressed surface Ig and to see if EBV-infected cells were limited to a specific isotype. IgD^- B cells were isolated from PBMC previously depleted of IgD^+ B cells. Cells expressing various immunoglobulin isotypes were then positively selected from the purified IgD^- B cells. We then measured the frequency of virus-infected cells in the selected population and in the depleted population. This experiment was performed for the IgG, IgA, and IgM isotypes. The Southern blot result for purified IgA^+ versus IgA^-, IgD^- B cells is shown in Figure 3. The estimated frequencies from all of the experiments are shown in Table 2. These experiments confirmed that the latently infected B cells express sIg. However, selective enrichment for any of the switched isotypes tested did not dramatically alter the frequency of virus-infected cells. It appears therefore that the virus-infected cells are evenly distributed throughout the memory subset but are excluded from naive B cells in the peripheral blood of healthy individuals. The restricted presence of EBV in the memory B cell subset in peripheral blood contrasts with the unrestricted infection of all B cells seen in vitro.

Table 2. IgD^- B Cells, Latently Infected with EBV in the Periphery of Persistently Infected Healthy Donors, Are Not Limited to a Specific Isotype

	Number of Infected Cells/10^7 Total B Cells				
Experiment	M+G+E+	A+	G+	M+	G+E+A+
1	675	700			
2	285	385	360		
3				350[a]	1600

[a] Insufficient cells were recovered for FACS analysis. This estimate is based on the assumption that the population is 100% pure and therefore may be an underestimate.

EBV Actively Replicates in Tonsillar Lymphoid Tissue of Persistently Infected Healthy Individuals

We have previously shown that viral replication is not detected in the peripheral blood (Decker et al., 1996); therefore, B cells must be infected elsewhere prior to exit into the periphery. It is known that viral replication occurs in tonsillar lymphoid tissue during acute infection, i.e., infectious mononucleosis (Anagnostopoulos et al., 1995). Infectious virus continues to be shed into the saliva throughout the lifetime of the host and this virus probably also originates from the mucosal lymphoid tissue; however, this has yet to be demonstrated due to the very small numbers of infected cells present. To overcome this problem, we have employed a DNA PCR–based in situ lysis gel technique (PCR Gardella gels) that we have developed and characterized previously (Decker et al., 1996). This technique can detect the viral DNA from a single infected cell and discriminate whether it is episomal and/or linear, the forms of the viral genome

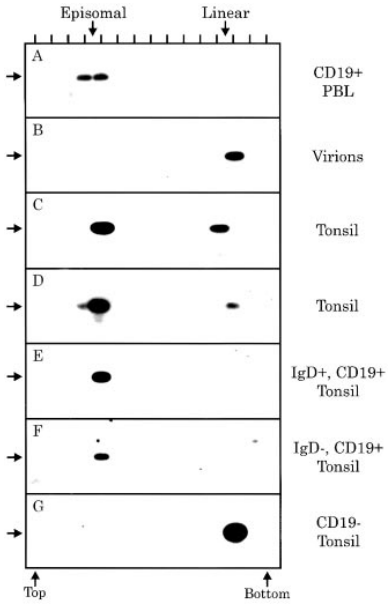


Figure 4. Lytic Replication of EBV Occurs in the Tonsil but Not the Peripheral Blood

PCR-modified Gardella gel analysis was performed on different cell subsets from tonsils. The Gardella gel is an in situ lysis gel that allows the episomal and linear forms of the viral DNA to be fractionated. The episomal DNA migrates more slowly than the linear DNA. After the gel is run, the lane containing the experimental samples is excised, sliced into a series of equal-size pieces, and DNA PCR for the viral genome is performed on DNA extracted from each slice. Southern blot analysis of the PCR products is shown. The position of episomal and linear DNA is denoted at the top. (A) PBL B cells, a positive control to indicate the migration of the episomal form of the viral DNA. (B) Virions, a positive control to indicate the migration of the linear form of the viral DNA. (C) The state of the viral genome in tonsillar lymphoid tissue. (D) Same as C but from a different donor. (E) IgD^+ tonsillar B cells. (F) IgD^- tonsillar B cells. (G) $CD19^-$ tonsillar B cells.

that are characteristic of latent or lytic infection, respectively. When this analysis was applied to tonsillar lymphoid tissue from healthy, persistently infected individuals (Figures 4C and 4D), linear and episomal forms of the virus were detected; therefore, viral replication is ongoing in this tissue. As controls for the migration rate of episomal and linear genomes, we used peripheral blood B (PBL-B) cells (Figure 4A) shown previously to contain only episomes (Decker et al., 1996) and purified virions (Figure 4B, lower panel) that only contain the linear form of the genome. In all, we have detected linear viral DNA in five out of seven tonsils analyzed.

Linear DNA can be generated artifactually in this method due to shearing of episomal DNA during processing. We do not believe that this is occurring, however, for several reasons. First, linear viral DNA was not present in five out of five peripheral blood samples that were processed in an identical manner. Second, the linear genomes from tonsils were present in a specific population of cells (see below). Third, linear viral DNA was only present when infected naive B cells were also detected (see below). The frequencies of virus-infected cells in PBL and tonsillar B cells were similar: 5–500 per 10^7 (Khan et al., 1996) and 20–100 per 10^7 (this paper) B cells, respectively. Therefore, the presence of linear viral DNA in tonsillar compared to PBL B cells was not due to loading more infected tonsillar B cells onto the gel.

We conclude therefore that viral replication is actively occurring in the tonsillar lymphoid tissue of healthy, persistently infected individuals.

EBV Latently Infected Cells Are Found in Both the IgD^- and IgD^+ Subsets of Tonsillar B Cells

Active viral replication is occurring in the tonsillar lymphoid tissue, and we know that EBV can infect all resting B cells, including naive B cells, in vitro. We would expect therefore that infected B cells of all types should be present in vivo at an area of active virus production. Specifically, and in contrast to the peripheral blood, where no infectious virus is being produced, we would expect both IgD^- and naive (IgD^+) B cell subsets to be infected in tonsillar lymph nodes. To test this prediction, we separated tonsillar B cells on the basis of sIgD expression, and the frequency of virus-infected cells in each population was again assessed by DNA PCR in conjunction with limiting dilution analysis. The result of one such analysis is shown in Figure 1C. EBV was detected in both the IgD^+ and IgD^- B cell subset. The frequency of infected cells in this case was calculated to be 40 per 10^7 in the IgD^+ B cells and 75 per 10^7 in the IgD^- B cells. Overall, we have detected EBV in the IgD^+ B cell subset from the tonsils of four out of five donors tested (Table 1). The range of frequencies was slightly lower in the IgD^+ subset (15–70 per 10^7) than the IgD^- subset (30–200 per 10^7), but this difference was not statistically significant. The exception was the tonsil in experiment 2 where the results were reminiscent of peripheral blood. EBV-infected cells were present in the IgD^- B cells, but so few signals were seen in the IgD^+ B cells that it was impossible to determine if this represented contamination with IgD^- cells or a very low frequency of infected IgD^+ cells.

For two tonsils (experiments 1 and 2 in Table 1), Gardella gel and phenotypic analysis were performed in parallel. The tonsil from experiment 1 had linear viral genomes and infected IgD^+ cells, whereas the tonsil in experiment 2 lacked both. Therefore, in these two samples there was an exact correlation between the presence of linear viral genomes and infected IgD^+ cells. It is possible that, at the time of tonsillectomy, the tonsil donor for experiment 2 was not shedding virus, thereby accounting for the lack of infected IgD^+ B cells.

We conclude that the picture of viral infection in mucosal lymphoid tissue represented by tonsils is different from that found in the periphery. In the periphery, the infection is quiescent with no viral reactivation and reinfection occurring and only latently infected, IgD^- memory cells present. By comparison, viral replication is actively underway in tonsillar lymphoid tissue and both naive (IgD^+) and IgD^- subsets of B cells are infected.

Both the IgD^- and IgD^+ Cells Are Latently Infected in the Tonsil

We have used DNA PCR to demonstrate that EBV DNA is associated with both the IgD^+ and IgD^- B cells in tonsils. However, lytic replication is also ongoing in the tonsillar tissue; therefore, the PCR signals could derive

from intact or fragmented virion DNA bound to the outside of the B cells. This would explain the even distribution of virus that we found among the tonsillar B cells. It would also explain why only infected IgD^- cells were found in the one tonsil that lacked viral replication. To establish if the populations were truly infected, we again resorted to PCR Gardella gels. As shown in Figures 4E and 4F, the covalently closed episomal form of the viral DNA was detected in both IgD^+ and IgD^- subsets. Since this assay is capable of detecting a single linear viral genome, we may conclude that both subsets are latently infected. This observation was also an important control because it rules out the possibility that the linear DNA seen in unfractionated tonsillar lymphoid tissue in Figures 4B and 4C was generated by artifactual shearing effects that occurred during processing and isolation of the tonsillar cells. If this had occurred, then linear viral DNA should also have been detected in the specifically purified populations of IgD^+ and IgD^- B cells. Linear viral DNA was associated with the $CD19^-$ population (Figure 4G), which includes non-B cells and terminally differentiated plasma cells that have lost their lineage markers.

We conclude therefore that the DNA PCR analysis was detecting intact viable genomes and that both the IgD^+ and IgD^- populations are latently infected.

Discussion

The molecular biology of EBV-driven B cell proliferation in vitro is beginning to be understood in detail. To understand how this process is regulated in normal infection and then disregulated, leading to pathogenic disease, it is necessary to study the mechanism of EBV persistence in vivo. We still know remarkably little about how this works. In this paper, we definatively show that EBV in peripheral blood has a tropism for persistence in a functionally specific subset of B cells—memory cells. This observation stands in contrast to the behavior of the virus in vitro where it is able to establish latency in virtually any type of mature B cell. The quantitative nature of our DNA PCR assay allows us to conclude that the association with memory cells is highly specific, since greater than 99% of the virus-infected B cells in the periphery were detected in the memory subset. This was confirmed by two less sensitive assays: RTPCR for LMP2a, the only latent transcript reproducibly shown to be expressed in the periphery, and the biological assay of spontaneous outgrowth. These results raise the possibility that EBV is using the biology of long-term B lymphocyte memory as a mechanism to sustain persistence.

In the periphery, EBV infection is always latent, transcriptionally quiescent, and limited to resting, IgD^- B cells. In mucosal lymphoid tissue, however, the situation is more dynamic because viral replication is ongoing. This virus is released into the saliva; however, it will also infect bystander B cells. Since EBV can infect any mature B cell, it was expected that we would find the virus in both IgD^+ and IgD^- tonsillar cells.

Our experiments fulfill two predictions, made in the Introduction, based on the assumption that EBV infection in vivo follows the pathways of antigen-driven activation. First, antigen can activate naive and memory B cells in the lymphoid tissue and EBV infects naive and memory B cells in the lymphoid tissue. Second, antigen-activated B cells only reenter the peripheral circulation as memory B cells and EBV recirculates in resting memory B cells. The consequent prediction from these observations is that EBV-infected B cells in lymphoid tissue differentiate in a manner that mimics the germinal center reaction that generates memory cells. A plausible mechanism for the establishment and maintenance of persistence can be constructed based on these ideas. When the virus first enters the host, during acute infection, it will infect and activate any mature B cell. These latently infected B cell blasts disseminate and amplify the viral genome, not as free virions, but as latent episomes within the proliferating cells. These B blasts can also differentiate in lymphoid tissue, allowing the virus, in the form of latently infected cells, to gain access to the memory subset. This continues until a strong CTL response arises to clear the infected B blasts, which are no longer required because life-time persistence has been established in the memory compartment. In fact, the highly proliferative blasts would be a threat to the host that the virus depends on for life-time persistence. Therefore, it is to the advantage of the virus that the blasts be rapidly destroyed once persistent infection of memory cells has been established. This conclusion is supported by the recent observations of Khanna et al. (1997) that the epitopes recognized by EBV-specific CTL are evolutionarily conserved. This confirms that there is a selective long-term advantage to the virus, favoring the destruction of infected B blasts.

If correct, then latently infected B cell blasts, like normally activated B cells, can transition from a proliferating to a resting state in vivo. Such a transition has never been shown in vitro for either infected or antigen-activated B blasts; however, a good deal is understood about how normal B cells achieve this in vivo (MacLennan, 1994; Liu et al., 1996). A simplified summary of this process is shown in Figure 5. B blasts, activated by antigen and T helper (Th) cells via CD40, enter the follicles of germinal centers to become highly proliferative centroblasts that have elevated levels of c-myc and express the germinal center–specific cell surface markers CD10 and CD77. After each cell division, a single resting cell, termed a centrocyte, is generated. Centrocytes express the pro-apoptotic genes Fas, bax, and p53 (Martinez-Valdez et al., 1996). Signals from antigen and Th cells drive them to terminally differentiate into plasma cells. When antigen becomes limiting, centrocytes either die rapidly from apoptosis or become memory cells. There is evidence to suggest that EBV-infected B blasts can become centrocytic if the critical transcriptional regulator EBNA2 is switched off (Polack et al., 1996) in the presence of an activated c-myc gene. Under these conditions, expression of the latent proteins is lost and the cells loose surface markers characteristic of B blasts, such as CD23, and acquire germinal center–specific markers, such as CD10 (Polack et al., 1996). If correct, then infected B blasts become germinal center cells by turning off the latent genes and proliferating

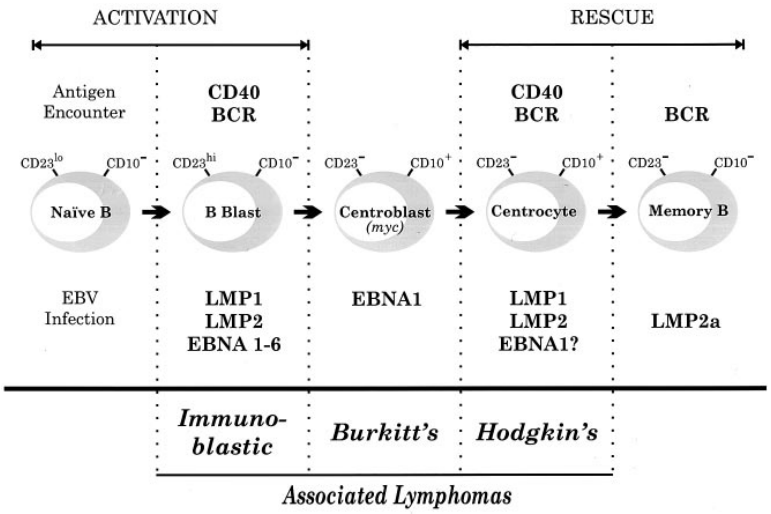


Figure 5. Diagrammatic Representation of the Steps Involved in Generating a Memory B Cell from a Naive B Cell and the Proposed Equivalents for EBV-Activated B Cells

The known EBV-positive lymphomas and their proposed origin from the different stages of B cell differentiation are also shown. The latent gene expression indicated for each stage is based on the known gene expression in the respective tumors. For details see the Discussion.

under the influence of cellular genes. How could such a cell replicate the viral episome and continue to differentiate? The solution is suggested by EBV+ Burkitt's lymphoma (BL), which is phenotypically identical to a germinal center B cell (MacLennan et al., 1988). The only viral gene expressed is EBNA1, the protein essential for replicating the viral episome, and it is expressed via a unique promoter, Qp (Nonkwelo et al., 1996; Schaefer et al., 1995). Qp may be regulated in such a way that if an infected B blast makes the transition to a centroblast by down-regulating the latent genes and expressing c-myc, EBNA1 expression will automatically be activated through Qp. If true, then BL is a tumor of an EBV-infected centroblast and the Qp/EBNA1 only form of transcription is the latency state characteristic of that cell.

If these EBV-infected centroblasts produce a centrocyte after each division, how are the centrocytes rescued from apoptosis? A solution is suggested by studies on another EBV+ tumor, Hodgkin's lymphoma (HL). It has recently been shown that HL is a tumor of the centrocytic or postcentrocytic stage of B cell differentiation (Kuppers et al., 1994). HL cells express LMP1 and LMP2 (Deacon et al., 1993) by an EBNA2-independent mechanism that is not currently understood. LMP-1 is believed to signal in a manner that mimics CD40 and related members of the TNF receptor family which are critical in rescuing centrocytes. LMP2 contains the same signaling motif as the BCR complex (see below) and can provide a BCR-related rescue signal (Caldwell et al., 1998). Therefore, in principle LMP1 and 2 could provide the rescue signals that allow the centrocyte to survive and differentiate into a resting memory B cell that expresses only LMP2a.

It is unclear how the relative numbers of normal memory B cells expressing the various isotypes are regulated. It is interesting therefore that EBV, in the periphery, is distributed throughout the memory subset while being excluded from all naive cells. EBV is believed to enter and be released through mucosal epithelium; therefore, it was conceivable that the virus was limited to the IgA^+ subset, which preferentially homes to this tissue. Although this was not the case, we did find IgA^+, EBV-infected cells. It is possible that these are the cells that will return EBV to mucosal epithelium, guaranteeing that infectious virus is released into saliva.

Once EBV enters the memory pool, the frequency of infected cells is stable for at least 6 years (Khan et al., 1996; D. T.-L., unpublished data). Maintenance of this stability may rely on the same mechanisms that sustain long-term memory. B cell memory is extremely long lived (reviewed in Tough and Sprent, 1995), with recall being detected decades after primary exposure to an antigen. Memory B cells only divide once every several months (Schittek and Rajewsky, 1990) but seem to require frequent stimulation through a functional BCR for survival (Gray and Skarvall, 1988; Lam et al., 1997). The origins of this signal in normal B cells are unclear, since it is highly unlikely that antigen can persist for decades. We have speculated previously that resting memory B cells latently infected by EBV may be maintained by signaling through LMP2a (Miyashita et al., 1997). This is the only latent gene consistently found to be expressed in these cells and has an intact ITAM (Beaufils et al., 1993), the signaling motif found in the BCR. More recently, it has been shown that LMP2a can indeed provide a rescue signal that replaces the BCR for development of B cells in the bone marrow of LMP2a transgenic mice (Caldwell et al., 1998). At present, we cannot measure the lifetime of latently infected cells in the periphery. Therefore, we cannot distinguish whether individual cells are extremely long lived or are being continuously replaced through sporadic proliferation and/or new infection. The only study of latently infected cells in individuals where viral replication was blocked by acyclovir (Yao et al., 1989) was not carried out long enough to effectively test if infection was necessary for long-term persistence.

Although differentiation is an attractive model to explain the selective presence of EBV in memory B cells in the periphery, there are other possible explanations. Specifically, it could simply be that the infected IgD^+ cells generated in the lymphoid tissue are too short lived to enter the periphery or that they fail to differentiate and are lysed by CTL. It should be possible to distinguish these various possibilities by careful analysis of B lymphoid subsets and by studies on immunosuppressed

individuals. For example, we found that both the IgD^+ and IgD^-,$CD19^+$ populations in tonsils are latently infected. It will be important to know whether the IgD^- cells include all B cells from centroblast through to memory cells or are restricted only to memory cells. If the latter is true, it would suggest that virus-infected cells do not differentiate and that the infected IgD^+ cells are absent from the circulation because they are short lived. If virus-infected cells are present throughout the gamut of differentiating B cell types, it will be important to know if latent gene expression in these populations follows that predicted from the model in Figure 5.

Many other questions remain to be addressed about the nature of the infection that is ongoing in the lymphoid tissues. It will be interesting to see if viral replication is restricted to mucosal lymphoid tissue since EBV is presumably only released onto mucosal surfaces. This includes further characterization of the cells that replicate the virus. In tonsils from donors with acute infection, these cells are terminally differentiated B cells that have lost their lineage markers (Anagnostopoulos et al., 1995). This is consistent with our observation that the cells replicating the virus in the tonsils of persistently infected individuals are $CD19^-$. If true, this would imply that EBV-infected cells are capable of differentiating into either memory or plasma cells and would implicate virtually every aspect of mature B cell biology in the mechanism of EBV persistence.

In conclusion, EBV persistence in vivo provides a unique opportunity to study and understand how potentially pathogenic viruses have evolved complex strategies to integrate their biology with that of the host cells they occupy.

Experimental Procedures

Primary Cells and Cell Lines

Namalwa (ATCC) is an EBV+ BL line. It contains one or two copies of the EBV genome and was used as a positive control for all PCR reactions. BJAB is an EBV− B cell lymphoma that was used as a negative control for all experiments and as carrier cells when the PBMC number tested was less than 1×10^4 cells. IB4 is an EBV+ lymphoblastoid cell line used as a positive control for LMP2a RT-PCR.

Heparinized peripheral blood (240 ml) obtained from healthy donors was layered onto Ficoll-Hypaque (Pharmacia) and centrifuged at 2000 rpm for 30 min at 25°C. Buffy coats were removed and washed two times with PBSA (1× PBS/0.5% BSA) at 1200 rpm for 15 min. PBMCs were then resuspended at a concentration of 2×10^7 cells/ml in the same buffer.

Tonsils were obtained from patients undergoing routine tonsillectomies, primarily for obstructed breathing disorders, at the New England Medical Center. Tonsils were minced in PBSA and the resulting suspension was passed through silkscreen to remove any connective tissue. The cell suspension was diluted to 1×10^8 cells/ml, layered onto Ficoll-Hypaque, and processed as above.

Magnetic Bead Separations

PBMCs and tonsillar mononuclear cells were resuspended to 2×10^7 cells/ml in PBSA as 1 ml aliquots. Biotinylated αIgD antibody (Southern Biotec cat #2030–08) was added to each tube (0.015 μg for PBMCs and 0.060 μg for tonsillar cells) and incubated on a rotator at 4°C for 30 min. All tubes were washed two times with PBSA and resuspended to 180 μl in the same buffer. 20 μl of streptavidin-coated microbeads (Miltenyi) was added to each and incubated for 15 min at 4°C. Cells were again washed and resuspended in 500 μl for separation using MACS columns (Miltenyi) and kept at 4°C at all times. The MACS column (AS type) was prepared by rinsing with 10 ml of PBSA and then inserted into a varioMACS magnet. The flow rate of the column was adjusted by attaching a 25G needle to the base of the stopcock. Cells were then loaded onto the column, and the negative fraction was collected. The cells were washed through by applying 3 ml of PBSA to the column while still attached to the magnet. The retained population was then washed by removing the column from the magnet and injecting 2 ml of PBSA from the bottom of the column using the side syringe supplied. The 25G needle was then replaced with a 23G needle, the column was reinserted into the magnet, and cells were allowed to flow through. These cells were collected as the wash fraction and discarded. The column was again rinsed with 3 ml of PBSA as before to remove any remaining nonspecifically bound cells. The column was then removed from the magnet, the 23G needle removed, and the column washed with 10 ml of PBSA to elute the retained cells. For separation of other cell populations, the same general protocol was performed using the following concentrations of biotinylated antibody: αCD19 (our lab), 0.018 μg for PBMC, 0.072 μg for tonsil; αIgA (Southern Biotec), 0.25 μg for PBMC; αIgG and αIgM (Southern Biotec), 0.025 μg for PBMC. For analysis of memory B cells expressing different isotypes, IgD^+ B cells were first depleted from whole PBMC as described above, and the remaining IgD^- B cells were isolated by positive selection using the Multi-Sort kit (Miltenyi). Flow cytometric analysis was used to determine the purity of the cell populations before and after MACS separation. The positively selected populations were always greater than 85% pure and usually greater than 95% pure.

FACS Analysis and Antibodies

All fractionated populations were analyzed using a Becton Dickinson FACScan with Lysis II software. After separations, all fractions were stained with an antibody to the appropriate isotype conjugated to PE (Southern Biotec) as well as αCD20-FITC (DAKO). FACS analysis allowed for determination of both the recovery and purity of the isolated populations. As negative controls, MOPC21 (IgG1 isotype control, Sigma), 1a2 (IgG2a isotype control, this laboratory), and MOPC121 (IgG2b isotype control, Sigma) were used.

PCR and PCR Product Analysis

Isolated populations were aliquoted at the desired number into a 96-well V-bottom microtiter plate (Immulon). The plate was centrifuged at 1500 rpm for 15 min at 4°C and the supernatant aspirated. 10 μl of a lysis solution containing 0.45% Tween-20, 0.45% NP-40, 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), and 0.5 mg/ml Proteinase K was added to each well and the plate incubated for at least 2 hr at 55°C. After incubation, the plate was centrifuged quickly to remove condensation from the lid of the plate. PCR was performed in a final volume of 50 μl per reaction. 5 μl of cell lysate was added to each PCR reaction. The PCR and Southern blotting conditions used were exactly as previously described (Miyashita et al., 1995).

To determine the absolute number of infected cells in each population, a limiting dilution analysis was performed as described previously (Miyashita et al., 1995; Khan et al., 1996). The DNA PCR can detect the presence of a single viral genome in as many as 10^6 uninfected cells. Poisson statistics were used to calculate the frequency of EBV-infected cells. To exclude the possibility of external contamination of the DNA PCR, we included eight negative DNA samples per analysis. Furthermore, the lack of contamination could be confirmed since the PCR signals always fractionate consistently, for example into the IgD^- subset of PBL-B cells, and the signals titrated out, i.e., they were weaker and less frequent with fewer cells.

LMP2a RT-PCR

RNA was purified from up to 5×10^6 cells with Trizol (GIBCO) as described by the manufacturer. RNA pellets were resuspended in 10 μl of RNAse free H_2O and subjected to cDNA synthesis by adding 2 μl of random hexamers (50 ng/μl) and incubating at 70°C for 10 min. The tube was then quickly cooled on ice and quickly spun. 1 μl of 10 mM dNTPs, 2 μl of 100 mM DTT, 2 μl of 25 mM $MgCl_2$, and 2 μl of 10× RT Buffer (500 mM KCl and 200 mM Tris [pH 8.4]) were added to the tube and incubated at 25°C for 5 min. 50 U of Superscript reverse transcriptase (GIBCO-BRL) was added and the

tube was further incubated at 25°C for 10 min followed by incubation at 42°C for 50 min. Following cDNA synthesis, reactions were incubated at 70°C for 15 min to inactivate the reverse transcriptase. cDNA was ethanol-precipitated, washed, and resuspended in 10 μl of HPLC H_2O. LMP2a PCR was subsequently performed on the entire cDNA sample using amplimers and reaction conditions as described previously. In brief, the PCR contained 10 mM Tris (pH 8.3), 2.0 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTP, and 10 pM of each amplimer. The "hot start" method was used, and the reaction temperatures were 95°C for 15 sec, 49°C for 15 sec, and 72°C for 30 sec, with a total of 40 cycles followed by a 5 min 72°C extension step. PCR products were resolved on a 2% Nuseive, 1% agarose gel and transferred to Nytran for Southern blotting as described by the manufacturer. Blots were probed using PCR product for LMP2a derived from the IB4 cell line. This product was random primed labeled and products were detected as previously described (Miyashita et al., 1997).

Gardella Gel Analysis

PCR-modified Gardella gel analysis was performed as previously described (Decker et al., 1996). In brief, intact cells were lysed in situ and linear genomes were resolved from circular genomes on a 0.75% agarose gel. Each lane is sliced into many sections from which DNA is subsequently purified for EBV-specific DNA PCR. Linear DNA migrates faster than episomal DNA, and the exact migration point can be defined using virions as a source of linear DNA and PBL-B cells as a source of episomal DNA.

Spontaneous Outgrowth Assay

Peripheral blood cell populations were tested for spontaneous outgrowth as described (Lam et al., 1994). 1×10^5 cells were aliquoted into wells of a microtiter plate containing 9×10^5 cord blood cells. Cells were grown at 37°C with 5% CO_2 and fed with RPMI-1640/10% FCS twice weekly. At 1 month, all wells were scored for outgrowth by microscopic examination as well as performing the EBV-specific DNA PCR described above on an aliquot taken from each well to confirm the presence of the virus.

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