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Is EBV Persistence In Vivo a Model for B Cell Homeostasis?

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Summary

We have measured the absolute numbers of EBV-infected B cells in the peripheral blood of healthy persistently infected individuals. Single measurements on a panel of 15 healthy individuals demonstrate that the frequency varies over a wide range from 1–50 per 10^6 B cells. Repeat measurements over 1–3.5 years on several individuals whose frequencies varied over a 10-fold range showed that the variation does not represent the fluctuation in the frequency that can occur within an individual; rather, the frequencies are specific to the individual. The frequency within an individual measured over time is stable and contributes less than 10% to the variance seen in the whole population. These measurements suggest that the level of EBV-infected B cells is tightly regulated and we propose that the same homeostatic mechanisms that regulate the levels of normal B cells also regulate B cells latently infected with EBV.

Introduction

Epstein–Barr virus (EBV) is a member of the herpesvirus group (reviewed by Kieff, 1996; Rickinson and Kieff, 1996). These viruses are characterized by the ability to persist for the life of the infected host. The molecular and cellular mechanisms underlying this persistence are only now beginning to be understood. Much thinking about EBV persistence has been fueled by observations on tumor cells and in vitro infected cell lines. Studies in vivo have been hampered by the very low frequencies of virus-infected B cells and the fact that variable numbers of genome copies are present in infected cells depending on the form of the infection. Previous studies have employed either biological assays, termed spontaneous outgrowth (Rickinson et al., 1985; Yao et al., 1985, 1989, 1991; Lam et al., 1991), or DNA polymerase chain reaction (PCR) (Saito et al., 1989; Ambinder et al., 1990; Telenti et al., 1990; Wagner et al., 1992). Spontaneous outgrowth depends on the ability to detect the release of infectious virus through immortalization of bystander cells. However, we do not know how heterogeneous the infected cell population is, how it varies over time and between individuals, or the efficiency of the outgrowth assay at detecting different forms of infection. Therefore, this assay cannot be used to estimate the true number of infected cells or to compare measurements made within or between individuals. On the other hand, the assays used for the PCR studies were only sensitive enough to measure the relative genome copy number. However, it is known that latently infected cells in vitro can contain multiple copies of the viral genome that can range from 5–500 (Sugden et al., 1979) and that cells replicating the virus contain thousands of genomes (Summers and Klein, 1976). Thus, the PCR assays could only compare relative burdens of viral DNA. They could not measure the actual numbers of infected cells in an individual and could not, therefore, be used meaningfully to compare measurements of the number of infected cells either within or between individuals. For example, it is well established that the viral genome burden increases in immunosuppressed individuals (Saito et al., 1989; Telenti et al., 1990). But the PCR-based assays could not distinguish what part of this was due to an absolute increase in the numbers of latently infected cells and what part was due to a switch to viral replication, which would also increase the number of viral genomes per cell. To resolve these issues, we set out to develop a DNA PCR-based assay that would allow us to detect a single viral genome in the presence of as many as 10^6 uninfected cells. Using this assay, it was possible to demonstrate that the virus was present in vivo in B cells that had a cell surface phenotype ($CD23^-$, $CD80(B7)^-$) that had not been observed previously (Miyashita et al., 1995). Subsequent studies demonstrated that these cells are latently infected (Decker et al., 1996), resting and express the *LMP2A* gene (E. M. M. et al., unpublished data). EBV latency in a resting B cell had not been described previously and raises the question as to how the virus establishes itself in this population of cells and how its numbers are maintained.

During the course of our studies on the phenotype of EBV-infected B cells in vivo, we measured the frequency in a small panel of donors. When the measurements were repeated on two individuals 2 years later, they showed remarkably little variation over time, raising the possibility that the frequency was regulated and individual specific. We have now tested this prediction by measuring frequencies in a larger panel of individuals over the course of 1–3.5 years. This study reports the absolute numbers of EBV-infected cells in a panel of healthy individuals and substantiates the prediction that the frequency is individual specific, stable over long periods of time, and therefore regulated.

Results

The Absolute Frequency of EBV-Infected Cells in a Healthy Population over Time

We have measured the frequency of EBV-infected cells in the B cells of a panel of 15 healthy donors. We have previously shown that all of the infected cells in healthy individuals reside in the $CD19^+$ B cell fraction of the E rosette–negative population. Therefore, the frequencies were measured after depletion of T cells by rosetting to sheep red blood cells or enrichment of B cells by positive selection with a CD19 monoclonal antibody. The frequency of infected cells was then measured using a

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Table 1. Frequency of EBV-Infected Cells in a Population of Healthy Donors over Time

	Frequency/10^7 B cells over time (years)						
Donor	0	0.5	1.0	2.0	2.5	3.0	3.5
1	40	—	—	—	—	43	—
2	25						
3	30						
4	>10						
5	290	625[B]	—	250	—	220[B]	—
6	100	—	—	—	—	72	86
7	460	—	—	—	500[B]	250[B]	64
8	25						
9	40	25	—	90	—	64	—
10	10						
11	90[B]	—	96[B]				
12	70	—	95				
13	85[B]						
14	25						
15	265[B]						

Frequencies were estimated on E^- cells and were calculated from the frequency of EBV-infected cells in the population and the percentage of B cells as estimated by FACS analysis. In some cases, the frequencies were measured on purified B cells and these cases are denoted with a B. Since the values for the frequencies demonstrated a skewed distribution, we generated a data set with a normal distribution by taking the natural logarithm of the values. This allowed the estimation of the standard deviation (S), and the variance (S^2) for the ln data from the whole population denoted S^2_T. To calculate the best estimate of the variance in measurements within individuals, the values for S^2 for repeat measurements on each individual were combined to give a pooled estimate, S^2_W. For details see Experimental Procedures.

limiting dilution DNA PCR analysis correcting for the percentage of B cells as assessed by FACS analysis. Table 1 summarizes the frequencies of EBV-infected B cells in the peripheral blood for the panel of 15 healthy donors. The frequencies ranged from 10–460 per 10^7 B cells. In Table 1 we also summarized the accumulated data from multiple estimates on seven of these individuals over the course of a 1–3.5 year period. By visual inspection, it appeared that the variation in the frequency measurements within individuals was much smaller than between individuals, suggesting that the frequency may be stable and individual specific. To see whether this conclusion could be validated by statistical analysis, we calculated the relative contribution of the variance in repeated observations made on the same individuals to the variance in observations made between individuals. The distribution of values between and within individuals was definitely skewed; therefore, we calculated the natural log of the values (Winer, 1971), which generated a data set that had a normal distribution. Analysis of the ln data derived from Table 1 yielded a mean value of 4.14 with a standard deviation (S_T) of 1.10, giving a variance for values on the total population (S^2_T) of 1.210.

To calculate the best estimate of the variance in measurements within individuals, we combined all of the data from the individuals for whom we had repeat measurements over time (for details see Experimental Procedures). This gave a pooled estimate of the variance in measurements within individuals (S^2_W) (Winer, 1971) of 0.097. The variance between individuals (S^2_B) can then be derived by subtracting the variance within individuals ($S^2_W = 0.097$) from the total population variance ($S^2_T = 1.21$), i.e., $1.21 - 0.097 = 1.113$.

From this analysis, we may conclude that >90% (1.113 of 1.21) of the variance seen in the whole population can be accounted for by variance between individuals and <10% (0.097 out of 1.21) is due to the variance seen in multiple measurements over time in the same individual. Therefore, we may conclude that the frequency is individual specific and stable over time.

Is the Assay Sensitive Enough to Detect a Single Cell Containing 1–2 Copies of the Viral Genome?

To substantiate the analysis made above, we performed three control experiments to establish the sensitivity and reproducibility of the PCR assay. First, a limiting dilution analysis was performed using the Namalwa cell line, which is known to have one or two copies of the viral genome. If the assay detects single events then, according to the Poisson distribution, a straight line should be generated when the number of cells per sample is plotted against the logarithm of the fraction of negative samples and this was found to be the case (data not shown). The sensitivity and reproducibility of the assay were also tested by using the fluorescence-activated cell sorter to sort single Namalwa cells into wells of a microtiter plate and showing that the PCR was 96% reliable (23 of 24 samples were positive; data not shown). In control sorts, the cell sorter was 98% efficient at delivering fluorescence-labeled beads into the wells, therefore it is likely that the one negative result in the PCR can be accounted for by failure to deliver a cell into the well rather than failure of the PCR. Last, the reproducibility of the assay for detecting single copies of the viral genome was tested by demonstrating that the PCR could reproducibly (>99%) detect the DNA from one viral genome when diluted with the DNA from 10^6 uninfected cells (data not shown). These control experiments confirm that the assay will detect a single viral genome in a single cell and demonstrate that the PCR is reproducible, i.e., that there is no technical error inherent in the protocol that causes it to fail more than 2% of the time when low genome copies are being measured.

How Accurate Are the Frequency Measurements and How Stable Is the Frequency in an Individual?

An example of the results for purified B cells from one donor are shown in Figure 1. We have used both E^-

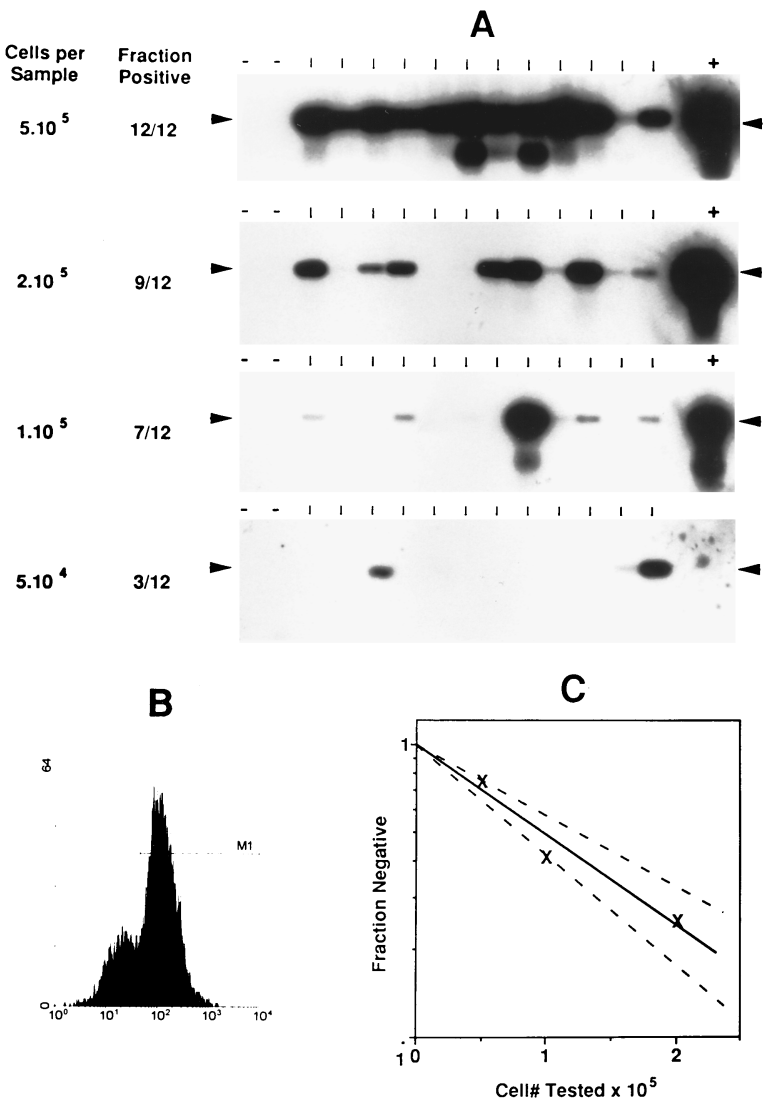

Figure 1. An Example of an Analysis of Peripheral Blood Mononuclear B Cells for the Presence of EBV

B cells were prepared from the peripheral blood using the MACS magnetic fractionation method with the pan-B cell antibody against CD19, as described in Experimental Procedures. DNA PCR analysis was performed on limiting dilutions of the purified B cells and FACS analysis was performed to estimate the purity of the B cells.

(A) Southern blot of the PCR products. The cell numbers tested were the following: 5×10^5, 2×10^5, 1×10^5, and 5×10^4 per sample. Lanes with experimental samples are labelled with a vertical line, positive controls with a plus, and negative controls with a minus.

(B) FACS analysis of the purified B cell population used for the limiting dilution analysis. The cells were stained for the pan-B cell marker CD20.

(C) The results from (A) are plotted using a semilog scale and demonstrate that the dilution analysis follows a Poisson distribution. Therefore, the frequency was estimated by calculating the number of cells for which the fraction of negative samples was 0.37. The ± error was calculated by drawing lines through the most extreme high and low data points (designated with dotted lines) and calculating frequencies from these lines. This analysis yields a raw frequency of 71 ± 14 infected cells per 10^7 total cells. FACS analysis revealed that the purified B cell population was 74% B cells; therefore, the corrected frequency was 96 ± 19 per 10^7 B cells.

cells and purified B cells and the frequency was always calculated based on the limiting dilution analysis and the percentage of B cells in the preparations, as assessed by FACS analysis, since we have shown that all of the infected cells in peripheral blood are B cells (Miyashita et al., 1995). Figure 2 shows the data obtained from four different donors. Taking the most extreme data points from each set of measurements, it is possible to calculate a maximum and minimum value for the frequency. From these values, it is possible to make a conservative estimate that the error in the frequency measurement is no more than ±30%. This was confirmed in a separate experiment in which the E^- cells from the same donor (donor 12) were assayed independently three times, using three serial dilutions each time. The values obtained were 120 ± 40, 90 ± 30, and 95 ± 50. Taking the extreme values, the largest error in a single estimate was no more than ±50%; however, comparison of the actual frequencies, measured in the three assays, suggests an error closer to 20% (97 ± 20). Since an error of ±30%–50% is sufficient to account for the variance (S^2_W) within individuals, it is not possible to conclude with certainty that the variation seen in measurements within individuals; for example, the high value of 625 at 0.5 years for donor 5, is significant. Therefore, within the experimental error of the assay (≤ ±30%), the frequencies are stable over time.

Discussion

In this paper, we report that the absolute frequency of EBV-infected B cells in the peripheral blood of a panel of healthy donors is between 1–50 per 10^6 B cells. We have also performed repeat measurements over the course of 1–3.5 years on several of these donors whose individual frequencies varied over a 10-fold range. These measurements showed that this variation in frequency does not reflect the variation that occurs within an individual; rather, a particular individual has a specific and stable frequency over time, such that it remains unchanged within the sensitivity of the assay for at least 1–3.5 years. This suggests tight regulation of the level of EBV-infected B cells. How are they regulated? Recently, an emerging picture has been forming of how

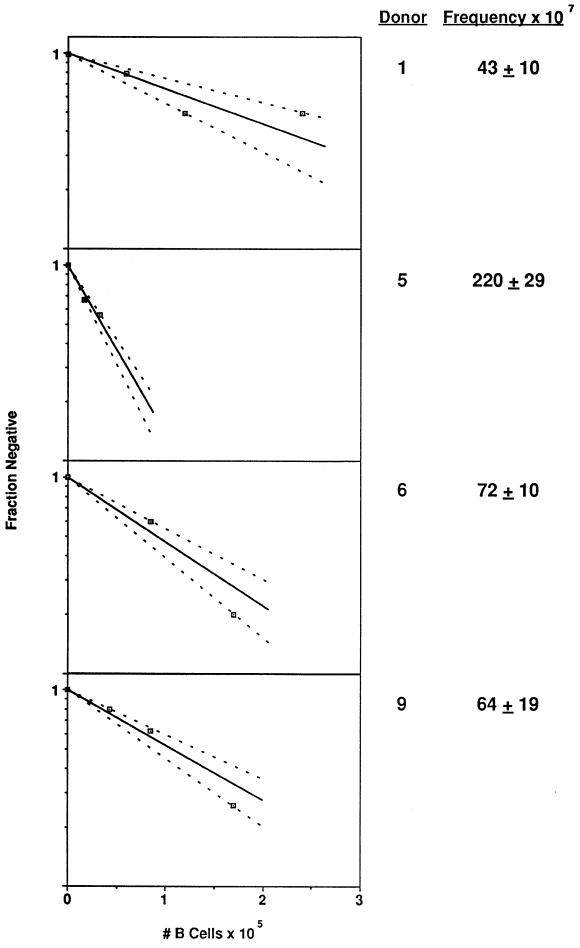

Figure 2. Analysis of the Frequency of EBV-Infected B Cells in the Peripheral Blood of Four Donors

Limiting dilution analysis of the kind exemplified in Figure 1 was performed and the fraction of negative samples was plotted against the number of B cells per sample at each dilution. The number of B cells per sample was estimated from the total number of cells tested, and the percentage of B cells based on FACS analysis after staining for the pan-B cell marker CD20. Since we have established the validity of the Poisson distribution (Figure 1; data not shown), the frequency was estimated by calculating the number of B cells for which the fraction of negative samples was 0.37. The ± error was calculated by drawing lines through the most extreme high and low data points (designated with dotted lines) and calculating frequencies from these lines.

EBV persists in vivo. The infected cells in the peripheral blood are latently infected and resting (Miyashita et al., 1995; Decker et al., 1996) and do not express the growth-promoting latent genes (Qu and Rowe, 1992; Tierney et al., 1994; Chen et al., 1995). They do express LMP2A, explaining the absence of viral replication (Decker et al., 1996), since LMP2A is believed to block fortuitous viral reactivation (Miller et al., 1995). Although LMP2A is a known target for CTL (Masucci and Ernberg, 1994), the cells may not be lysed because they lack B7 (Miyashita et al., 1995), which is required to activate memory CTL responses (Azuma et al., 1992). Therefore, the resting B7-negative circulating B cell, whose frequency we have measured in these studies, is the proposed candidate to be the site of long-term viral persistence. We have suggested the term "latency program" to describe this novel form of EBV latency (Thorley-Lawson et al., 1996), distinguishing it from latent gene expression associated with immortalization (the growth program), usually referred to as latency 3 (Rowe et al., 1987).

How do these resting cells maintain a constant frequency? Suggestive evidence for a mechanism comes from studies on the EBNA-1 protein. EBNA-1 is essential for the replication of the latent episomal viral genome (Yates et al., 1985) and contains a domain that prevents it from being processed and presented as a CTL target (Levitskaya et al., 1995). Therefore, EBNA-1 can be expressed in a proliferating B cell without being detected by the immune response. Thus, it is likely that the latently infected resting B cells sporadically go through periods of proliferation in which only EBNA-1 is expressed, a condition that we refer to as "EBNA-1 only" latency.

Seen in this light, EBV persistence is a balance between a pool of LMP2A$^+$ resting B cells and periodic transition through a proliferating EBNA-1 only state, driven by physiologic signals, to replenish the pool of latently infected resting B cells. This is analogous to the maintenance of B cell memory and raises the possibility that long-term persistence by EBV in the B cell compartment exploits and is a model for long-lived B cell memory. In the case of EBV infection, the absolute numbers of EBV-infected cells that enter the B cell pool are probably a function of a number of factors, including age and severity of infection, efficiency of the immune response in clearing the virus and virus-infected cells, and the genetic background of the individual. The immunological equivalent would be the establishment of memory B cells to a specific antigen. The absolute frequency of these cells would depend on the severity and frequency of exposure to the antigen, the efficacy of the immune response, and the genetic background of the individual. Thus, the fraction of B cells that are EBV infected or recognize a specific antigen both vary considerably between individuals while being maintained as part of a fairly constant population of total B cells.

Adoptive transfer experiments have led to the conclusion that memory B cells in mice only survive for 10–12 weeks in the absence of antigen. Periodic exposure to antigen is required for them to be long lived (Gray and Skarvall, 1988), giving rise to memory responses that last for decades in the human. Similarly, the population of resting B cells persistently infected with EBV appears to be maintained stably over long periods of time. This raises the question of whether fortuitous stimulation by cognate antigen plays a role in EBV persistence or whether the signals supplied by antigen are replaced by the actions of the growth-promoting latent genes.

In this paper, we have also presented a detailed analysis of the DNA PCR assay. This was done both to demonstrate that it can detect a single genome in a single EBV-infected cell and to substantiate our claim to measuring absolute numbers of infected cells in vivo. The reproducibility, sensitivity, and accuracy of this assay allows us to measure the frequency of EBV-infected cells and show that it is stable over time to an accuracy within the margin of error of the assay $\leqslant \pm 30\%$. By contrast, the variation seen within the population is clearly much

greater and cannot be accounted for by the variance in measurements within individuals, which contributed less than 10% of the total variance. This leads us to the conclusion that the frequencies are individual specific. If the error of the assay is ~30%, this is not due to false negatives. We do not obtain a significant level of negative results (≤2%) in control experiments in which we have tested multiple samples that are known to be positive. We have also controlled for this in another way during actual measurements of frequencies by showing that samples that were negative for EBV were still positive for an internal control sequence, the *bcl-2* gene (data not shown).

It is unclear at this time whether the most extreme values for the frequency within an individual represent genuine fluctuations. The most obvious example of this is the value of 625 for donor 5 at 0.5 years. The intrinsic error for the assay that we have measured would not be sufficient to account for this unusually high value, raising the possibility that there may have been a transient increase in the number of infected cells in this individual. However, the error may be larger in measurements taken over long periods of time. Not withstanding, it is striking that even for this individual the frequency returns to a value remarkably close to the original in subsequent measurement 2 and 3 years later.

Previous estimates of the frequency of EBV-infected cells in the peripheral blood of healthy donors have depended on the biological assay of spontaneous outgrowth that relies on the infected cells releasing infectious virus that is then detected as immortalization of bystander B cells. We do not know what fraction of the cells undergo this process, the efficiency of the assay, or the extent to which these parameters vary from time to time or from individual to individual. Nevertheless, many inferences about the nature of EBV persistence have been drawn using this assay, therefore it would be of interest to know how closely the values measured by outgrowth correlate with the more precise and reproducible PCR method. The most extensive use of this assay has been by Yao et al. (1985, 1989, 1991). These authors have reported that the number of cells giving rise to spontaneous outgrowth is stable for several months in a given individual with values consistent with frequencies ranging from around 20 to <0.1 per 10^6 B cells (Yao et al., 1985). This suggest that the outgrowth assays underestimate the frequency of infected cells, particularly at the low end. Furthermore, comparison is hindered because the dilutions reported do not always follow a Poisson distribution (Yao et al., 1991), making estimates of frequency problematic. This probably occurs because the cord blood filler cells used are more efficiently immortalized than the adult donor cells, leading to more efficient outgrowth at lower donor cell numbers.

Outgrowth assays and DNA PCR have also been used to demonstrate an increase in the viral burden in the peripheral blood of immunosuppressed individuals that has generally been interpreted as an increase in latently infected cells (Yao et al., 1985; Saito et al., 1989; Telenti et al., 1990). Analysis of a large group of immunosuppressed patients with our assay will allow definitive statements to be made about the exact mechanisms that contribute to the overall increase in viral burden. Specifically, we will be able to distinguish a true increase in the number of infected cells from contributions made by viral reactivation. In the long run, this should provide insights into the effects of immunosuppression on EBV persistence and hopefully define parameters that may have predictive value in understanding which particular individuals are at risk for EBV-associated neoplasia.

Experimental Procedures

Cell Lines

The Namalwa cell line (American Type Culture Collection) is an EBV-positive Burkitt's lymphoma. It contains either one or two integrated copies of EBV. It was used for all experiments that tested the sensitivity of the PCR protocol. It was also included in all experiments as a positive control. BJAB (gift of Dr. E. Kieff) is an EBV-negative B cell lymphoma. It was used to provide filler cells when performing limiting dilution analysis. A minimum of five samples of 10^6 BJAB cells alone were also included in every experiment to act as a negative control for PCR. The cells were maintained in 10% fetal bovine serum (Biowhittiker), RPMI 1640 (Mediatech) at 37°C in an atmosphere of 5% CO_2.

Cell Fractionation

The donors in this study were all EBV-positive, ranging in age from 25–60 years. They included three women and twelve men: two Hispanics, four Asians, and nine Caucasians.

Peripheral blood (usually 120 ml) was drawn from healthy donors using heparinized syringes. The blood was layered onto Ficoll–Hypaque (Pharmacia). Buffy coats were removed and washed with Heparin (1 U/ml) in 1% fetal calf serum, RPMI 1640. E^- cells were prepared by depleting peripheral blood mononuclear cells (PBMCs) of T cells (E^+) using neuraminidase-treated (GIBCO BRL) sheep red blood cells (gift from Dr. A. Rabson) as described previously (Hurley and Thorley-Lawson, 1988). Typically, E^- cells contained 20%–40% B cells. B cells were prepared by positive selection from whole PBMCs using a monoclonal antibody to the B cell–specific marker CD19 (Dako) and the MACS bead system. First, buffy coats were removed and washed with RPMI 1640 with 1% fetal calf serum and heparin (1 U/ml). PBMCs were resuspended in phosphate-buffered saline supplemented with 5 mM EDTA, 0.5% BSA (PBSA) at 2×10^7 cells/ml and aliquoted into 1.5 ml microcentrifuge tubes at 1 ml/tube. Biotinylated antibody was added to each tube and tubes were put on a rotator at 4°C for 30 min. The cells were then washed two times with PBSA and MACS streptavidin microbeads (Miltenyi), diluted with phosphate-buffered saline to 200 μl, added to each tube, and incubated for 15 min on ice. The cells were then washed once and resuspended in PBSA. MACS type AS separation columns (Miltenyi) were used according to the instructions of the manufacturer. In brief, the column was washed with 10 column volumes of PBSA. The magnetically stained cell suspension was then applied to the top of the column and allowed to pass through. The column was then washed with 5 column volumes of PBSA. The collected passthrough was the negative fraction. The column was removed from the magnetic separator, back-flushed with a side syringe, replaced back on the magnetic separator, and again washed with 5 column volumes of PBSA. The column was then removed from the magnetic separator and washed with 5 column volumes of PBSA. The cells that eluted were the positive fraction. The flow rate of the column was controlled by using a 23G needle. These preparations were always >75% B cells and usually >90% B cells.

The percentage of B cells in the population to be analyzed was assessed by staining with an antibody to the B cell–specific marker CD20 (Dako) directly coupled to fluorescein isothiocyanate. As a negative control, MOPC 21 (IgG1 isotype control, Sigma, St. Louis, Missouri) was used and propidium iodide was used to distinguish live from dead cells. Samples were analyzed using a Becton-Dickenson FACScan with Lysys II software.

For analysis of single Namalwa cells, single cells were sorted with a FACStar Plus cell sorter directly into the wells of a 96-well

V-bottomed plate that already contained 10^6 BJAB cells in 0.2 ml of medium.

DNA PCR Analysis for EBV DNA

Either non-T (E^-) PBMCs or purified B cells were used for analysis by PCR. DNA was isolated using a modification of the protocol of Coen (1990). Cells were pelleted and resuspended with 100 μL proteinase K digestion buffer (1 mg/ml proteinase K, 20 mM EDTA [pH 8.0], 20 mM Tris [pH 7.4], 0.5% SDS). Cell lysates (100 μL) were incubated at 55°C for 4–18 hr. The lysates were extracted with 100 μL each of phenol and chloroform-isoamyl alcohol (24:1). The organic phase was back-extracted with 50 μl digestion buffer (without proteinase K) and then extracted twice with an equal volume of chloroform-isoamyl alcohol (24:1). The aqueous phase was mixed with 50 μL of 10 M ammonium acetate and 2.5 vol of 100% ethanol and stored overnight at −20°C. The precipitated DNA was pelleted in a microcentrifuge for 15 min at 4°C and 70% ethanol was used to wash away excess salt. The pellets were air dried and resuspended in 50 or 25 μl high pressure liquid chromatography H_20 (Aldrich) and either stored at −20°C or used directly in PCR.

Primers (22 bp) were made from the W repeat region of EBV. They were 5′-CTTTAGAGGCGAATGGGCGCCA-3′ and 5′-TCCAGGGCCT TCACTTCGGTCT-3′ and amplified the region between 14068 and 14562 to give a product of 494 bp. To determine whether the extracted DNA was a suitable template for PCR, i.e., test for false negatives, we used primers against the single copy cellular gene, *bcl-2*. These were 5′-CTTTAGAGAGTTGCTTTACGTG-3′ and 5′-TC CATATTCATCACTTTGACAA-3′ and generated a PCR product that was 277 bp long (Wyatt et al., 1992).

PCRs were performed in a final volume of either 50 or 100 μL with up to 4 μg of DNA per reaction. The DNA was denatured for 10 min at 95°C "hot start," while amplification mixture, containing 20 pmol of each primer, 200 μM dNTPs (Pharmacia), 50 mM KCL, 10 mM Tris [pH 8.3], 2.0 mM $MgCl_2$, 2.0 U Taq polymerase (Perkins Elmer, Branchburg, New Jersey) was added. In later experiments, Taq extender (Stratagene) was also included and the mixture was added prior to the hot start. Both modifications significantly improved the strength of the signals obtained. The reactions were either amplified in the DNA Thermal Cycler 4800 or GeneAmp PCR System 9600 (Perkins Elmer). For the DNA Thermal Cycler 4800, GeneAmp PCR tubes were used with an overlay of mineral oil. The DNA was denatured for 15 s at 95°C, annealed for 15 s at 64°C, and extended for 30 s at 72°C for a total of 30 cycles with the last cycle having an extension for 5 min of 72°C. For the GeneAmp PCR System 9600, MicroAmp Reaction tubes were used and no mineral oil was required. The DNA was denatured for 15 s at 94°C, annealed and extended for 45 s at 66°C for a total of 30 cycles. The amplification of *bcl-2* used all the same reagents except the primers and the final $MgCl_2$ concentration was 1.5 mM. These reactions were amplified in the DNA Thermal Cycler 4800 for 15 s at 95°C, for 15 s at 55°C, and 30 s at 72°C for a total of 30 cycles. For each set, there were 36 samples of which 6–10 would be negative controls containing 10^6 BJAB cells, 1 would be a no DNA control, and 1–3 positive controls, usually 60–10^4 Namalwa cells.

Southern Blotting of the PCR Products

The PCR products were fractionated on a large (24 cm × 20.5 cm) 2% or 3% Nusieve, 0.6% or 1% SeaKem Agarose gel (FMC). $\phi \times$ 174 DNA digested with HinfI or HaeIII (NEB) was used as molecular weight markers. The products were then transferred by capillary action onto Nytran (Schleicher and Schuell), following the instructions of the manufacturer. The membranes were probed with a PCR product from the W repeat of either IARC 304 or Namalwa, labeled with α-P^{32} dATP and dCTP (3000 Ci/mMol; ICN or Dupont, NEN Research Products) by random priming according to the instructions of the manufacturer. (Boerhinger–Mannheim). The membranes were hybridized and washed following the recommended protocol for Nytran.

Statistical Analysis

To estimate the frequencies, a limiting dilution analysis was performed with at least three dilutions of cells with 8–12 samples per dilution (for example see Figure 1). Since the distribution of values for the whole population was definitely skewed (see Table 1), we calculated the natural log of the values (Winer, 1971), which generated a data set that had a normal distribution giving a variance (S^2_T) for the total population of 1.124.

Since we had repeat measurements on several individuals, we wished to calculate the best estimate of the variance in measurements within individuals (S^2_W) by pooling all of this data. To do this, the variance (S2) from the ln values of repeat measurements on each individual over time from Table 1 were calculated. These were then combined to give a pooled estimate according to the formula:

$$S^2_W = \frac{(n_1 - 1)\, S_1^2 + (n_2 - 1)\, S_2^2}{n_1 + n_2}$$

where n equals number of measurement on a given individual, S equals the standard deviation of those measurements, and S^2 equals the variance (Winer, 1971).

∴ From the data in Table I

$$S^2_W = \frac{(0.0495)^2 + 3(0.471)^2 + 2(0.165)^2 + 3(0.333)^2 + 3(0.558)^2 + (0.042)^2 + (0.212)^2}{2 + 4 + 3 + 4 + 4 + 2 + 2}$$

$$= 0.097$$

The relative contribution of variance within individuals (S^2_W) and between individuals (S^2_B) to the total variance in the population (S^2_T) is calculated (Winer, 1971) according to the formula:

$$S^2_T = S^2_W + S^2_B$$

Since $S^2_W = 0.097$ (see above)

and $S^2_T = 1.210$ (from Table 1)

∴ $S^2_B = 1.113$

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