Immunity, Vol. 9, 405–411, September, 1998, Copyright ©1998 by Cell Press

Epstein-Barr Virus LMP2A Drives B Cell Development and Survival in the Absence of Normal B Cell Receptor Signals

Robert G. Caldwell*, Joanna B. Wilson,†
Steven J. Anderson*, and Richard Longnecker*‡
*Department of Microbiology-Immunology
Northwestern University Medical School
Chicago, Illinois 60611
†Division of Molecular Genetics
Institute of Biomedical and Life Sciences
University of Glasgow
Glasgow G11 6NU
United Kingdom

Summary

Epstein-Barr virus (EBV) establishes a persistent latent infection in peripheral B lymphocytes in humans and is associated with a variety of malignancies and proliferative disorders. Latent membrane protein 2A (LMP2A) is one of only two viral proteins expressed in latently infected B lymphocytes in vivo. LMP2A blocks B cell receptor (BCR) signal transduction in vitro by binding the Syk and Lyn protein tyrosine kinases. To analyze the significance of LMP2A expression in vivo, transgenic mice with B cell lineage expression of LMP2A were generated. LMP2A expression results in the bypass of normal B lymphocyte developmental checkpoints allowing immunoglobulin-negative cells to colonize peripheral lymphoid organs, indicating that LMP2A possesses a constitutive signaling activity in nontransformed cells.

Introduction

Epstein-Barr virus (EBV) is a nearly ubiquitous human oncogenic herpesvirus associated with numerous proliferative disorders, including Burkitt's lymphoma, Hodgkin's disease (HD), AIDS-associated immunoblastic lymphoma, oral hairy leukoplakia, and nasopharyngeal carcinoma (NPC) (for review, Rickinson and Kieff, 1996). B lymphocytes infected with EBV in vitro are immortalized and subsequently termed lymphoblastoid cell lines (LCLs). These EBV-transformed LCLs contain EBV episomes and nine virus-encoded proteins. Six are EBV nuclear antigens (EBNAs), EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNALP (for review, Kieff, 1996). Three are the integral latent membrane proteins (LMPs), LMP1 (Kieff, 1996), LMP2A, and LMP2B (for review, Kieff, 1996). In healthy individuals, EBV typically establishes a persistent latent infection in which the virus can be detected in resting, nonproliferating peripheral B lymphocytes (Miyashita et al., 1995, 1997). These latently infected cells express only two virally encoded genes, namely LMP2A and EBNA1 (Qu and Rowe, 1992; Tierney et al., 1994; Chen et al., 1995; Miyashita et al., 1997). Further, LMP2A is expressed in tumor cells in several EBV-associated malignancies including NPC and HD (for review, Rickinson and Kieff, 1996).

LMP2A contains twelve hydrophobic membrane-spanning regions and aggregates in the plasma membrane in characteristic patches (Longnecker and Kieff, 1990; Longnecker et al., 1991). The cytoplasmic amino terminus of LMP2A contains eight tyrosine residues, including a consensus immunoreceptor tyrosine activation motif (ITAM) domain (Longnecker and Kieff, 1990). The LMP2A ITAM, when phosphorylated, constitutively binds the Syk protein tyrosine kinase (Fruehling et al., 1996). In addition, LMP2A binds specific Src family protein tyrosine kinases and other cellular proteins (Longnecker et al., 1991; Burkhardt et al., 1992). In EBV-transformed B cells grown in vitro, the LMP2A amino terminal cytoplasmic domain functions to block BCR signal transduction, preventing normal calcium flux and the accumulation of tyrosine-phosphorylated proteins following BCR cross-linking (Miller et al., 1994, 1995; Longnecker and Miller, 1996). Inasmuch as this signaling block prevents the activation of lytic replication in EBV-transformed cells, a role for LMP2A in establishing or maintaining latency in vivo has been proposed (Miller et al., 1994, 1995; Longnecker and Miller, 1996). However, the relevance of LMP2A expression in human peripheral B cells has been difficult to examine due to the paucity of EBV latently infected peripheral B cells (Yao et al., 1985; Lewin et al., 1987; Lam et al., 1991; Yao et al., 1991; Miyashita et al., 1995). To analyze the significance of LMP2A expression in vivo, transgenic mice with B cell lineage expression of LMP2A were generated. LMP2A expression results in the bypass of normal B lymphocyte developmental checkpoints allowing immunoglobulin-negative cells to colonize peripheral lymphoid organs, indicating that LMP2A possesses a constitutive signaling activity in nontransformed cells.

Results

Transgene Construction and Expression

The Eμ-LMP2A transgene was constructed by inserting chimeric genomic and cDNA LMP2A sequences downstream of the immunoglobulin heavy chain promoter and intronic enhancer (Eμ). Two independent transgenic lines, designated TgE and Tg7, were analyzed. Southern hybridization of LMP2A cDNA sequences to BamHI-digested genomic tail DNA identified a 2300 bp band predicted from the transgene sequence (data not shown). Both lines exhibited no gross developmental defects and can survive for greater than 12 months in a barrier facility. LMP2A expression was detected in purified splenic B cells by immunoblot analysis (Figure 1A) and in bone marrow cells by RT-PCR (Figure 1B).

LMP2A Redirects B Cell Development

To examine effects of LMP2A expression on peripheral B cell populations, flow cytometric analysis for expression of CD19 and IgM was performed on splenic cells. TgE and Tg7 splenic cells displayed unique alterations

‡To whom correspondence should be addressed (e-mail: r-longnecker@nwu.edu).

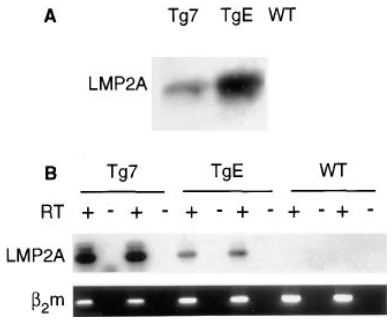

Figure 1. LMP2A Expression in Eμ-LMP2A Mice

(A) Immunoblot of LMP2A expression in trangenic spleen samples. (B) RT-PCR analysis of LMP2A expression in mouse bone marrow samples. cDNA was prepared from two independent bone marrow samples of each mouse genotype indicated in the presence (+) or absence (-) of reverse transcriptase. cDNA was subjected to PCR using either LMP2A or beta-2 microglobulin (β_2m)-specific primers. The amplified LMP2A-specific PCR products were visualized using ^{32}P radiolabeled oligonucleotide OL115. β_2m-specific PCR products were visualized by ethidium bromide staining and LMP2A immunoblots were performed as previously described (Fruehling et al., 1996).

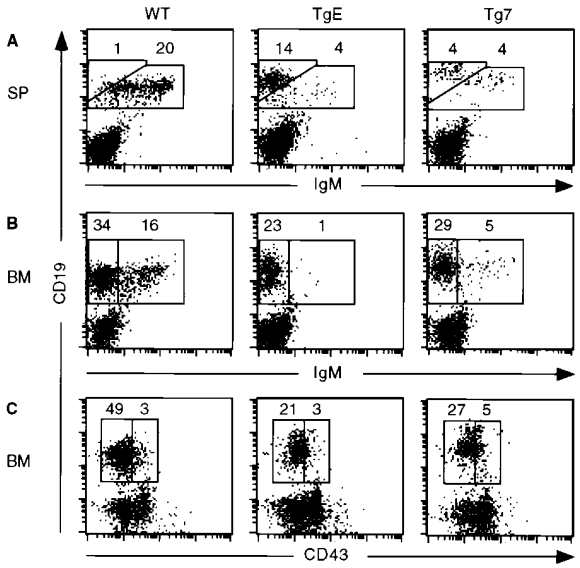

Figure 2. Analysis of B Lymphocytes in Eμ-LMP2A Mice by Flow Cytometry

Single cell suspensions of bone marrow (BM) and spleen (SP) samples were prepared and stained with the indicated antibodies. Plots show representative data from at least three independent experiments. Relative percentages of lymphocyte gated cells are indicated. Boxes delineating CD43$^+$ and CD43$^-$ staining cells were determined using RAG-1$^{-/-}$ bone marrow samples (Figure 4; Spanopoulou et al., 1994).

in surface phenotypes when compared to nontransgenic littermates. First, transgenic animals typically showed a reduction in the proportion and total number of CD19$^+$ B cells in the spleen (Figure 2A; Table 1). Second, and more strikingly, a high proportion of the CD19$^+$ cells in the transgenic spleens were negative for surface IgM (Figure 2A; Table 1). Such cells were virtually absent in the spleens of nontransgenic littermates. To verify that the CD19$^+$IgM$^-$ cells were from the B lineage, flow cytometric analysis was performed on splenic samples from transgenic and littermate control animals. Staining for CD3, pan-NK, or CD11b in combination with CD19 demonstrated that no more than background levels of staining were apparent for CD19 and each of the other markers (data not shown). Staining with B220 and CD19 indicated that all CD19$^+$ cells were also B220$^+$ (data not shown). These data indicate that the CD19$^+$IgM$^-$ cells observed in transgenic animals are not due to ectopic expression of LMP2A in non-B lineage cells resulting in the up-regulation of CD19. In addition, flow cytometric analysis staining for CD4 and CD8 of both splenic and thymic cell populations indicated no developmental defect in T lineage cells. In conclusion, these data suggest that LMP2A redirects B lymphocyte development in Eμ-LMP2A mice allowing IgM$^-$ B lineage cells to accumulate in the periphery.

To determine the stage at which B cell development was altered in Eμ-LMP2A mice, bone marrow cells from transgenic and nontransgenic animals were examined for expression of CD19, IgM, and CD43. In normal bone marrow, appropriate immunoglobulin heavy chain gene rearrangement and expression is required for transition from a CD19$^+$CD43$^+$ pro–B to a CD19$^+$CD43$^-$ pre–B cell stage (Rolink and Melchers, 1991). Subsequent rearrangement of immunoglobulin light chain genes and expression of both heavy and light chains at the cell surface allows for transition to the CD19$^+$IgM$^+$ immature B cell stage (Rolink and Melchers, 1991). The pattern of CD19 and IgM expression in transgenic bone marrow cells was similar to that seen in the spleen; namely, fewer CD19$^+$ cells were detected, with an especially dramatic reduction in the proportion of IgM$^+$ cells compared to nontransgenic animals (Figure 2B; Table 1). Similar to the control animals however, the majority of CD19$^+$ cells in the transgenic bone marrow did not express CD43 (Figure 2C; Table 1). The presence of CD43$^-$ B lineage cells lacking IgM expression in transgenic bone marrow suggested a potential defect at the pre-B stage of development.

LMP2A Selectively Blocks Immunoglobulin Heavy Chain Rearrangement

To determine if a defect in immunoglobulin gene rearrangement might underlie the failure of transgenic bone marrow cells to express IgM, we investigated the status of the immunoglobulin heavy (H) and kappa light chain genes (K) by using a PCR assay to detect ordered D-J_H, V-DJ_H, and V-J_K gene rearrangements (Schlissel et al., 1991). In genomic DNA from whole bone marrow of nontransgenic littermates, D-J_H and V-DJ_H, as well as V-J_K gene rearrangements were detected (Figures 3A–3C). Although D-J_H rearrangements in transgenic animals were detected at levels comparable to nontransgenic littermates (Figure 3A), V-DJ_H rearrangements were absent or detected at greatly reduced levels (Figure 3B). Surprisingly, transgenic V-J_K gene rearrangements were detected at the same level in transgenic and nontransgenic animals (Figure 3C). Together, these data indicate that LMP2A expression in precursor B cells alters normal B cell development, perhaps by

Table 1. Analysis of LMP2A Transgenic Mice

	Bone Marrow		Spleen	
Transgenic Line	Tg CD19$^+$/ Control CD19$^+$ (%)[a]	CD19$^+$IgM$^-$/ CD19$^+$ (%)[b]	Tg CD19$^+$/ Control CD19$^+$ (%)[a]	CD19$^+$IgM$^-$/ CD19$^+$ (%)[b]
Tg7$^+$ (n = 6)[c]	68% (48%–75%)	80% (66%–89%)	43% (23%–60%)	21% (7%–42%)
Control (n = 6)[c]	—[d]	60% (50%–66%)	—[d]	2% (1%–2%)
TgE$^+$ (n = 5)[e]	66% (53%–77%)	97% (96%–98%)	42% (14%–86%)	76% (69%–84%)
Control (n = 6)[e]	—[d]	59% (49%–65%)	—[d]	5% (4%–7%)
TgE$^+$RAG-1$^{-/-}$ (n = 4)[f]	60% (45%–87%)	100%[g]	54% (22%–112%)	100%[g]
RAG-1$^{-/-}$ (n = 3)[f]	30% (16%–35%)	100%[g]	—[h]	—[h]

Flow cytometric analysis was performed on bone marrow or splenic cell populations as described in the Experimental Procedures.
[a] Average reduction of CD19$^+$ cells in bone marrow or spleen of the transgenic lines was determined by dividing the total number of CD19$^+$ cells in the bone marrow or spleen of each transgenic by the total number of CD19$^+$ cells in the bone marrow or spleen of control animals.
[b] Average percent of CD19$^+$ cells that were IgM$^-$ in the bone marrow or spleen of the indicated murine lines was determined by dividing the CD19$^+$IgM$^-$ cell number in each line by the total number of CD19$^+$IgM$^-$ and CD19$^+$IgM$^+$ cells.
[c] Three different litters each composed of two Tg7 transgenics and two littermate controls were analyzed and used to derive the average value. The range of values is indicated in parentheses.
[d] Total CD19$^+$ cells in littermate control animals were set at 100%.
[e] Two different litters each composed of two TgE transgenics and two littermate controls and a third litter composed of one TgE transgenic and two littermate controls were used in the analysis to derive the average value. The range of values is indicated in parentheses.
[f] Two different litters, each also used for the TgE analysis composed of two TgE-RAG-1$^{-/-}$ animals and one or two RAG-1$^{-/-}$ animals were used in the analysis to derive the average value. The range of values is indicated in parentheses.
[g] All cells in the RAG-1$^{-/-}$ background are IgM$^-$ due to the absence of rearrangement in the RAG-1$^{-/-}$ background.
[h] There are no splenic B cells except background levels in RAG-1$^{-/-}$ animals.

transmitting signals normally attributed to the pre-BCR. Specifically, appearance of CD43$^-$ bone marrow cells that lack V-DJ$_H$ rearrangements suggests that signals from LMP2A may shut off V$_H$ recombination in a premature enforcement of allelic exclusion, while concomitantly allowing cells to progress to a CD43$^-$ stage.

LMP2A Alters B Cell Development in a RAG-Deficient Background

To directly test the possibility that LMP2A could provide developmental signals normally associated with expression of a functional heavy chain gene, namely transition from the pro–B to the pre–B cell stage (Rolink and Melchers, 1991), we examined the effects of Eμ-LMP2A transgene expression in a recombinase-deficient background. Recombinase activating gene 1 (RAG-1) null animals are unable to rearrange immunoglobulin genes and are characterized by a block in B cell development at the CD43$^+$ pro–B stage (Mombaerts et al., 1992; Spanopoulou et al., 1994). This block can be partially overcome by transgene-dependent expression of a functional heavy chain that allows B cell precursors to progress to the CD43$^-$ stage (Spanopoulou et al., 1994). TgE animals were bred to RAG-1$^{-/-}$ mice, and subsequent TgE$^+$RAG-1$^{-/-}$ were identified by PCR analysis of genomic tail DNA with specific oligonucleotide primers (data not shown). Flow cytometric analysis of splenic cells from TgE$^+$RAG-1$^{-/-}$ animals identified significant numbers of CD19$^+$ cells, a population absent in spleens of nontransgenic RAG-1$^{-/-}$ animals (Figure 4A; Table 1). Similarly, bone marrow of TgE$^+$RAG-1$^{-/-}$ animals exhibited a dramatic increase in CD19$^+$ cells when compared to the nontransgenic RAG-1$^{-/-}$ littermates (Figure 4B; Table 1). Examination of CD43 expression in these bone marrow samples revealed that unlike nontransgenic RAG-1$^{-/-}$ littermates, TgE$^+$RAG-1$^{-/-}$ bone marrow contained a large percentage of CD19$^+$ cells that did not express CD43 (Figure 4C; Table 2).

Transgenic Bone Marrow Cells Proliferate in IL-7-Dependent Growth Conditions

To determine if CD43$^-$ cells present in the transgenic bone marrow samples demonstrated the expected characteristics of normal pre–B cells, we examined the ability of these cells to respond to IL-7. Precursor B cell growth in IL-7-containing media in the absence of stromal cell contact requires progression beyond the CD43$^+$ stage, a transition dependent upon immunoglobulin heavy chain gene rearrangement and expression (Era et al., 1991; Spanopoulou et al., 1994). Bone marrow cells from TgE$^+$RAG-1$^{-/-}$, TgE$^+$RAG-1$^{+/+}$, and nontransgenic RAG-1$^{+/+}$ animals were cultured in vitro in IL-7-containing methylcellulose media. After 7 days, the number of colonies and total cells recovered from the three cultures was similar (data not shown), indicating that cells

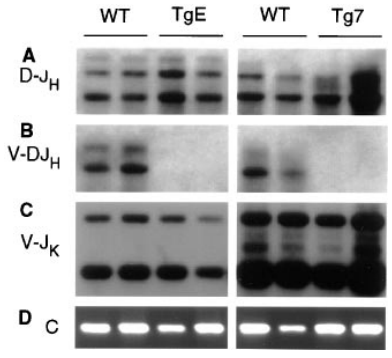

Figure 3. Detection of Immunoglobulin Gene Rearrangements in Eμ-LMP2A Bone Marrow Cells

PCR amplification and detection of D-J$_H$ (A), V-DJ$_H$ (B), and V-J$_K$ (C) gene segments using ^{32}P radiolabeled oligonucleotides as described. (D) Amplification of RAG-1 DNA sequences were used as a positive control (C) of DNA content. Two transgenic and two nontransgenic wild-type (WT) littermate control bone marrow samples were used for each genotype.

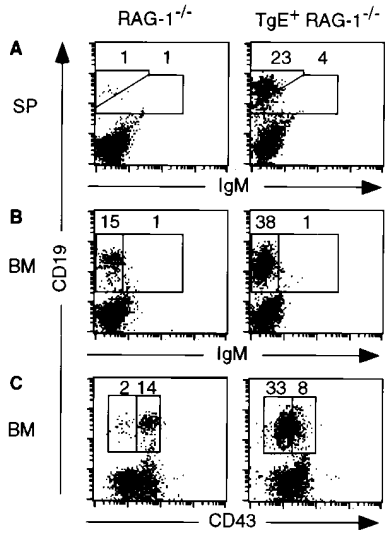

Figure 4. Analysis of B Lymphocytes in TgE × RAG-1$^{-/-}$ Animals

Single cell suspension of bone marrow and spleen cells from nontransgenic RAG-1$^{-/-}$ and TgE^{+}RAG-1$^{-/-}$ littermates were stained with the indicated antibodies and analyzed by flow cytometry as described in Figure 1. Plots show representative data from two independent experiments.

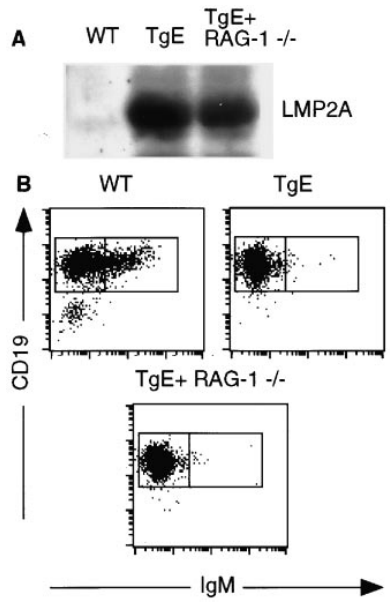

Figure 5. Analysis of Transgenic Cells Grown in IL-7-Supplemented Methylcellulose Media

(A) LMP2A expression from IL-7-stimulated bone marrow cultures. (B) Flow cytometry analysis of cells cultured in IL-7. Equivalent numbers of IL-7 cultured cells were stained with CD19- and IgM-specific antibodies and analyzed as in Figure 1. Plots show representative data from two independent experiments.

from the transgenic animals were able to survive and proliferate in response to IL-7. As previously reported (Spanopoulou et al., 1994), nontransgenic RAG-1$^{-/-}$ cells did not proliferate under these conditions (data not shown). LMP2A expression, detected by immunoblot analysis of cultured cells (Figure 5A), confirms that transgenic cells survive the culture conditions. Flow cytometric analysis of IL-7 cultures showed that virtually all cells from the three cultures were CD19^{+} (Figure 5B). However, whereas cultures from nontransgenic RAG-1$^{+/+}$ littermates contained numerous IgM^{+} cells, such cells were absent in TgE and TgE^{+}RAG-1$^{-/-}$ cultures (Figure 5B). Taken together, the in vivo and in vitro characteristics of the Eμ-LMP2A transgenic bone marrow cells indicate that LMP2A can provide development signals that mimic those initiating from an immunoglobulin heavy chain in the context of the pre-BCR.

Discussion

B lymphocytes from mice bearing targeted deletions of RAG-1, RAG-2, immunoglobulin heavy chain, or the Syk protein tyrosine kinase genes are unable to progress from the pro-B to pre-B developmental stage (Kitamura et al., 1991; Mombaerts et al., 1992; Shinkai et al., 1992; Cheng et al., 1995; Turner et al., 1995). In animals lacking peripheral immunoglobulin heavy chain gene expression by means of inducible Cre-*loxp*-mediated gene targeting, receptorless B lymphocytes are eliminated by apoptosis (Lam et al., 1997). These experiments illustrate the necessity for competent BCR signaling in all stages of B cell development, from early B cell ontogeny to peripheral B cell maintenance. Surprisingly, B cells lacking BCR expression are prominent in the bone marrow and periphery of the Eμ-LMP2A transgenic mice. Although these B cells exhibit many of the hallmarks of appropriately developing B lymphocytes, including downmodulation of CD43 expression, appropriate rearrangement of kappa light chain genes, IL-7 responsiveness, and colonization of splenic environments, they are unable to rearrange functional immunoglobulin heavy chain genes and subsequently do not express a pre-BCR. As well as providing a developmental signal that bypasses the requirement for heavy chain gene expression in bone marrow, LMP2A provides a survival signal to progenitor and peripheral B cells as evidenced by the increase in CD19^{+}IgM^{-} cells in spleens of Eμ-LMP2A transgenic mice. Most striking is that the Eμ-LMP2A transgene allows receptorless B cells to mature into CD43^{-} cells in the bone marrow that are able to colonize the spleen in a RAG-1$^{-/-}$ background.

Previous studies have shown that LMP2A forms tyrosine-phosphorylated aggregates in the plasma membrane, associates with the Syk and Lyn protein tyrosine kinases, and blocks BCR signal transduction in EBV-transformed lymphocytes in vitro (Longnecker et al., 1991; Burkhardt et al., 1992; Miller et al., 1994, 1995; Fruehling et al., 1996; Fruehling and Longnecker, 1997). In these cells, the potential for LMP2A to mimic rather than inactivate signal transduction pathways was not appreciated, especially since LMP2A is nonessential for EBV transformation (Longnecker et al., 1992, 1993a, 1993b). Data presented herein suggests that when expressed in progenitor mouse B lymphocytes in vivo, LMP2A can supersede the pre-BCR and redirect B cell development. This observation may have importance in EBV-associated malignancies. Although Reed-Sternberg cells in HD have been characterized as germinal

center–derived B lineage cells, some of these cells contain somatic mutations resulting in the absence of surface Ig expression, thereby indicating that these cells do not require BCR signaling for survival (Kanzler et al., 1996; Braeuninger et al., 1997). By mimicking signals derived from the BCR, LMP2A may provide a signal that would maintain these cells in the absence of a competent BCR. This may be an important first step in the development of HD.

The role of LMP2A in EBV latency has been the subject of much conjecture as the message for LMP2A is readily detected in immune competent hosts with EBV latent infections (Qu and Rowe, 1992; Tierney et al., 1994; Miyashita et al., 1997). Studies in vitro have indicated that LMP2A blocks normal BCR signal transduction, which prevents switch from latent to lytic replication following BCR signal transduction by surface immunoglobulin cross-linking (Miller et al., 1994, 1995). These observations have led to the hypothesis that the role of LMP2A in latent EBV infection is to prevent activation of lytic EBV replication by immunoglobulin-mediated signal transduction (Miller et al., 1993, 1994; Longnecker and Miller, 1996). This LMP2A function would be important in preventing lytic replication in latently infected lymphocytes as they circulate in the peripheral blood, bone marrow, or lymphatic tissues where they might encounter antigens, super antigens, or other ligands that could engage B cell receptors and activate EBV lytic replication. Data reported herein indicate another potential function for LMP2A in vivo, namely that LMP2A would provide a constitutive survival signal in mature B cells. This LMP2A-derived signal would obviate the necessity for BCR-induced survival signals in EBV latently infected peripheral B cells (Lam et al., 1997).

The site of EBV latency in immune competent human hosts is not fully delineated. Latency is not maintained by reinfection of circulating B lymphocytes in the oral epithelium. Acyclovir, a nucleoside analog that can inhibit lytic replication in the oral epithelium, has no effect on the number of latently infected B cells in the peripheral blood (Yao et al., 1989). Recent studies suggest that in peripheral blood, EBV may reside in memory B lymphocytes that are $CD19^+$, $CD23^-$, $B7^-$, sIg^+, and $sIgD^-$ (Miyashita et al., 1995, 1997; Babcock et al., 1998) and express EBNA1 and LMP2A (Qu and Rowe, 1992; Tierney et al., 1994; Miyashita et al., 1997; Babcock et al., 1998). Other potential sites of EBV latency may include bone marrow, lymph nodes, or other lymphoid organs, as EBV can be isolated from virtually any lymphoid tissue (for review, Rickinson and Kieff, 1996). It is intriguing to speculate that bone marrow cells may provide a reservoir for EBV-infected cells in humans. Engraftment of bone marrow cells can result in the loss of the resident virus or the appearance of a new virus strain from donor lymphocytes (Gratama et al., 1988). Progenitor B cells may be infected by EBV when circulating B cells containing the virus traffic to bone marrow. Progeny of latently infected bone marrow cells could generate the relatively stable number of EBV-infected lymphocytes observed in peripheral blood of latently infected individuals (Yao et al., 1985, 1991; Lewin et al., 1987; Lam et al., 1991; Miyashita et al., 1995). Interestingly, human fetal liver and bone marrow precursor B cells infected with EBV in vitro are similar to bone marrow cells from Eμ-LMP2A transgenic mice, in that they undergo $D\text{-}J_H$ but not $V\text{-}DJ_H$ gene rearrangements and hence do not express a functional BCR (Katamine et al., 1984). Theoretically, once latently infected bone marrow cells enter the periphery, constitutive signaling from LMP2A could provide a survival signal that would maintain these cells in the absence of competent BCR or the requirement for BCR signal transduction. Alternatively, the LMP2A survival signal may be important for EBV to gain access to or manipulate the memory B cell pool.

Whatever the site of EBV latency in the human host, peripheral B cells harboring the virus may undergo lytic replication when trafficking to the oral epithelium thereby allowing shedding of virus to naive hosts. In infected hosts, latent infection enables the virus to escape immune destruction, while periodic reactivation transfers virus back to epithelial tissue where the virus replicates. Future characterization of B lymphocytes from the Eμ-LMP2A transgenic mice will delineate the effects of LMP2A on normal B cell biology and elucidate the mechanisms underlying the persistence of EBV in latently infected humans.

Experimental Procedures

Mice

The Eμ-LMP2A sequences were constructed using the following cloning procedures. The SalI–NdeI fragment of p(μ/μ)gpt (GrosschedI and Baltimore, 1985) containing the E μ sequences was blunt-end cloned into the SmaI–EcoRV sites of pBluescript KS (Stratagene). This construct retains the immunoglobulin heavy chain promoter CAP site but has lost the associated initiation codon. To enhance transcriptional efficiency of the transgene, the SmaI–EcoRV fragment from pSAM3.1 (Dush et al., 1985) containing the first exon of the murine adenine phosphoribosyltransferase (APRT) gene was cloned into the HindIII site directly downstream of the immunoglobulin heavy chain promoter CAP site using HindIII linkers, thereby generating p162. This plasmid was cut in the 3′ multiple cloning site with ClaI and subsequently religated with a MunI–ClaI linker to generate pRL202. Due to the large size of the genomic LMP2A sequences, EBV sequences designed for this construct are a hybrid of cDNA and genomic sequences such that the LMP2A BalI–AvaI genomic fragment containing exons 2 through 8 was cloned into the corresponding BalI–AvaI cDNA fragment in pLMP2A (Longnecker et al., 1991) to generate pRL192. The EcoRI fragment of pRL192 containing the chimeric LMP2A sequences was cloned into the MunI site of pRL202 to generate pRL209. The SacI–KpnI fragment of pRL209 encompassing the immunoglobulin promoter/enhancer sequences, the APRT intron, and the cDNA/genomic LMP2A sequences was purified and microinjected into B6XSJL F1 single cell fertilized eggs as described (Hogan et al., 1994). The Eμ-LMP2A transgenic animals were bred to C57BL/6 animals for more than four generations as hemizygous animals at the Northwestern University Center for Experimental Animal Resources in accordance with university animal welfare guidelines. Wild-type C57BL/6 and $RAG^{-/-}$ animals were obtained from Jackson Laboratories.

Mouse Tail DNA Southern Hybridization

Mouse tail (1 cm) was digested overnight in tail lysis buffer (100 mM Tris [pH 8], 100 mM NaCl, 5 mM EDTA, 0.2% SDS, and 100 μg/ml proteinase K) at 55°C with occasional vortexing. The solution was centrifuged to pellet the cellular debris. The resulting solution was phenol/chloroform extracted, and genomic DNA was subsequently precipitated with absolute ethanol. Genomic DNA (10 μg) was digested with BamHI restriction endonuclese, subjected to standard gel electrophoreis in 0.8% agarose, and transferred to Genescreen Plus (NEN Dupont) per manufacturer's description. A 1,958 bp BamHI fragment from pLMP2A (Longnecker et al., 1991) encoding

the entire LMP2A cDNA sequence was isolated and utilized as an α^{32}P random-labeled probe for Southern blot hybridization as described (Marchini et al., 1993).

Isolation and Flow Cytometry Staining of Primary Cells

Bone marrow cells were flushed from femurs using cold staining buffer (10 mg/ml BSA, 1× PBS, 10 mM Hepes, and 0.1% NaAzide). Spleens were dissociated between frosted slides in staining buffer to prepare single cell suspensions. Red blood cells were lysed in red blood cell lysis buffer (Sigma). Approximately 2×10^6 cells were incubated in staining buffer with previously optimized concentrations of the indicated antibodies on ice for 15 min. Cells were washed and analyzed by flow cytometry using a Becton-Dickson FACScan and Cellquest analysis software. CCD3-FITC, CD4-PE, CD8-PE, CD11b-FITC, CD19-PE, CD43-FITC, B220-FITC, pan-NK-PE, and IgM-FITC were purchased from Pharmingen.

Purification of Splenic B Cells

Spleen cells (100×10^6) were resuspended in 10 ml staining media containing 1 ml of the following hybridoma supernatants: AT83A (anti Thy1.1), GK1.5 (anti-mouse CD4, ATCC # TIB 207), and 2.43 (anti-mouse CD8.2, ATCC # TIB210). Cells were incubated on ice for 30 min with occasional mixing. Cells were washed with staining buffer and resuspended in 9 ml prewarmed Low-Tox Media (Cedarlane Laboratories). Ice cold rabbit complement (1 ml) (Cedarlane Laboratories) was added to each sample and incubated at 37°C for 30 min with occasional mixing. The resulting purified B cells were washed twice in staining media and found to be 90% pure B cells by flow cytometry (data not shown). Equivalent numbers of purified B cells were subjected to LMP2A immunoblot analysis using rat monoclonal antibody 14B7 as described (Fruehling et al., 1996).

Bone Marrow cDNA Production

Total RNA was prepared from mouse bone marrow cells using Trizol Reagent (GIBCO-BRL). cDNA was prepared from mouse bone marrow RNA using 3′ Race System (GIBCO-BRL). 1 μl of a 1:5 dilution of the resulting cDNA was used for PCR as described.

Bone Marrow Genomic DNA Preparation

Bone marrow cells were lysed overnight in 1 ml of solution A (100 mM Tris [pH 7.5], 100 mM NaCl, 10 mM EDTA [pH 8.0], 1% Sarkosyl, and 200 μg/ml proteinase K) at 55°C. Lysate was extracted twice with phenol/chloroform, and DNA was ethanol precipitated and resuspended in TE (10 mM Tris and 1 mM EDTA [pH 8.0]). 250 ng was used for PCR as described.

PCR

PCR amplifications were performed in 25 μl reactions containing 1× PCR Buffer (Pharmacia), 1 U Taq polymerase (Pharmacia), 1 μM each oligonucleotide primer, 0.2 mM each dNTP, and described quantity of template material. The amplification cycle (15 sec at 94°C, 30 sec at 58°C, and 75 sec at 72°C) was repeated 25 times (bone marrow RT-PCR) or 30 times (bone marrow immunoglobulin PCR) followed by a single 15 min period at 72°C. Oligonucleotides for PCR reactions are as follows: OL113 and OL116 for LMP2A RT-PCR, β_2m sense and β_2m antisense (Takahama et al., 1995) for β_2-microglobulin RT-PCR, V_H558, D_HL, and J4 (Schlissel et al., 1991) for immunoglobulin heavy chain gene rearrangements, V_K degenerate (Schlissel and Baltimore, 1989) and OL109 for kappa light chain gene rearrangements and OL106 and OL121 for RAG-1 control PCR. PCR products were subjected to standard gel electrophoreis in 1.5% agarose, transferred to Genescreen Plus nylon membrane (NEN Dupont), and detected by hybridization to γ^{32}P-labeled oligonucleotide OL115 for LMP2A RT-PCR, J3 (Schlissel et al., 1991) for D-J_H and V-DJ_H rearrangements, and OL110 for V-J_K rearrangements. The sequence of previously unpublished primers is as follows:

OL106 TACCCTGAGCTTCAGTTCTGCACC,
OL107 TGACTGTGGGAACTGCTGAACTTT,
OL109 TCCCTCCTTAACACCTGATCTGAG,
OL110 CGTTTTATTTCCAGCTTGGTCCCC,
OL113 AAGGCATTTACGTTCTGGTGATGC,
OL115 TTGCTCTTCGTGCTCTTCATGTCC,
OL116 GAAGAGTATGCCAGCGACAATCAG,
and OL121 GAGTTTCAGTGCTCGTTGAGTCAG.

Methocellulose Culture

5×10^5 cells were plated in 3 ml Methocult M3630 methocellulose media (Stem Cell Technologies) per manufacturer's instructions. After 7 days of culture, cells were counted, washed three times in staining media, and subjected to flow cytometry as described above, and LMP2A immunoblot analysis using rat monoclonal antibody 14B7 as described (Fruehling et al., 1996).

Acknowledgments

We thank Luz Longan for breeding and maintaining the transgenic animal colony and Lynn Doglio and the Northwestern University/CMIERTransgenic Facility for generating transgenic founder animals. R. G. C. is supported in part by the Northwestern University Carcinogenisis Training Grant 5T32CA09560 from the National Institutes of Health. S. J. A. is supported by Public Health Service grant GM55626. R. L. is supported by Public Health Service grants CA62234 and CA73507 from the National Cancer Institute. R. L. is a scholar of the Leukemia Society of America.

Received June 30, 1998; revised August 8, 1998.

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