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Three Pathways of Epstein-Barr Virus Gene Activation from EBNA1-Positive Latency in B Lymphocytes

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Previous studies on Epstein-Barr virus (EBV)-positive B-cell lines have identified two distinct forms of virus latency. Lymphoblastoid cell lines generated by virus-induced transformation of normal B cells in vitro, express the full spectrum of six EBNAs and three latent membrane proteins (LMP1, LMP2A, and LMP2B); furthermore, these lines often contain a small fraction of cells spontaneously entering the lytic cycle. In contrast, Burkitt's lymphoma-derived cell lines retaining the tumor biopsy cell phenotype express only one of the latent proteins, the nuclear antigen EBNA1; such cells do not enter the lytic cycle spontaneously but may be induced to do so by treatment with such agents as tetradecanoyl phorbol acetate and anti-immunoglobulin. The present study set out to determine whether activation of full virus latent-gene expression was a necessary accompaniment to induction of the lytic cycle in Burkitt's lymphoma lines. Detailed analysis of Burkitt's lymphoma lines responding to anti-immunoglobulin treatment revealed three response pathways of EBV gene activation from EBNA1-positive latency. A first, rapid response pathway involves direct entry of cells into the lytic cycle without broadening of the pattern of latent gene expression; thereafter, the three "latent" LMPs are expressed as early lytic cycle antigens. A second, delayed response pathway in another cell subpopulation involves the activation of full latent gene expression and conversion to a lymphoblastoidlike cell phenotype. A third response pathway in yet another subpopulation involves the selective activation of LMPs, with no induction of the lytic cycle and with EBNA expression still restricted to EBNA1; this type of latent infection in B lymphocytes has hitherto not been described. Interestingly, the EBNA1$^+$ LMP$^+$ cells displayed some but not all of the phenotypic changes normally induced by LMP1 expression in a B-cell environment. These studies highlight the existence of four different types of EBV infection in B cells, including three distinct forms of latency, which we now term latency I, latency II, and latency III.

Epstein-Barr virus (EBV) is carried by more than 95% of adults as a largely asymptomatic infection. Primary infection with EBV normally occurs through salivary exchange in the oropharynx, and this is believed to result in virus replication in stratified squamous epithelial cells and in the subsequent infection of trafficking B lymphocytes. Following primary infection, whether symptomatic or silent, the virus persists in the healthy host for life through mechanisms that are not fully understood. Although some form of latent infection in epithelial cells represents one possible strategy for virus persistence, most recent studies favor the concept of virus carriage in the lymphoid compartment via the long-term survival of non-productively infected B cells (15, 52). Accordingly, there is renewed interest in the different types of virus-cell interactions that can occur in EBV-infected B lymphocytes, and in this context much can be learned from in vitro models.

The best-characterized EBV-B cell interaction is that shown by lymphoblastoid cell lines (LCLs) established following experimental infection of resting B cells in vitro. These growth-transformed cells are to a large extent nonpermissive for virus replication and express just nine viral proteins (reviewed in reference 23), including six nuclear antigens (EBNA1, EBNA2, EBNA3a, EBNA3b, EBNA3c, and EBNA-LP) and three membrane proteins (LMP1, LMP2A, and LMP2B). In LCLs, all six EBNA mRNAs are differentially spliced from long transcripts initiated either from the Cp promoter or from the adjacent Wp promoter at the "leftward" end of the viral genome (42, 51), while the LMP mRNAs are transcriptionally controlled by promoters at the "rightward" end of the genome that are responsive to transactivation by EBNA2 (10, 24, 50, 56). Coordinate expression of the EBNAs and LMPs in experimentally infected B cells leads not only to growth transformation but also to a dramatic change in the cellular phenotype, with upregulation of several B-cell surface activation markers (i.e., CD23, CD30, CD39, and CD70) and adhesion molecules (LFA-1, ICAM-1, and LFA-3) as well as of the cellular oncogene *bcl-2*. Gene transfection studies have attributed certain facets of the above phenotypic change to the effects of individual latent proteins. Thus, EBNA2 and EBNA3c can upregulate expression of some of the cellular activation antigens, while LMP1 has multiple effects, including upregulation of the cellular adhesion molecules, of CD23, and of the *bcl-2* oncogene (18, 47–49).

A second form of latent infection in B cells is exemplified by certain EBV-positive Burkitt's lymphoma (BL) cell lines which express only EBNA1 and no other viral proteins (17, 39). At the transcriptional level, these cells show expression of EBNA1 mRNA initiated exclusively from an Fp promoter which is located about 50 kb downstream of the Cp and Wp promoters used in LCLs (42). These BL lines are also phenotypically distinct from LCLs since they display undetectable or very low levels of the B-cell activation antigens and cellular adhesion molecules that are abundantly expressed in LCLs and instead express certain other markers (e.g., CD10 and CD77) that are not seen on LCLs (16, 34). This cellular phenotype, designated the group I BL cell surface phenotype, is a characteristic of all BL tumors in

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vivo and during initial culture in vitro (34). However, many established BL tumor cell lines progress upon serial passage in vitro to display a group III cell surface phenotype, which is similar to that displayed by normal LCLs. This switch from a group I to a group III cellular phenotype is associated with induced expression of the full range of nine latent viral proteins that are expressed in LCLs (39). At the transcriptional level, the activation of latent gene expression is reflected by a switch from Fp promoter usage to Cp and/or Wp promoter usage for initiation of EBNA transcripts (42).

Another interesting feature of the above phenotypic drift is that while all group I BL lines generally display a tightly latent EBV infection, group III lines often contain a small proportion of cells in the lytic cycle, as do LCLs (17). This suggested that the natural route from EBNA1$^+$ latency into the lytic cycle in B cells may involve, as a necessary intermediate step, transient acquisition of full virus latent gene expression and an LCL-like cellular phenotype. We began the present series of experiments specifically to examine this question by following the response of group I BL cell lines to reported inducers of the lytic cycle. In so doing, we identified three distinct pathways of EBV gene activation from EBNA1$^+$ latency.

MATERIALS AND METHODS

Cell lines. Seven of the BL lines used in this work had been well characterized previously and were selected because they were known to display a group I cell surface phenotype and to express EBNA1 in the absence of the other latent viral proteins (17, 18, 39). These lines were Chep-BL, Eli-BL, Mutu-BL, Odhi-BL, Wan-BL, BL37, and WW2-BL. The Akata-BL line, which had not previously been characterized with respect to cell surface phenotype and pattern of latent viral gene expression, was kindly provided by K. Takada (Nihon University School of Medicine, Tokyo, Japan) as a control cell line sensitive to anti-immunoglobulin (anti-Ig)-mediated effects. All cultures were grown in RPMI-1640 medium buffered with bicarbonate and supplemented with 10% fetal calf serum and were passaged by twice-weekly 1:5 subculturing.

Induction of BL lines by TPA or by ligation of surface Ig. For stimulation of cultures with phorbol 12-myristate 13-acetate (TPA; obtained from Sigma), cells were harvested and resuspended at 3×10^5 cells per ml in fresh medium containing 3×10^{-8} M TPA. Cultures were harvested at 72 h for analysis of EBV gene expression. Cells to be stimulated with anti-Ig were resuspended in medium containing 0.2% dialyzed rabbit anti-human IgG (Dakopatts; A407) or rabbit anti-human IgM (Dakopatts; A409), depending on the type of Ig expressed by the BL line being induced. The Akata-BL and Wan-BL lines both expressed IgG, while all the other lines expressed IgM.

Monoclonal antibodies. The following monoclonal antibodies were used for the detection of EBV antigens in this work: CS.1-4 and S12 (anti-LMP1) (27, 36); PE2 (anti-EBNA2) (53); BZ1 (anti-BZLF1) (55); R3 [anti-EA(D) proteins encoded by BMRF1] (31); 72A1 and 2L10 (anti-gp340 late membrane glycoprotein encoded by BLLF1) (19, 33); and V3 (anti-VCA protein encoded by BcLF1) (46). Monoclonal antibodies to cellular proteins included bcl-2 100/124 (anti-Bcl-2) (32); AC2 (CD39) (38); MHM6 (CD23) (38); and TS2/9 (CD58, anti-LFA-3) (43).

Western immunoblotting. The procedure for Western blotting has been described fully elsewhere (40). Briefly, cells were solubilized by sonication in gel sample buffer, and the equivalent of 10^6 cells was loaded into each track of discontinuous 7.5% or 10% polyacrylamide–sodium dodecyl sulfate (SDS) Laemmli gels. The electrophoresed proteins were transferred onto nitrocellulose filters, which were subsequently blocked with milk and incubated overnight either with monoclonal antibodies (1:10 dilution of hybridoma culture supernatant) or with selected human sera (1:100 dilution). When monoclonal antibodies were used as probes, the filters were subsequently incubated for 1 h with a rabbit anti-mouse IgG. Specifically bound antibodies were detected with ^{125}I-protein A and autoradiography. Human sera used as probes for Western blots included serum EE, which reacts predominantly with various lytic cycle antigens, including the BZLF1 protein and the 45- to 55-kDa proteins of the EA(D) complex; serum AMo, which reacts with EBNA1 but none of the other EBNAs; and serum PBe, which reacts with all six EBNAs.

Immunofluorescence assays. Cell smears were air-dried and fixed at −20°C for 10 min either with a mixture of methanol-acetone (1:2, vol/vol) or with acetone alone. Single- and two-color immunofluorescence studies on these fixed cells were performed exactly as described elsewhere (41). Briefly, routine monochromatic fluorescence was performed by a three-step method which involved incubation with monoclonal antibody, then with polyclonal goat anti-mouse IgG antibodies conjugated with fluorescein isothiocyanate (FITC), and finally with polyclonal rabbit anti-goat Ig antibodies which were also conjugated with FITC. For dichromatic fluorescence assays, a simple two-step procedure was used in which two monoclonal antibodies (one of the IgG1 subclass, and another of the IgG2a subclass) with different specificities were pooled for the first step and a mixture of FITC-conjugated goat antibodies to mouse IgG1 and rhodamine-conjugated goat antibodies to mouse IgG2a was used for the second step.

For cell surface phenotyping, viable cells were incubated with monoclonal antibodies to cell surface antigens and then with FITC-labeled goat anti-mouse IgG antibodies, as described previously (41). The stained cells were analyzed on a Becton Dickinson FACS 440.

Detection of LMP1 and LMP2 mRNAs. Total RNA was prepared from cells and analyzed for the presence of specific EBV latent transcripts by reverse transcription and polymerase chain reaction (PCR) amplification by methods described in detail elsewhere (21). Briefly, cDNA was synthesized from 2 μg of total RNA with 5 U of avian myeloblastosis virus reverse transcriptase and 100 pmol of the transcript-specific oligonucleotide 3′ primer. Thereafter, cDNAs were amplified by PCR following the addition of 100 pmol of the relevant 5′ primer and 2.5 U of *Taq* polymerase. The products were analyzed by electrophoresis on 3% agarose gels, Southern blotting, and probing with the relevant oligonucleotide probe which had been end-labeled with [^{32}P]ATP and T4 polynucleotide kinase. The combinations of oligonucleotide primers and probes used were in each case designed to amplify across defined splice junctions in EBV-encoded mRNAs, thus allowing specific detection of RNA transcripts in the presence of contaminating EBV genomic DNA. LMP1 transcripts were detected by using a 3′ primer spanning exons 3 and 2 (coordinates 168956 to 168965 and 169042 to 169051; sequence ATACCTAAGACAAGTAAGCA), a 5′ primer for exon 1 (coordinates 169490 to 169471; sequence ACACACTGCCCTGAGGATGG), and a probe for exon 2 (coordinates 169081 to 169100; sequence ACAATGCCTGT CCGTGCAAA). For the detection of LMP2A and LMP2B transcripts, a common 3′ primer for the common exon 3

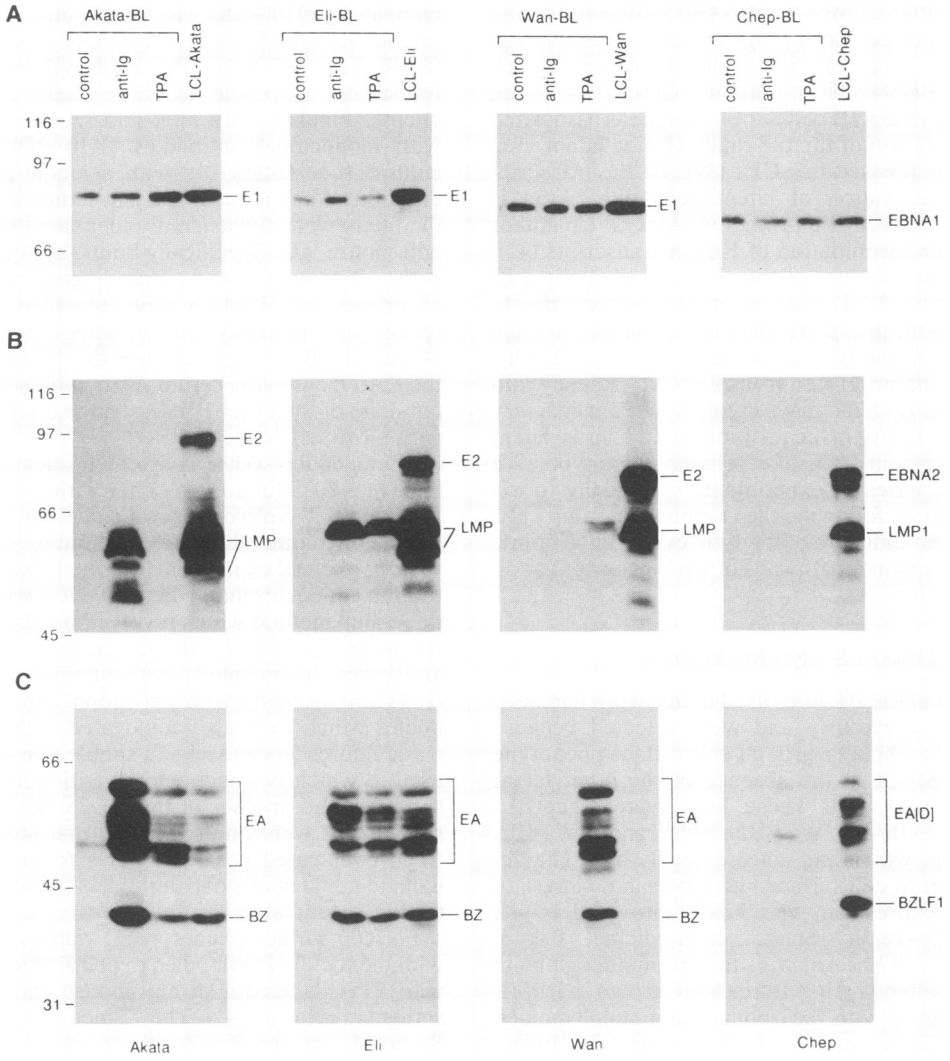

FIG. 1. Western blot analysis of EBV gene expression of four group I BL cell lines treated either with anti-Ig for 24 h or with TPA for 3 days. For each BL line, treated BL cells were compared with control untreated cells and with a reference normal LCL transformed with the same BL-derived virus. Proteins from 10^6 cells were separated by SDS-PAGE, and identical sets of blots were probed with (A) a human serum, AMo, which reacts with EBNA1, (B) a pool of monoclonal antibodies, PE2 and CS.1-4, which react with EBNA2 and LMP1, respectively, or (C) a human serum, EE, which has unusually high titers of antibodies reactive with the BZLF1 protein and the EA(D) complex of lytic cycle proteins. Sizes of protein standards are shown in kilodaltons.

(coordinates 380 to 361; sequence CATGTTAGGCAAATT GCAAA) and a common exon 2 probe (coordinates 62 to 81; sequence ATCCAGTATGCCTGCCTGTA) were used. The 5′ primer used to detect the LMP2A transcripts was from the unique coding exon 1 (coordinates 166874 to 166893; sequence ATGACTCATCTCAACACATA), while the 5′ primer used to detect the LMP2B transcript was from the unique noncoding exon 1 (coordinates 169819 to 169838; sequence CAGTGTAATCTGCACAAAGA).

RESULTS

Responsiveness of group I BL cell lines to anti-Ig and to TPA. Preliminary experiments were performed with the Akata-BL line as a control line known to be sensitive to two inducers of the lytic cycle, anti-Ig and TPA (8, 45). The cellular phenotype and virus latent gene expression in Akata-BL cells had not previously been characterized, but we found that the line actually displayed a group I cell surface phenotype and expressed EBNA1 (Fig. 1A) in the absence of detectable expression of EBNA2 and LMP1 (Fig. 1B) and of the other EBNAs (data not shown). Akata-BL was therefore incorporated into the panel of eight group I BL lines studied in this work. While the activation of Akata-BL via ligation of surface Ig is known to be rapid, producing synthesis of early lytic cycle viral proteins within 2 to 3 h (45), induction of several different EBV-positive lines with TPA typically takes 24 to 48 h (57). Therefore, in the initial series of experiments, the eight group I BL lines were each cultured either for 24 h with anti-Ig or for 72 h with TPA. The cells were then harvested for analysis of EBV gene expression by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting. For each BL line, the induced and control noninduced BL cells were compared with a reference normal

LCL transformed with the same BL virus isolate and expressing the full spectrum of latent proteins.

The results in Fig. 1 show that Akata-BL was activated into the lytic cycle very efficiently by anti-Ig and to a lesser extent by TPA (Fig. 1C), a result consistent with published data (8). In addition, it was also noted that activation via anti-Ig induced expression of LMP1 to a level comparable to that expressed in the corresponding LCL, whereas EBNA2 was not induced (Fig. 1B): in this particular experiment, TPA induced barely detectable levels of LMP1, but in other experiments in which activation of the lytic cycle was more efficient, the coinduction of LMP1 was more marked (data not shown). Similar results were obtained with the Eli-BL line, except that here the treatments with anti-Ig and TPA both induced lytic cycle antigens with similar efficiency (Fig. 1C). As with the Akata-BL line, both treatments of Eli-BL also induced the expression of LMP1 but not of EBNA2 (Fig. 1B). Three lines (Mutu-BL, BL37, and Wan-BL) were activated with varying efficiency by TPA to express lytic cycle antigens and LMP1 but were unresponsive to anti-Ig. This pattern of results is illustrated by the Wan-BL line in Fig. 1. Another three lines (WW2-BL, Odhi-BL, and Chep-BL) were poorly responsive both to TPA and to anti-Ig, whether assessed by lytic cycle induction or by LMP1 expression (see Chep-BL line, Fig. 1).

In responsive group I BL cell lines, therefore, both types of treatment activated not only the EBV lytic cycle but also the expression of a latent viral protein, LMP1. We chose to investigate this phenomenon in more detail by concentrating on the induction of the Akata-BL and Eli-BL lines via anti-Ig. This choice was made because, compared with that of TPA, the effect of anti-Ig is rapid and synchronous. Furthermore, TPA was often toxic to the BL cells at the concentration required to induce the lytic cycle, sometimes reducing viability by up to 50% at day 3, whereas anti-Ig generally had little effect upon the viability of the cultures. The ensuing studies identified three distinct pathways of activation mediated by anti-Ig.

Pathway 1: direct entry into the lytic cycle with induction of LMP1 as a lytic cycle antigen. Akata-BL cells were treated with anti-IgG, and replicate cultures were harvested at several time points over a period of 74 h for analysis by Western blotting. The pattern of results obtained in three separate time course experiments is illustrated by the representative results of one experiment shown in Fig. 2. Expression of the immediate-early protein BZLF1 was detected within 2 to 5 h postactivation (Fig. 2B), followed by expression of a full-size LMP1 protein by 7 to 9 h (Fig. 2C). In this particular experiment, a smaller LMP1 protein was also detected several hours after the full-size LMP1 was induced (Fig. 2C), and it was tentatively designated truncated LMP1 (Tr-LMP1) because it may have initiated from an internal methionine codon in the LMP1 gene, as reported for TPA-induced B95.8 LCL cells (20). In the present work, the full-length LMP1 protein was consistently induced, while the Tr-LMP1 was not always detected.

Immunofluorescence analysis of Akata-BL at the single-cell level showed that induction of LMP1 was confined to cells which had entered the lytic cycle. Cell smears were stained with monoclonal antibodies specific for the immediate-early BZLF1 protein, the EA(D) product of the BMRF1 gene, the VCA product of the BcLF1 gene, and the LMP1 and EBNA2 latent proteins. Representative results obtained in one of several experiments are illustrated in Fig. 3. Between 2 and 9 h after the addition of anti-IgG, there was a sequential induction of the BZLF1, EA, LMP1, and VCA

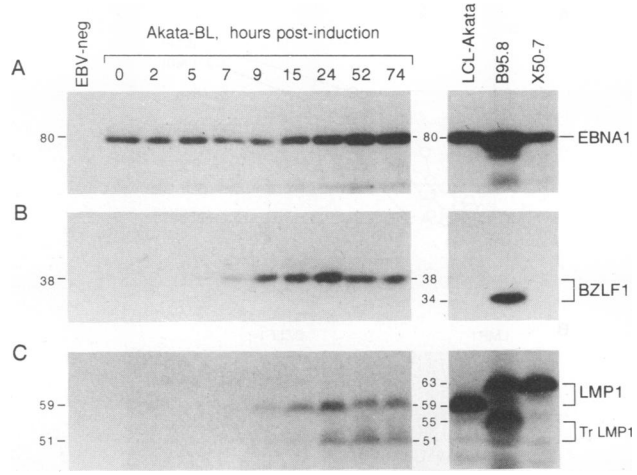

FIG. 2. Western blot analysis showing the kinetics of EBV activation in Akata-BL cells following treatment with anti-IgG. Replicate cultures of the Akata-BL line treated with anti-IgG were harvested at the time points indicated. Protein extracts from reference cell lines were also separated on the same gel and included an EBV-negative B-lymphoma line (BJAB), a normal LCL transformed with Akata virus (LCL-Akata), an EBV-positive marmoset LCL which contained a proportion of cells in the lytic cycle (B95.8), and a tightly latent human LCL transformed with B95.8 virus (X50-7). Three identical blots were probed either with (A) human serum AMo, which reacts with EBNA1, (B) monoclonal antibody BZ1, which reacts with the BZLF1 protein, or (C) monoclonal antibodies CS.1-4, which react with LMP1. Sizes are indicated in kilodaltons.

proteins (Fig. 3A). Dichromatic staining for LMP1 and BZLF1 at 9 h postinduction showed that the LMP1 protein was expressed exclusively in the $BZLF1^+$ (i.e., lytic cycle) cell population (Fig. 3B); these LMP^+ $BZLF1^+$ cells did not express EBNA2 (Fig. 3C). LMP1 was therefore a genuine component of the lytic cycle, and its expression was independent of EBNA2. This first response pathway was also observed in anti-IgM-treated Eli-BL cells (see below).

In order to confirm that the full-size LMP1 protein observed above was indeed the product of latent LMP1 transcripts, total RNA preparations were analyzed for the presence of specific mRNA by reverse transcription and PCR amplification. The 2.8-kb latent LMP1 transcript that is abundantly expressed in latently infected LCLs from the EDL1 promoter (20) was reverse-transcribed with a 3′ primer which spanned the exon 3-exon 2 splice junction, and PCR amplification was carried out after the addition of a 5′ primer from exon 1. Note that this combination of primers does not detect the unspliced mRNA transcript which is driven from the EDL1a promoter in TPA-induced B95.8 cells and which is thought to encode the Tr-LMP1 protein (20). These primers amplified a 381-bp reverse-transcribed product, as expected, in the three LCLs tested (Fig. 4A). There was a barely detectable amplification of this LMP1 product in the control Akata-BL line itself, but following induction of these cells for 24 h with anti-Ig, a 381-bp product was reproducibly obtained at a level comparable to that obtained with LCLs (Fig. 4A).

It has been reported that LMP2 is usually coexpressed with LMP1 in latent infection (14) and that its expression can be elevated by TPA (26). We therefore sought to determine whether LMP2A and/or LMP2B mRNAs were induced in the present context. Reverse transcription of total cell RNA

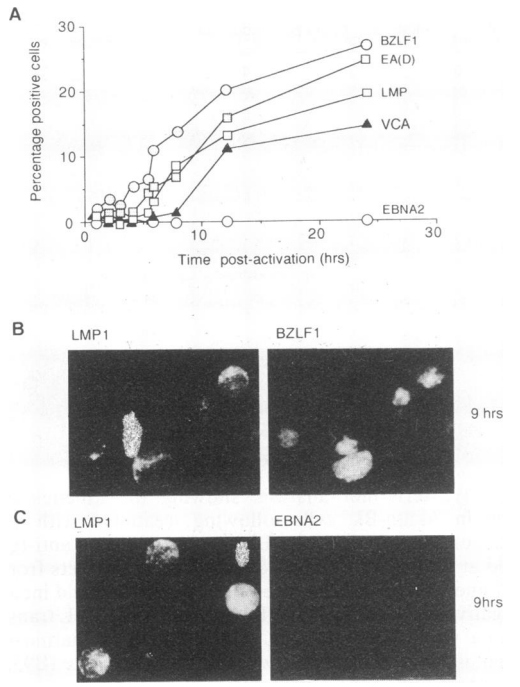

FIG. 3. Immunofluorescence analysis of EBV gene activation in the Akata-BL line following treatment with anti-IgG. (A) Kinetics of EBV gene activation analyzed with the following monoclonal antibodies: BZ1, specific for the BZLF1 protein; R3, specific for proteins of the EA(D) complex encoded by BMRF1; V3, specific for the VCA protein encoded by BcLF1; CS.1-4, specific for LMP1; and PE2, specific for EBNA2. (B) Dual staining with antibodies to LMP1 and to BZLF1 at 9 h postactivation; LMP1 expression was detected by using the S12 monoclonal antibody and a rhodamine-conjugated anti-mouse IgG2a second-step antibody, while BZLF1 expression was detected by using the BZ1 monoclonal antibody and an FITC-conjugated anti-mouse IgG1 second step. (C) Expression of LMP1 (detected with S12 and anti-IgG2a) in the absence of EBNA2 (assayed with PE2 and anti-IgG1) at 9 h postactivation.

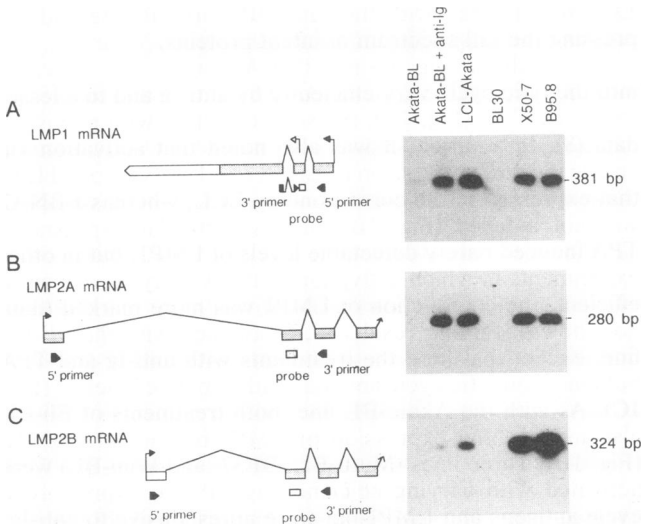

FIG. 4. Detection of latent viral transcripts for LMP1 (A), LMP2A (B), and LMP2B (C) by reverse transcription and PCR amplification in Akata-BL cells after activation with anti-IgG for 24 h. The design of the primer-probe combinations used is illustrated schematically (not to scale) to the left of the Southern blots: the coordinates of the primers and probes are given in the Materials and Methods section. The coding sequences of the spliced transcripts are indicated by shaded areas, while open areas indicate noncoding sequences. The location of the promoter sequences and the direction of transcription are indicated by the bent arrows: for the LMP1 transcript, the solid arrow indicates the EDL1 promoter which drives the 2.8-kb mRNA encoding the full-length protein, while the hollow arrow indicates the location of the cryptic EDL1a promoter for the unspliced lytic mRNA which encodes a Tr-LMP1 and which is not detected by the chosen combination of primers and probe. Total RNA preparations were reverse-transcribed with the 3′ primers indicated and then amplified after the addition of 5′ primers. Specific amplified products were detected by Southern blotting with the probes indicated. The Southern blots shown included amplified material from uninduced Akata-BL cells as well as from four reference cell lines: a normal LCL transformed with Akata-BL virus, LCL-Akata; an EBV-negative cell line, BL30; and the LCLs X50-7 and B95.8.

was carried out with a 3′ primer from the exon 3 common to both LMP2A and LMP2B mRNAs, and the products were amplified after the addition of a 5′ primer from the unique first exon of either the LMP2A or the LMP2B mRNA. Specific products of 280 kb (LMP2A) or 324 kb (LMP2B) were detected by Southern blotting with a probe from the common exon 2. These combinations of primers and probes revealed that both LMP2A and LMP2B transcripts were indeed induced in Akata-BL cells following activation with anti-Ig (Fig. 4B and C), exactly paralleling the observations on LMP1 transcription.

Pathway 2: switch to an LCL-like (EBNA2$^+$ LMP$^+$) form of latency. Extension of the time course over which anti-Ig activation of Akata-BL cells was followed revealed the existence of a second, delayed response pathway. As shown in Fig. 5A, maximal expression of lytic cycle antigens was obtained by 12 to 24 h postactivation, and after a further 24 h, a small subpopulation of EBNA2$^+$ cells was detected. Dichromatic staining revealed that the EBNA2$^+$ cells present by 48 h did not coexpress either LMP1 (Fig. 5B) or the late lytic cycle antigen gp340 (Fig. 5C) and were therefore clearly distinct from those cells previously activated into the lytic cycle. However, by 72 h, 20 to 30% of the EBNA2$^+$ cells had activated LMP1 expression (Fig. 5D), and a small minority of these EBNA2$^+$ LMP1$^+$ cells expressed lytic cycle antigens (data not shown), just as would be expected of cells moving into an LCL-like form of infection. This second response pathway was also observed with anti-IgM-treated Eli-BL cells (see below).

Pathway 3: switch to a novel (EBNA2$^-$ LMP1$^+$) form of latency. Activation of the Eli-BL line by treatment with anti-IgM was followed as for the Akata-BL line, and here the results revealed three different response pathways. Compared with Akata-BL, a smaller proportion of the Eli-BL cells were activated directly into the lytic cycle, and the kinetics of this response were slightly slower. Thus, the first BZLF1$^+$ cells appeared at 9 to 12 h (Fig. 6A), at which time these cells were LMP1$^-$ (Fig. 6B). Just as with the Akata-BL, however, progression of these cells through the lytic cycle was accompanied by the induction of LMP1 as a lytic cycle antigen, so that by 24 h all the BZLF1$^+$ cells coexpressed LMP1 (Fig. 6C). Delayed induction of EBNA2 was again observed by 48 h in a small distinct subpopulation of cells (Fig. 6A) subsequently shown to be moving towards an LCL-like form of infection.

The most striking feature distinguishing the Eli-BL response from that shown by anti-Ig-treated Akata-BL cells

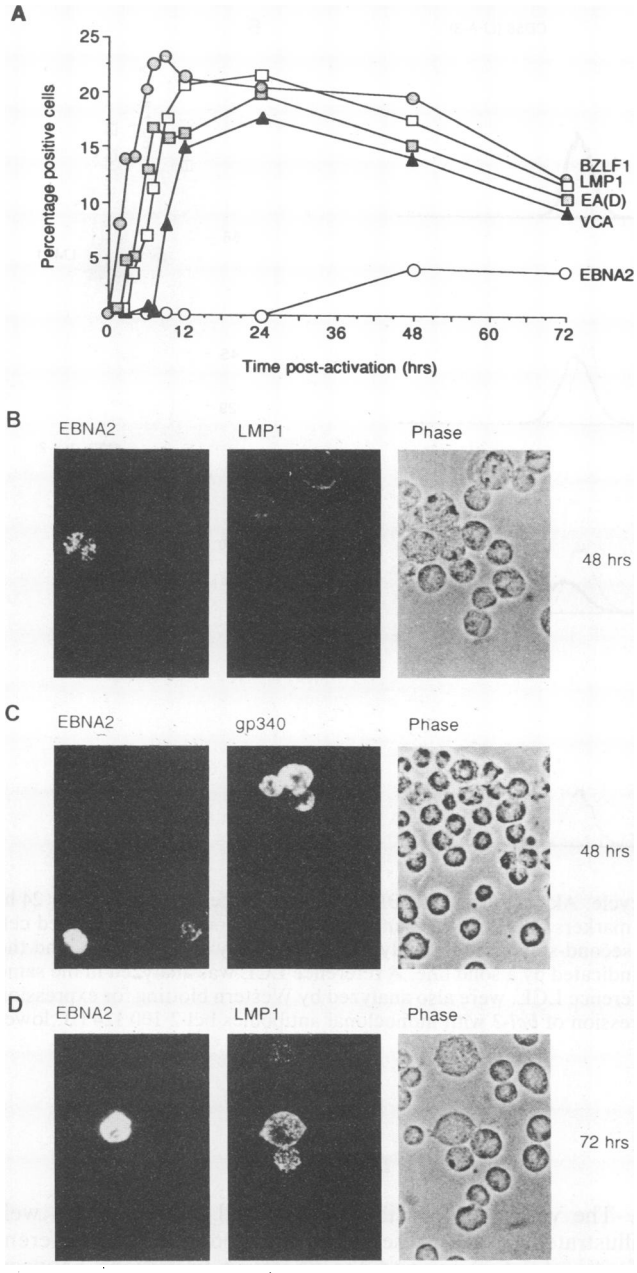

FIG. 5. Extended immunofluorescence analysis of EBV gene activation in the Akata-BL line following treatment with anti-IgG for up to 72 h. (A) Kinetics of activation determined as described in the legend to Fig. 3A. (B to D) Examples of dichromatic fluorescence assays with the PE2 (anti-EBNA2) IgG1 monoclonal antibody together with the IgG2a monoclonal antibody S12 (anti-LMP) or 2L10 (anti-gp340 late membrane antigen). (B) Staining for EBNA2 and LMP1 at 48 h postactivation; (C) staining for EBNA2 and gp340 at 48 h postactivation; (D) staining for EBNA2 and LMP1 at 72 h postactivation. For each pair of immunofluorescence photographs, the same field is shown on the right under phase-contrast illumination.

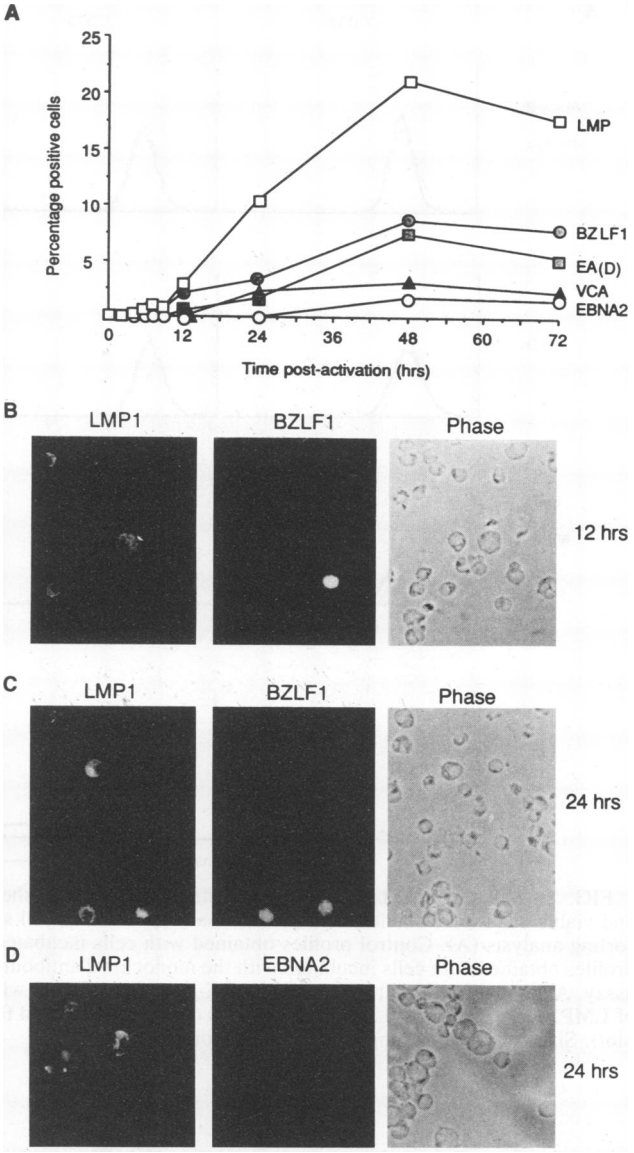

FIG. 6. Immunofluorescence analysis of the kinetics of EBV gene activation in the Eli-BL line following treatment with anti-IgM for up to 72 h. (A) Kinetics of activation determined as described in the legend to Fig. 3A. (B and C) Results of dichromatic immunofluorescence assays for LMP1 (S12; IgG2a monoclonal antibody) and BZLF1 (BZ1; IgG1 monoclonal antibody) at 12 h and 24 h, respectively, after treatment with anti-IgM. (D) Staining for LMP1 (S12) and a lack of staining for EBNA2 (PE2; IgG1 monoclonal antibody) at 24 h after treatment with anti-IgM.

was the accumulation between 12 and 48 h postactivation of large numbers of LMP1$^+$ cells which were neither in the lytic cycle (Fig. 6A, B, and C) nor expressing EBNA2 (Fig. 6A and D). This third response pathway was a dominant feature of the Eli-BL response, whether activated by anti-Ig or by TPA, but was never observed in activated Akata-BL cultures. Analysis of mRNA from the Eli-BL cells at 24 h postactivation showed that, as with the induced Akata-BL cells (see Fig. 4), LMP1 was being expressed from the characteristically spliced latent LMP1 mRNA and that the LMP2A and LMP2B transcripts were also coinduced with LMP1 (data not shown). However, because two distinct LMP1$^+$ populations were present in the Eli-BL cultures at this time, it was not possible to ascertain whether LMP2A and LMP2B were expressed in both or just one of these populations.

Phenotypic effects of LMP1 expressed in different response

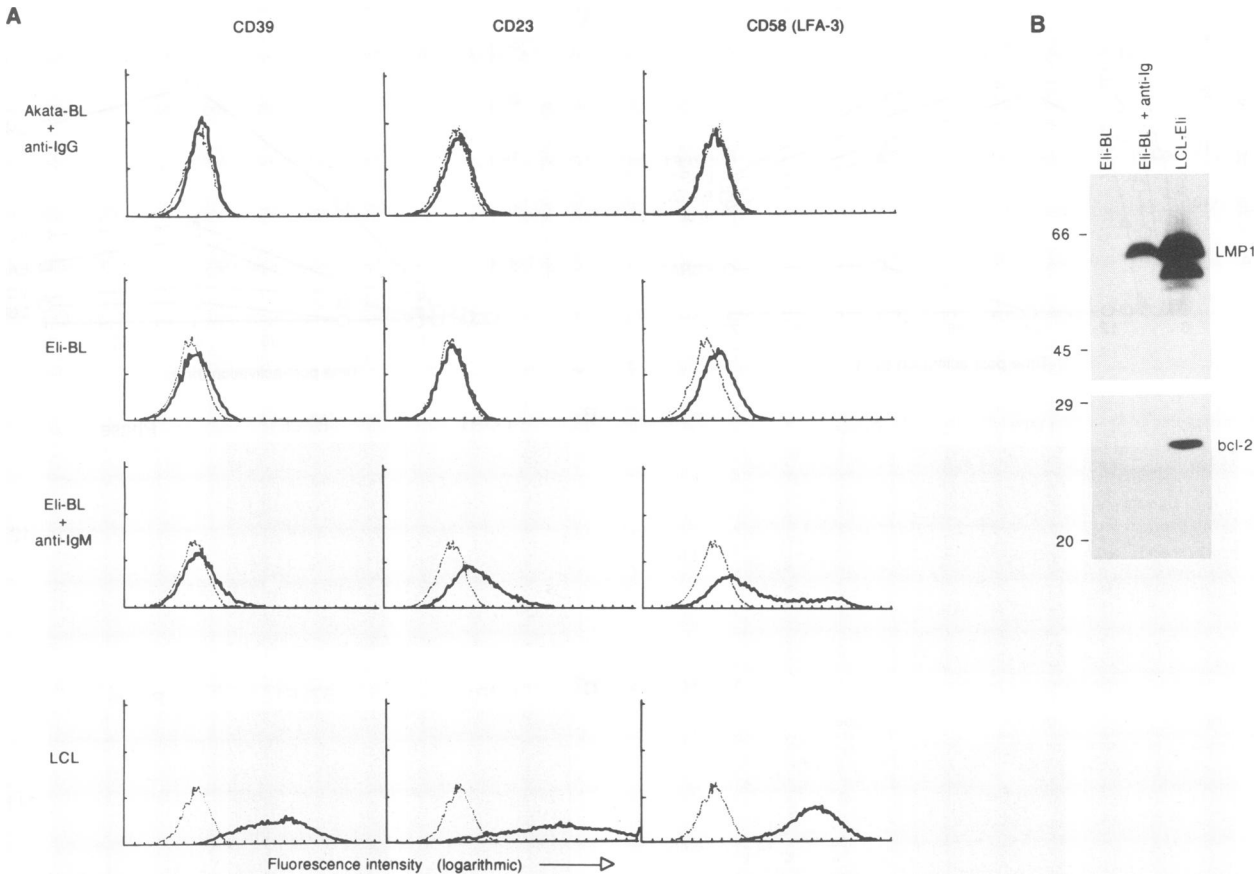

FIG. 7. Effect of LMP1 expression on cellular phenotype in the lytic cycle. Akata-BL and Eli-BL cells were treated with anti-Ig for 24 h, and viable cells were stained with monoclonal antibodies to cell surface markers (CD39, CD23, and LFA-3) for fluorescence-activated cell sorting analysis (A). Control profiles obtained with cells incubated with second-step antibody only are indicated by a dashed line, and the profiles obtained with cells incubated with the monoclonal antibodies are indicated by a solid line. A reference LCL was analyzed in the same assay. Aliquots of the control and induced Eli-BL cells, together with a reference LCL, were also analyzed by Western blotting for expression of LMP1 with monoclonal antibodies CS.1-4 (B, upper blot) and for expression of *bcl-2* with monoclonal antibodies bcl-2 100/124 (B, lower blot). Sizes of protein standards are shown in kilodaltons.

pathways. In a B-lymphocyte environment, LMP1 can mediate a number of changes in cellular phenotype. These include upregulation of cellular adhesion molecules such as LFA-3 (47), of some but not all cell surface activation antigens (e.g., CD23 but not CD39 [47, 49]), and of the cellular oncogene *bcl-2* (18). We therefore investigated whether such changes were induced by LMP1 when expressed either in the lytic cycle (the dominant response pathway in Akata-BL) or in the EBNA2$^-$ LMP1$^+$ form of latency (the dominant response pathway in Eli-BL). Anti-Ig-mediated activation of about 20% of Akata-BL cells into the lytic cycle did not cause upregulation of any of the above cell surface markers (Fig. 7A) or of the *bcl-2* oncogene (data not shown). In contrast, anti-Ig-induced Eli-BL contained a subpopulation cells staining for LFA-3 at an intensity similar to that observed on LCLs and for CD23 at a lower intensity, while there was no induction of CD39 (Fig. 7A). Dichromatic staining for LMP1 and LFA-3 and for LMP1 and CD23 confirmed that the observed surface changes occurred in LMP1$^+$ cells (data not shown). Interestingly, these cells did not show demonstrable activation of *bcl-2* expression (Fig. 7B).

DISCUSSION

The variety of possible EBV-B cell interactions is well illustrated by the present in vitro models. These different forms of infection, and the observed transitions between them, are summarized in Fig. 8. As an aid to discussion, we have introduced a new nomenclature for the different forms of latency that have now been identified. Thus, latency I (Lat I) is exemplified by group I BL cells expressing EBNA1 in the absence of the other latent viral proteins. Latency III (Lat III) is exemplified by group III BL lines and LCLs expressing all nine latent viral proteins. To these previously characterized forms of latency, we can now add latency II (Lat II), exemplified by those anti-Ig-induced Eli-BL cells which coexpress EBNA1 and LMPs in the absence of the other EBNAs.

Most earlier studies on the experimental activation of the lytic cycle were performed with B cells showing a Lat III form of infection (23). In contrast, the present work describes a detailed analysis of the kinetics of lytic cycle induction from Lat I. The most important finding in this context is that such cells can be activated directly into the lytic cycle, bypassing the Lat III form of infection. In these

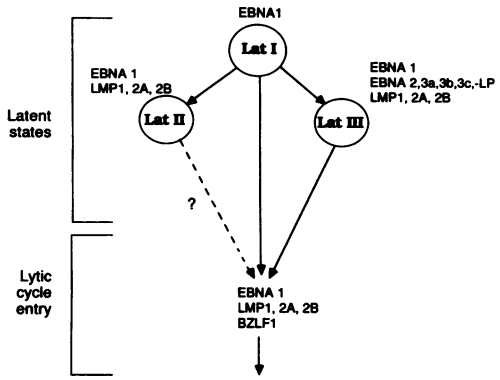

FIG. 8. Schematic illustration of pathways of EBV gene activation from the EBNA1-positive (Lat I) form of latency.

activated cells, the continued absence of EBNA2, EBNA3a, EBNA3b, EBNA3c, and EBNA-LP proteins could be demonstrated by Western blotting (Fig. 1 and data not shown); furthermore, transcriptional analysis showed that EBNA1 mRNA continued to be expressed from the Fp promoter and that the Cp/Wp promoter necessary for expression of the other EBNA mRNAs remained silent (24a). As with induction into the lytic cycle from Lat III, entry of cells into the lytic cycle from Lat I involved BZLF1 expression as the first detectable viral event. In this context, it is noteworthy that both TPA (7) and ligation of surface Ig (2, 8) appear to induce the lytic cycle via cellular signalling pathways which induce protein phosphorylation by protein kinase C. Because the BZLF1 promoter region is itself directly responsive to such phosphoproteins (13, 25), these protein kinase C substrates represent prime candidates for initiating BZLF1 transcription; thereafter, the BZLF1 protein augments its own expression via distinct autoresponsive promoter elements (12, 44), irreversibly initiating the cascade of lytic cycle gene expression (11, 22).

The present work further demonstrated that switching B cells from Lat I directly into the lytic cycle involved induction of LMP1, and probably LMP2A and LMP2B, as early lytic cycle antigens. This observation needs to be set against earlier findings, all with Lat III cell systems, implicating LMP1 as a lytic cycle-associated protein. Thus, Hudson et al. (20) first observed the appearance of a new 2.6-kb "lytic" LMP1 mRNA in TPA-induced B95.8 cells. This new mRNA was predicted to encode a truncated (N-terminally deleted) LMP1 protein (20) which has been tentatively identified in some other Lat III lines containing a lytically infected cell subpopulation (1, 3, 6, 36), but which has never been localized to that particular subpopulation. On the other hand, the existing level of full-length LMP1 protein can be increased by TPA treatment of Raji cells (3) and of LCLs (36); but when studied at the single-cell level, this increase was seen generally throughout the population and was not restricted to cells in the lytic cycle (36). These reported effects of TPA on LMP1 expression in Lat III cell systems may therefore be independent of lytic cycle induction per se. In contrast, our present results in Lat I cell systems (in which there is no basal expression of LMP1) clearly show that the full-length protein is consistently upregulated as a component of the early lytic cycle.

The delayed induction of EBNA2, both in Akata-BL and in Eli-BL cells at 48 h after treatment with anti-Ig, occurred in a subpopulation of cells distinct from that induced into the lytic cycle (Fig. 5 and 6). A similar bifurcation of latent and lytic gene activation was recently reported for the Rael group I BL line treated with the demethylating agent 5'-azacytidine (28). Transcriptional analysis showed that this late expression of EBNA2 coincides with activation of the Cp and Wp promoters (24a) that are functional in LCLs (42, 51). Furthermore, these EBNA2$^+$ cells subsequently progressed to express LMP1, with only a minority then entering the lytic cycle (Fig. 5). Together, these observations strongly suggest that the EBNA2$^+$ subpopulation is in the process of switching from Lat I to a Lat III form of infection, a route already observed in previous studies monitoring spontaneous progression of BL lines from a group I to a group III cellular phenotype on serial passage (17, 34, 39).

Perhaps the most interesting response pathway identified in the present work is that leading from Lat I to a form of latency, Lat II, not hitherto recognized in B cells. To date, the only examples of this EBNA1$^+$ LMP$^+$ form of infection have involved EBV-associated tumors of non-B-cell origin, namely, nasopharyngeal carcinoma (9, 54) and Hodgkin's disease (30). Here, we show that under certain circumstances B lymphocytes can support a Lat II form of infection and that, at least in this in vitro system, LMP can be expressed as a latent antigen independently of EBNA2-mediated transactivation (10, 50). Since LMP1 has the potential to alter several facets of the B-cell phenotype (18, 47, 49), we questioned to what extent such LMP1-associated changes accompanied the switch from a Lat I to a Lat II form of infection. Interestingly, some of the expected changes, e.g., upregulation of LFA-3 and CD23, were observed, whereas another change, upregulation of *bcl-2*, was not (Fig. 7). In contrast, the switch from Lat I into the lytic cycle, although also associated with induction of LMP1 expression, did not alter any of the above phenotypic properties. We interpret this latter observation to be a consequence of the fact that entry of herpesvirus-infected cells into the lytic cycle results in rapid inhibition of cellular metabolism.

We suggest that all three forms of latency here identified in an in vitro system may have a natural counterpart within the EBV-infected B-cell pool in vivo. One important constraint on survival of infected B cells is the existence of potent virus-specific cytotoxic T-cell responses in all healthy EBV-carrying individuals. In this context, recent studies show that such T-cell responses are rarely, if ever, directed towards target epitopes derived from EBNA1, but are preferentially directed against the other latent proteins (29a). The restricted expression of EBV latent genes in the Lat I form of latency, together with the associated downregulation of cellular adhesion molecules, would enable such cells to avoid the T-cell immunosurveillance mechanism (16, 35). Likewise, activation from Lat I directly into the lytic cycle via the pathway identified in the present work would also be able to proceed in the face of the prevailing T-cell response. Hence, Lat I cells could provide a reservoir both for virus persistence as a latent infection and for efficient virus reactivation. Switching to the Lat III form of infection clearly also occurs with regularity in healthy EBV carriers, since cellular and humoral responses to the full spectrum of latent proteins are stably maintained (4, 5, 29, 37). Activation to Lat III presumably permits a burst of cell proliferation, but any cell progeny which then retained this form of infection would be susceptible to immune system elimination. At present, it is not clear under what circumstances the Lat II form of infection may occur in B cells in vivo or what particular advantages such an infection may confer. Coex-

pression of EBNA1 and LMPs may provide a proliferative advantage in certain situations, although, as with the Lat III form of infection, such cells are likely to be subject to immune system control. Therefore, just as immunologically compromised individuals have provided examples of Lat III infection in B-cell lymphomas (53), it would be interesting to screen the same group of patients for evidence of B-cell proliferations with a Lat II form of infection.

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