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Up Regulation of the Epstein-Barr Virus (EBV)-Encoded Membrane Protein LMP in the Burkitt's Lymphoma Line Daudi after Exposure to *n*-Butyrate and after EBV Superinfection

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The Burkitt's lymphoma line Daudi carries a nontransforming Epstein-Barr virus (EBV) strain that has a deletion in the *Bam*HI WYH region of the genome coding for the EBV nuclear antigen 2 (EBNA-2). Daudi cells fail to express the EBV-encoded latent membrane protein (LMP) (D. Ghosh and E. Kieff, J. Virol. 64:1855–1858, 1990). We show that LMP expression can be up regulated by exposure to *n*-butyrate and by superinfection with the B95-8 (B virus)- and P3HR1 (P virus)-derived EBV strains. Two LMP polypeptides of 60 and 48 kilodaltons (kDa) were detected in immunoblots of Daudi cells that had been exposed to 3 mM *n*-butyrate for 24 h. The intensity of the 48-kDa LMP increased during 72 h, in parallel with the appearance of early antigen-positive cells. The 60-kDa LMP was expressed at a low level and remained constant. Superinfection of Daudi cells with B and P virus induced the 60-kDa LMP within 3 h. In addition, P virus induced the 48-kDa LMP at a low level. The B virus-encoded EBNA-2 and EBNA-5 were detected 12 h after superinfection. The B virus-encoded 63-kDa LMP was coexpressed with the endogenous LMP after 48 h. Inactivation of the virus by UV illumination abolished the expression of the B virus-encoded antigens but did not affect the induction of the endogenous LMP. The B-cell activation marker CD23 was up regulated by B virus superinfection but not by *n*-butyrate exposure. CD23 was also expressed at a higher level in a stable B virus-converted subline, E95A-Daudi, that was EBNA-2 positive and coexpressed the Daudi virus- and B virus-encoded LMP. The results suggest that LMP expression is regulated by the interaction of cellular and viral factors. Binding of the virus to its membrane receptor might be involved in the triggering of cellular control mechanisms. Viral gene products are not directly involved in this function but may contribute to create a permissive cellular environment for LMP expression.

Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) carry multiple episomal copies of the viral genome and express a limited set of viral genes. Nine EBV-encoded proteins have been identified. Six of them are nuclear antigens (EBNA-1 to EBNA-6) (7). The other three, the latent membrane protein (LMP) (10) and two terminal proteins (42), are associated with the cell membrane.

LMP is encoded by spliced exons from the BNLF-1 open reading frame (16). It associates with the cytoskeletal protein vimentin (26) and shows a patchy localization in the cell membrane (27). Stable LMP expression in two EBV-negative Burkitt's lymphoma (BL) lines, Louckes and BL-41, was paralleled by an increase of cell size and intracellular free calcium and by up regulation of B-cell activation markers and cell adhesion molecules (46). The transfected cells become sensitive to lysis by EBV-specific major histocompatibility complex class I-restricted cytotoxic T lymphocytes, generated by stimulation with autologous EBV LCLs (36). LMP can also induce anchorage independence and tumorigenicity in rodent fibroblasts (4, 44). The structural similarity between LMP and the rhodopsin receptor family has been taken to suggest that LMP may serve as a growth factor receptor (30, 43).

LMP mRNA represents the most abundant viral transcript in EBV-transformed LCLs (10). Hudson et al. (18) detected two LMP mRNA species in B95-8 cells. The most abundant transcript encodes a 63-kilodalton (kDa) protein. A second transcript is found only in cells that have entered the lytic cycle (18, 40). It encodes a 48-kDa protein that lacks the N terminus and four of the transmembrane domains, cannot associate with the cytoskeleton, and does not transform rodent fibroblasts (45).

EBV-positive BL biopsies and derived cell lines that have maintained the BL phenotype in vitro do not express LMP and EBNA-2 to EBNA-6 (39, 41) but regularly express EBNA-1. EBNA-2 to EBNA-6 and LMP may be up regulated during prolonged in vitro propagation, in parallel with a shift towards a more "LCL-like" phenotype (41). Rescue of EBV from BL cells by cocultivation with normal B cells leads to the full expression of all seven antigens in the derived LCLs, confirming that the lack of EBNA-2 to EBNA-6 and LMP expression is dependent on the BL cell phenotype (8a, 31, 39). The down regulation of these proteins in the BL cell was associated with a high level of viral DNA methylation, in contrast to LCLs where the EBV genome is unmethylated (8a, 31).

It has been shown that EBNA-2 is required for LMP expression (1, 35; R. Fåhraeus, A. Jansson, A. Ricksten, Q. A. Sjöblom, and L. Rymo, Proc. Natl. Acad. Sci. USA, in press), although exceptions have been found (6, 33). We wished to examine the requirements for LMP expression in the EBNA-2-defective BL line Daudi (20) that is LMP negative at the protein and mRNA levels (14). We report that LMP expression can be induced by *n*-butyrate and also by exposure to infectious or UV light-inactivated EBV.

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MATERIALS AND METHODS

Cell lines. The EBV-positive BL line Daudi (20, 23) was maintained in RPMI 1640 medium supplemented with 2 mM glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 5% heat-inactivated fetal calf serum (complete medium). For *n*-butyrate treatment, exponentially growing cells were resuspended at a concentration of 5×10^5/ml in complete medium containing 3 mM *n*-butyrate (B 5887; Sigma Chemical Co., St. Louis, Mo.).

Virus. Culture supernatants from mycoplasma-negative EBV producer lines B95-8 (B virus) (34) and P3HR1 (P virus) (17) were used as a source of infectious virus.

The virus preparations were filtered (pore size, 0.45 μm) and assayed for EBV-binding activity on the EBV-negative lymphoma line BJAB by measuring the percentage of membrane antigen-positive cells. Briefly, 1×10^6 cells were incubated for 1 h at 4°C with 1 ml of the viral preparation. Cells were washed twice with cold phosphate-buffered saline (PBS) and incubated for 1 h on ice with a 1:50 dilution of a previously characterized (24) fluorescein-conjugated plasma from a BL patient (MUTUA) containing anti-viral capsid antigen (VCA) titers of 1:320, early antigen diffuse (EAD) and early antigen restricted (EAR) titers of 1:10, and EBNA titers of 1:40. The cells were washed four times with cold PBS, and smears were fixed in methanol-acetone in a ratio of 2:1 and counterstained with Evans blue. The virus preparation gave 70% membrane antigen-positive cells.

When indicated, samples of the virus were exposed to UV illumination with a Philips Hg lamp at a distance of 20 cm from a lamp providing 10 J/m^2 per s, as determined by a Latarjet dosimeter. An illumination of 2,000 J was found to abolish the EBNA-inducing capacity of B virus. For the present experiments, the B virus was irradiated with 4,000 J (UV-B virus).

EBV superinfection. Daudi cells were suspended at concentration of 5×10^6/ml in the different virus preparations. After 1 to 2 h of incubation at 37°C under occasional agitation, the cells were transferred to complete medium and resuspended at 1×10^6/ml. After culture at 37°C in a 5% CO_2 atmosphere for the indicated periods of time, the cells were distributed in aliquots that were used for detection of EBV antigens by immunofluorescence and immunoblotting and for analysis of surface marker expression.

Detection of EBV antigens. (i) Detection by immunofluorescence. EBNA was detected by anticomplement immunofluorescence staining (37) by using previously characterized sera from EBV-infected individuals. The same sera were absorbed with P3HR-1 and Daudi cells for the specific staining of EBNA-2 (8). Early antigen (EA) was detected by direct immunofluorescence (15) by using fluorescein isothiocyanate-conjugated immunoglobulin G from a BL patient (ESTER).

(ii) Detection by immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western immunoblotting were performed as described previously (31). Cells (10^7) were dissolved in 1 ml of electrophoresis sample buffer. Proteins were separated by discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the method of Laemmli (25) and blotted on nitrocellulose paper (Schleicher & Schuell, Inc., Dassel, Federal Republic of Germany). The efficiency of the protein transfer and the position of molecular weight markers was visualized by staining the filters with Ponceau S (Sigma). Excess protein-binding sites were blocked by incubation of the blotted filters for 1 h in PBS containing 5% dried skimmed milk (PBS-milk). The filters were subsequently incubated with the specific antibodies diluted in PBS-milk for 2 h at room temperature or 16 h at 4°C. After incubation with the first antibody, the filters were washed in PBS-milk and subsequently incubated with the appropriate alkaline phosphatase-conjugated second antibody (Bio-Rad, Kemila Sollentuna, Sweden) for 2 h at room temperature. After three washes in PBS containing 0.5% Tween, the specifically bound second antibody was detected by fast-red salt precipitation in the presence of alpha naphthyl phosphate. A previously characterized serum from an EBV-seropositive donor (PG serum: VCA, 1:1,280; EAD, 1:640; EAR, 1:20; EBNA, 1:320; and EBNA-2, 1:80) was used for detection of EBNAs. LMP was detected by the S-12 monoclonal antibody (MAb) (30) provided by D. Thorley-Lawson (Department of Pathology, Tufts University School of Medicine, Boston, Mass.) The MAb JF186 (11) was used for the detection of EBNA-5 in B-virus superinfected cells. EA was detected by E5.7 MAb (29) provided by G. Pearson (Department of Microbiology, Georgetown University School of Medicine, Washington, D.C.). The human serum was used at 1:50 dilution, and the MAbs were used at 1:1,000, 1:50, and 1:10 dilutions of affinity-purified culture supernatants.

Analysis of B-cell markers. The following MAbs were used for the analysis of B-cell markers: B-2 (CD-21) for the EBV receptor; MHM24 (CD-11a) and TS-2 (CD-58) for cell adhesion molecules; and LB-1 and AC-2 (CD-39) and MHM-6 (CD-23) for B-cell activation markers. The percentage of positive cells and the intensity of expression were assessed by fluorescence-activated cell sorter analysis after indirect immunofluorescence staining.

RESULTS

Induction of LMP by *n*-butyrate. As reported earlier (28), exposure of Daudi cells to 3 mM *n*-butyrate induced the productive viral cycle. After 72 h of treatment, EAs were detected by immunofluorescence in about 15% of the cells. Two major EA proteins of 42 and 46 kDa were detected in immunoblots probed with the MAb E5.7 (Fig. 1A). The intensity of both bands increased between 24 and 48 h, in parallel with the increment of EA-positive cells. Only the 42-kDa EA band was detected after 72 h.

The LMP-specific MAb S-12 detected two bands of 60 and 48 kDa in immunoblots of *n*-butyrate-treated Daudi cells (Fig. 1B). The intensity of the 60-kDa band was low and remained at the borderline of detectability during the 72-h observation period. In contrast, the intensity of the 48-kDa band increased in parallel with the increment in the percentage of EA-positive cells (from 1 to 15%) and the intensity of the EA bands.

Induction of LMP by EBV infection. Daudi cells expressed LMP after infection with P and B virus preparations.

P virus induced both the 60- and 48-kDa LMP. The two bands became detectable between 3 and 12 h after infection (Fig. 2A). The intensity of the 60-kDa band increased with time, whereas the 48-kDa band was expressed at a constantly low level (Fig. 2A). Because of the similarities in molecular weight, we could not decide whether the endogenous and P virus-encoded LMP were coexpressed.

B virus induced exclusively the 60-kDa Daudi virus-encoded LMP (Fig. 2B). This was regularly detected 3 h after infection, reached the maximal expression after 15 h, and remained stable until the termination of the experiment. The B virus-encoded 63-kDa LMP appeared 48 h after superinfection and was coexpressed with the Daudi virus-

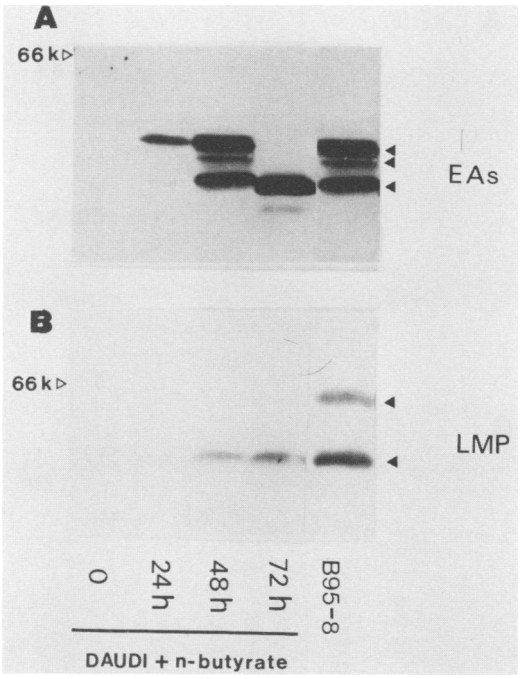

FIG. 1. Time course of induction of EA and LMP by *n*-butyrate. Daudi cells were exposed to 3 μM *n*-butyrate, and samples were collected every 24 h. Western blots were probed with the LMP-specific MAb S-12 (B) and the anti-EA MAb E5.7 (A). At 72 h, EAs were detected by immunofluorescence in 15% of the cells.

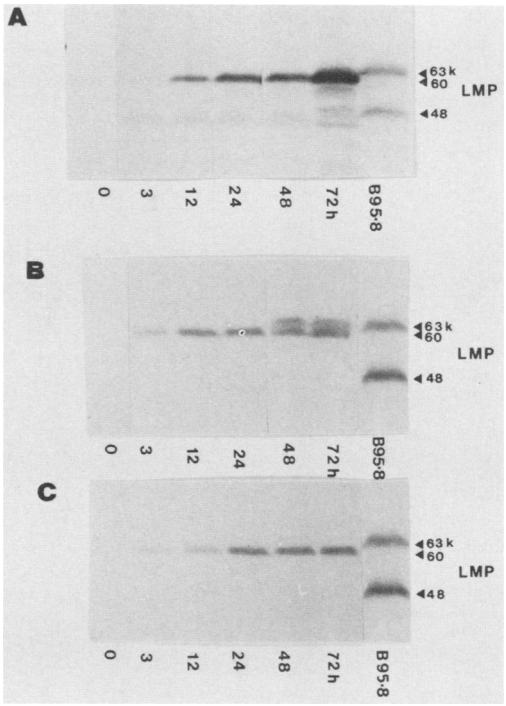

FIG. 2. LMP induction by EBV superinfection. Daudi cells were superinfected with P virus (A), B virus (B), and UV-inactivated B virus (C). The blots were probed with S-12 MAb.

encoded 60-kDa LMP (Fig. 2B). The same time course of 60-kDa LMP expression was observed when Daudi cells were infected with B virus exposed to UV light (Fig. 2C). The UV-irradiated B virus preparation did not induce the B virus-encoded LMP (Fig. 2C) nor EBNA-2 (data not shown), confirming the inactivation of the corresponding gene.

Expression of B virus-encoded proteins in superinfected Daudi cells. Daudi cells do not express EBNA-2 because of the deletion of a large portion of the *Bam*HI WYH region of the viral genome (20). They express a single 30-kDa EBNA-5 band that can be detected with polyvalent human sera (48) but which does not react with the JF-186 MAb raised against the B virus-encoded EBNA-5 polypeptide (11).

The B virus-encoded EBNA-2 and EBNA-5 were detected in immunoblots 12 h after infection (Fig. 3A and B). At this time, EBNA-2 was detected in 6% of the cells by anticomplement immunofluorescence staining. Expression of EBNA-3 and EBNA-4 increased significantly 3 h after infection (Fig. 3A). Because of the similar size and poor resolution in the high-molecular-weight area of the blots, we could not determine whether these antigens were encoded by B virus or by the Daudi virus. However, superinfection with UV-treated B or P virus did not induce any change on the expression of the endogenous Daudi virus-encoded EBNA-3 and EBNA-4 (data not shown).

The B virus-encoded 63-kDa LMP appeared after 48 h (Fig. 2B) and was coexpressed with the endogenous 60-kDa LMP. In accordance with previous reports (21), superinfection with B virus did not increase the percentage of EA-positive cells from the background level (approximately 1%).

A 100% EBNA-2-positive subline of Daudi, E95A-Daudi, was obtained by cloning in limiting dilution. The clone expressed high levels of EBNA-2 (Fig. 4A) and coexpressed the B virus- and Daudi virus-encoded LMP (Fig. 4B). The Daudi virus-encoded 60-kDa LMP band was consistently expressed at a lower level. Both the Daudi virus- and the B virus-encoded LMP species were up regulated by exposure of E95A-Daudi to 3 mM *n*-butyrate. It is noteworthy that the 48-kDa LMP was not induced by *n*-butyrate treatment of E95A-Daudi.

Induction of B-cell activation markers. The B-cell activation marker CD23 was induced by superinfection of Daudi with B95-8 virus but not by treatment with *n*-butyrate (Fig. 5). A significant increase of fluorescence intensity was observed after 24 h. The number of positive cells increased from 26 to 56% during the 72 h of the experiment. The E95A-Daudi line expressed high levels of CD23 (67% of the total population). Other B-cell activation markers such as CD-39 (LB-1/AC-2), CD21 (B-2), and the adhesion molecules LFA-1 (CD11a) and LFA-3 (CD58) were not significantly affected (data not shown).

DISCUSSION

Earlier findings have suggested that both cellular and viral factors can influence the expression of the EBV-encoded membrane protein LMP. The influence of cellular factors is manifested by the differential expression of LMP in the three major EBV-carrying cell phenotypes LCL, BL, and nasopharyngeal carcinoma (9, 39, 41) and in cell hybrids between EBV-positive BL cells and EBV-negative B- and non-B-cell lines (6). Viral control can be exemplified by the *trans* activating role of EBNA-2, as shown by the expression of LMP in B virus- but not P virus-converted sublines of EBV-negative BLs (35) and by the induction of LMP expression after EBNA-2 transfection into P virus-converted cells

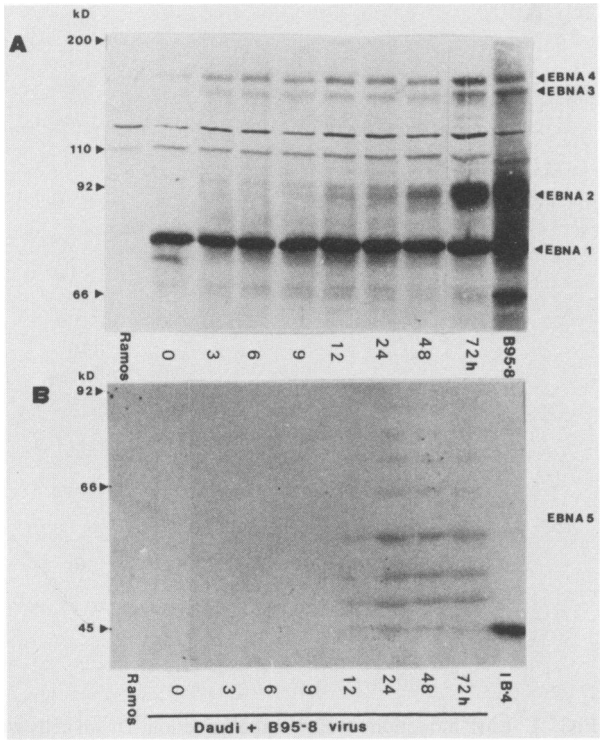

FIG. 3. Expression of B virus-encoded antigens after superinfection of Daudi cells. Blots of total cell extracts collected at different times after B virus superinfection were probed with the EBV-positive human serum PG (A) and the anti-EBNA-5 MAb JF186 (B). Ramos is an EBV-negative BL line. B95-8 and IB-4 are EBV-positive LCLs.

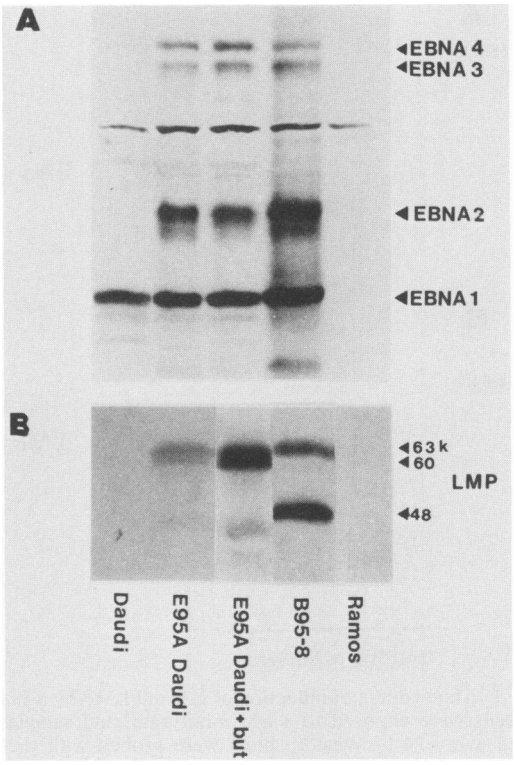

FIG. 4. Expression of EBV antigens in the E95A-Daudi line. E95A-Daudi cells were exposed to 3 μM *n*-butyrate, and the sample was collected after 48 h. (A) EBNA-1 to EBNA-4 were detected with the PG serum. (B) LMP was detected with the MAb S-12.

(1). In order to dissect the relative contribution of cellular versus viral factors, we have investigated the requirements for LMP expression in the Daudi BL line that carries an EBNA-2 gene-deleted EBV strain (20) and is LMP negative at the protein and mRNA levels (14).

We have shown that LMP can be induced in Daudi cells by *n*-butyrate. This confirms that the Daudi virus encodes an intact LMP gene but fails to express it due to a regulatory phenomenon.

Exposure to *n*-butyrate and superinfection with P virus induced two LMP species of 60 and 48 kDa. B virus superinfection induced only the 60-kDa LMP. Expression of the 48-kDa protein was associated with entry of some cells into the lytic cycle, as shown by the similar time course of EA and 48-kDa LMP induction and by the parallel intensification of the EA- and LMP-specific bands in immunoblots. Correlation between the low-molecular-weight LMP expression and entry of the cells into the lytic cycle has been previously reported (40). The failure to express the 48-kDa LMP after B virus superinfection is in line with the well-documented (21) inability of the B virus to induce the productive cycle in superinfected EBV-carrying cells. It is likely that the 48-kDa protein corresponds to the shorter LMP transcripts initiated at the lytic promoter ED-L1A in B95-8 cells (18).

The present data confirm our previous findings showing that EBNA-2 is not always required for LMP expression (6) and support the notion that cellular factors play an important role for the control of the LMP gene. LMP was up regulated by infecting Daudi cells with the EBNA-2-deleted P virus and by UV-inactivated B virus that could not induce any detectable EBNA-2. Both the P and B viruses induced the endogenous Daudi LMP 3 h after superinfection, long before any exogenous virus-encoded antigen (e.g., establishment of B virus infection) could be demonstrated by immunological methods, suggesting that EBV-induced LMP expression is not determined by the onset of a secondary infection. It is possible that the virus particles carry structural components that, after being introduced into the cell, can activate LMP expression. Alternatively, the binding of the virus to the CR2 receptor may by itself be sufficient to trigger the cellular mechanisms that activate LMP expression in Daudi cells.

Our previous finding that LMP is suppressed in the hybrid derived from the fusion of the EBV-carrying LMP-positive BL line Raji and the EBV-negative BL line Ramos suggests that at least certain BL cells can inhibit the expression of LMP independently of EBNA-1 to EBNA-6 (6). The negative regulation of LMP appears to be dependent on the phenotype of the BL cell. B virus-converted Ramos, BL41, and BL28 sublines express EBNA-2 but not LMP, except in the sublines in which EBV conversion was accompanied by a marked shift toward a more LCL-like phenotype (33).

It has recently been demonstrated that the 5′-flanking region of the LMP gene contains a constitutive positive transcriptional element that is controlled by adjacent negative regulatory sequences (12; Fåhraeus et al., in press). Transcription initiated from the ED-L1 promoter was completely repressed in EBV-negative DG75 cells transfected with reporter vectors that contained the LMP regulatory region. The activity of the promoter could be induced by

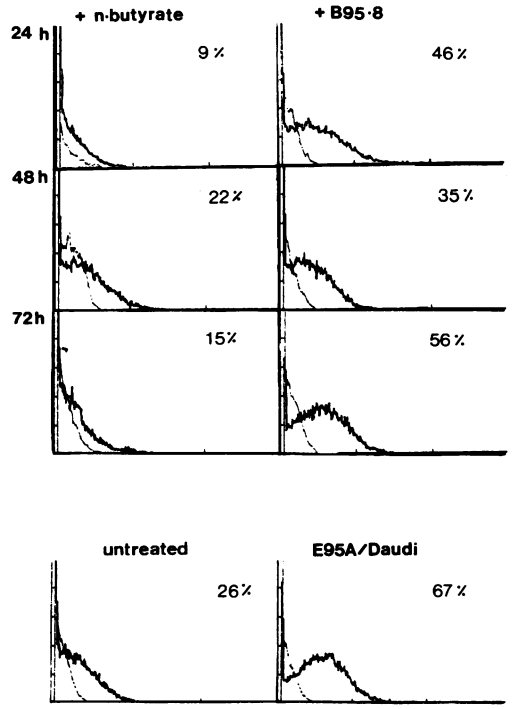

FIG. 5. Expression of the B-cell activation marker CD23, determined by fluorescence-activated cell sorter analysis of cells stained with the MHM6 MAb.

cotransfection with EBNA-2 expression vectors. Moreover, deletion of a portion of the LMP regulatory region resulted in a high level of transcription, even in the absence of EBNA-2. The results suggested that EBNA-2 induces the expression of LMP in EBV-infected B cells by blocking the function of a transcriptional repressor.

Conceivably, the activity of the repressor may be influenced by the stage of B-cell activation. Both P virus and UV-inactivated B virus can induce the expression of B-cell activation markers, BCGF receptors, and leukocyte inhibitory factor production in resting B cells (13, 32). This type of B-cell activation was inhibited by virus-neutralizing antibodies, indicating that it is triggered by the binding of the virus to the CR2 receptor. B cells could also be activated by cross-linking CR2 with anti-gp140 antibodies (32).

Superinfection of Daudi cells with the intact virus induced the expression of the B virus-encoded EBNA-2 and EBNA-5. EBNA-3 and EBNA-4 were not induced after superinfection with UV-irradiated B virus superinfection, suggesting that different mechanisms control LMP expression and the other nuclear antigens.

The endogenous and B virus-encoded LMP proteins were coexpressed in Daudi cells superinfected with the intact virus, while the low-molecular-weight LMP was not induced by *n*-butyrate in the E95A-Daudi cells. It has been reported that not all the cell lines that enter into the lytic cycle express the low-molecular-weight LMP. Great variability has been observed among B95-8-transformed LCLs (40) and has been related to the stage of activation or differentiation of the particular cell. It is likely that a similar explanation can be applicable to the lack of 48-kDa induction in *n*-butyrate-treated E95A-Daudi cells.

The high expression of EBNA-2 in the E95A-Daudi cell line suggests that although EBNA-2 is not an absolute prerequisite for LMP expression in Daudi cells, it may contribute to the creation or stabilization of an LMP-permissive cellular environment. EBNA-2 can *trans* activate cellular genes, including CR2 (5, 22), cell adhesion molecules (46a), and the B-cell activation marker CD23 (47). We have found that CD23 is up regulated in Daudi after B virus superinfection but not after *n*-butyrate treatment, indicating that a high expression of the 48-kDa LMP protein does not affect the levels of CD23 expression. CD23 was also expressed at an increased level in the E95A-Daudi line, confirming that it has progressed towards a more activated phenotype.

The difference in the time course of the endogenous and B virus-encoded LMP expression in superinfected Daudi cells also deserves some comments. The B virus-encoded LMP did not appear until 36 h after infection, i.e., after EBNA-2 and EBNA-5. These antigens appear with a time course similar to that observed after primary infection of normal B lymphocytes (3, 38). It is likely that the expression of the B virus-encoded LMP in Daudi cells can be influenced, at least in part, by the same factors that determine the sequence of viral gene expression after primary infection. It has been suggested that circularization of the viral genome may be required for LMP expression. Hurley and Thorley-Lawson (19) have shown that circularization occurs within 16 to 20 h after infection, subsequent to EBNA expression but prior to LMP expression. If circularization of the viral genome were a limiting factor for LMP expression in normal B cells, this would not influence the rapid *trans* activation of the endogenous LMP in the Daudi cell line that constitutively carries about 100 copies of the EBV episome (2).

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