Latent Membrane Protein of Epstein-Barr Virus Induces Cellular Phenotypes Independently of Expression of Bcl-2

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The stable expression of the Epstein-Barr virus (EBV) latent membrane protein (LMP) in certain EBV-negative Burkitt's lymphoma cell lines correlates with an increased expression of the oncogene Bcl-2 (S. Henderson, M. Rowe, C. Gregory, D. Croom-Carter, F. Wang, R. Longnecker, E. Kieff, and A. Rickinson, *Cell* 65:1107–1115, 1991). This finding is consistent with a model in which Bcl-2 contributes to the immortalization of B cells mediated by EBV. We therefore asked whether the expression of Bcl-2 protein correlates with the induction of three cellular phenotypes induced by or associated with LMP. The expression of Bcl-2 in primary B cells infected with the B95-8 strain of EBV varied between 1 and 1.8 times that in uninfected cells when 50% of the cells were infected, expressed LMP, and incorporated 20-fold more [³H]thymidine than did uninfected cells. This finding indicates that induced proliferation of these primary cells is not sufficient to induce Bcl-2. We found that BALB/c 3T3 cells and their derivatives transformed by LMP do not express Bcl-2 detectably. The expression of LMP at high levels in lymphoid cells is cytotoxic and correlates with an increased expression of Bcl-2 following stable selection for the introduced LMP gene; 2 days after transfection, control vector- and LMP-transfected populations, however, express equal levels of Bcl-2 protein. We also analyzed transient expression of LMP in an EBV-negative Burkitt's lymphoma cell line. Infection of BJAB cells with the B95-8 strain of EBV results in an increase in Bcl-2 expression with a time course similar to that of LMP expression, and LMP alone transiently induces an increase in Bcl-2 expression in these cells. We interpret these observations to indicate that increased expression of Bcl-2 is unlikely to contribute to the ability of EBV to immortalize primary B cells and that both the transformation of rodent cells and the cytotoxicity mediated by LMP are independent of Bcl-2.

Epstein-Barr virus (EBV) is a human herpesvirus causally associated with Burkitt's lymphoma (BL) and nasopharyngeal carcinoma. The biological activity of EBV that is likely to underlie its contribution to these malignancies is exemplified by its ability to induce the unlimited proliferation of human B cells in vitro. A small subset of viral genes is expressed in latently infected (immortalized) B cells. One of the latently expressed proteins (latent membrane protein [LMP], encoded by the BNLF-1 open reading frame and also termed LMP-1) is likely to have an important role in the initiation and/or maintenance of the immortalized state. LMP, in the absence of any other viral gene products, functions as an oncogene in rodent cells; it induces anchorage-independent and tumorigenic growth of both Rat-1 (30) and BALB/c 3T3 (3) cells. The expression of LMP in certain EBV-negative BL cell lines yields some phenotypes similar to those observed in primary B cells upon infection with EBV. LMP-positive derivatives of BJAB and Louckes cells (EBV-negative BL cell lines) express increased levels of the B-cell activation marker CD23 and decreased levels of CD10; LMP-positive derivatives of BL41 cells (EBV negative) express increased levels of CD39 as well as decreased levels of CD10 (32). The expression of LMP in Louckes cells also correlates with an increase in the levels of the cell adhesion molecules LFA-1, LFA-3, and ICAM-1; these cells exhibit increased homotypic cellular adhesion, presumably

as a result of elevated levels of adhesion molecules (31). Thus, the expression of LMP in certain EBV-negative BL cell lines can mimic some phenotypic changes seen upon infection of B lymphocytes with EBV.

Recent work has identified a correlation between the expression of EBV latent genes and the expression of the Bcl-2 oncogene in BL cell lines (8, 11). Bcl-2 is an oncogene identified in follicular lymphomas containing a chromosomal translocation between the Bcl-2 gene and the immunoglobulin heavy-chain locus (5–7, 25, 27). The expression of Bcl-2 has been shown to promote the survival of B-cell precursors (28) and of memory and immunoglobulin-secreting B cells (19) and to block programmed cell death in B cells (20). The oncogenic activity of Bcl-2 is thought to result from its ability to promote cell survival rather than cell proliferation. Gregory et al. (8) showed that EBV-positive BL cell lines that express the full complement of latent gene products are resistant to induced apoptosis. The introduction of individual latent genes (LMP, EBNA-1, and EBNA-2) into certain EBV-negative BL cell lines followed by selection for their expression demonstrated a correlation between the expression of LMP and both resistance to induced apoptosis and the expression of Bcl-2 (11). The expression of Bcl-2 in the cell lines was shown to be sufficient for the anti-apoptotic effect (11). It was concluded that the expression of LMP in EBV-positive BL cell lines results in the induction of Bcl-2 and that the expression of Bcl-2 confers a survival function upon the cells. These observations are consistent with a possible role for Bcl-2 in the immortalization of primary B cells by EBV. The link between EBV and increased expression of Bcl-2 in certain BL cell lines indicates that EBV may

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supply a survival function to the cells by inducing the expression of Bcl-2.

We asked whether cellular phenotypes associated with or induced by LMP correlate with changes in the levels of Bcl-2. We found that the expression of Bcl-2 does not correlate with the induction by LMP of anchorage-independent growth or of cytotoxicity and that the expression of Bcl-2 in primary human B cells is not significantly altered by infection with EBV under conditions in which LMP expression is easily detected.

MATERIALS AND METHODS

Cells. BALB/c 3T3 is an adherent murine cell line (1) that was obtained from the American Type Culture Collection and grown in Dulbecco's medium plus 10% bovine calf serum (Hyclone). BJAB is an EBV-negative BL cell line (13) that was grown in RPMI plus 10% fetal bovine serum (Hyclone). HH514 is derived from the P3HR1 clone of the Jijoye BL cell line (21) and was maintained in RPMI plus 10% bovine calf serum (Hyclone). B95-8 is a marmoset cell line transformed in vitro by EBV (18) and was maintained in RPMI plus 10% bovine calf serum. All cells were grown with 200 U of penicillin and 200 µg of streptomycin per ml at 37°C in a 5% CO₂ atmosphere.

Preparation of virus. B95-8 cells (initial density, 10⁶/ml) were maintained in the presence of 20 ng of tetradecanoyl phorbol acetate per ml–3.5 mM sodium butyrate for 5 days. Cells were removed by centrifugation, and the supernatant was filtered sequentially through 0.8- and 0.2-µm-pore-size cellulose acetate filters. This preparation of virus was either used in this form or concentrated 10-fold by filtration with 76-mm-diameter polyvinylidene difluoride filters (molecular weight cutoff, 500,000) (Molecular/Por type F; Amicon).

Plasmids. LMP expression vectors were described previously (10). In brief, plasmids used in cytotoxicity experiments contained the LMP, NΔ43, or CΔ55 genes driven by the human cytomegalovirus (CMV) immediate-early promoter and enhancer, the origin of plasmid replication of EBV, and the hygromycin phosphotransferase gene as a selectable marker; the control vectors for these experiments were identical but lacked LMP sequences. The EBNA-2 expression vector pEΔA8 was described previously (24). A mutant EBNA-2 expression vector was derived from pEΔA8 by insertion of a termination codon into the EBNA-2 open reading frame (after one-third of the open reading frame at a *Bst*EII site) to generate a truncated, nonfunctional EBNA-2 protein identical to that encoded by p615, as described by Hammerschmidt and Sugden (9).

Antibodies. The affinity-purified anti-LMP antiserum generated in rabbits was described and characterized previously (2). 3F11 is a hamster anti-mouse Bcl-2 monoclonal antibody to murine Bcl-2 (29). 6C8 is a hamster anti-human Bcl-2 monoclonal antibody that recognizes human Bcl-2, and TN3 is a hamster anti-tumor necrosis factor alpha monoclonal antibody (12).

B-cell purification. B cells were purified from human blood as described previously (33). Fractions of adult peripheral blood obtained from the Red Cross were fractionated over Renografin-Ficoll, and adherent cells in the leukocyte fraction were removed by plating at 37°C on tissue culture plates. Surface immunoglobulin (sIg)-positive (sIg⁺) cells were purified from nonadherent cells by panning with rabbit anti-human Fab (33). The resulting purified cells were typically >80% sIg⁺, as determined by staining of live cells for sIg with rabbit anti-human Fab. The sIg⁺ cells isolated by

this method were unlikely to be activated by sIg cross-linking, since the cells were selected with anti-human Fab fragments; no stimulation of [³H]thymidine uptake in freshly isolated sIg⁺ cells was observed, unless the cells were infected with EBV.

Infection of B cells. Purified B cells were infected on the day following their purification. Cells were incubated at room temperature with a filtered viral supernatant at a density of 2 × 10⁵/ml for 2 h with continuous rocking. Cells were then isolated by centrifugation and plated in RPMI plus 10% fetal bovine serum for the duration of the experiment. The efficiency of infection was assayed by EBNA staining with sera from an EBV-positive donor (23). In general, 50% of the cells were found to be infected under these conditions, indicating that they were infected with a multiplicity of 0.7 infectious particles per cell.

[³H]thymidine uptake. Cells (10⁶/ml) were labeled for 24 h with 20 µCi of [³H]thymidine per ml in complete medium. After a wash, cell pellets were lysed, precipitated with 10% trichloroacetic acid, filtered, and counted by liquid scintillation. Uninfected B cells incorporated ~1,000 cpm/5 × 10⁵ cells; infected cells incorporated ~30,000 cpm/5 × 10⁵ cells by day 6 postinfection. The time course and level of incorporation of [³H]thymidine were a function of the multiplicity of infection used and were similar to those previously documented (16).

Western immunoblot analysis of LMP and Bcl-2. The expression of LMP was monitored by Western blot analysis with an affinity-purified anti-LMP antiserum as previously described (17). The expression of Bcl-2 was monitored by Western blot analysis as previously described (17) by a three-step staining procedure. Blots were incubated first with a 1:100 dilution of a hamster anti-human (6C8) or hamster anti-mouse (3F11) Bcl-2 monoclonal antibody, next with 3.33 µg of biotinylated anti-hamster immunoglobulin G (Vector Laboratories) per ml, preadsorbed for 60 min at room temperature with 5 mg of liver acetone powder (Sigma) per ml, and then with Vectastain ABC-alkaline phosphatase reagent (Vector), prepared in accordance with the manufacturer's instructions. These blots then were developed as described previously (17). Molecular mass markers of 105, 80, 46, 35, and 27 kDa were from Stratagene. LMP and Bcl-2 migrated with apparent molecular masses of 62 and 25 kDa, respectively.

Transfections. HH514 and BJAB cells were transfected by electroporation. Approximately 10⁷ cells were electroporated with 20 µg of plasmid DNA by use of the settings of three capacitor banks, 1,500 V, and maximal R-adjust (14). Electroporated cells were pooled and plated on growth medium overnight, and then surviving cells were isolated by fractionation over Renografin-Ficoll.

Cytotoxicity assay. LMP-mediated cytotoxicity was assayed with HH514 cells as previously described (10).

Immunofluorescence staining and flow cytometric analysis.

(i) **Double staining for LMP and Bcl-2 in HH514 cells.** Cells were purified over Renografin-Ficoll prior to being stained. Cells (10⁶/100 µl) were incubated with primary antibodies (1:25 rabbit anti-LMP antiserum and 1:40 6C8 [anti-Bcl-2]) diluted in phosphate-buffered saline (PBS)–1% bovine serum albumin (BSA)–0.3% saponin (PBS/BSA/saponin) at 0°C for 30 min. After a wash in the same buffer, cell pellets were incubated with a secondary antibody (1:40 biotinylated anti-hamster immunoglobulin G preadsorbed with 5 mg of liver acetone powder per ml) diluted in PBS/BSA/saponin for 30 min at 0°C. Pellets then were washed and incubated with a tertiary antibody (1:100 streptavidin-RED613 [GIBCO/

BRL]-1:40 goat anti-rabbit immunoglobulin G conjugated to fluorescein isothiocyanate) in PBS/BSA/saponin for 30 min at 0°C. Pellets then were washed in PBS-1% BSA, resuspended in the same buffer at a concentration of 10^6 /ml, and analyzed by use of an EPICS Profile II flow cytometer.

(ii) **Double staining for sIg and Bcl-2 in B cells.** Cells were stained as described above, except that the primary antibody was rabbit anti-human Fab (1:100) instead of anti-LMP antiserum.

The irrelevant control antibody for Bcl-2 staining was a hamster anti-tumor necrosis factor alpha monoclonal antibody (TN3) and was used at a dilution of 1:30; the irrelevant control antiserum for LMP staining was an affinity-purified rabbit anti- β -galactosidase antiserum and was used at a dilution of 1:10; the irrelevant control antiserum for sIg staining was a rabbit preimmune serum and was used at a dilution of 1:100. In the histograms shown in Fig. 3, specific Bcl-2 staining obtained with 6C8 was staining that lay to the right of the vertical line; specific sIg staining was staining that lay above the horizontal line. In the histograms shown in Fig. 7, specific Bcl-2 staining obtained with 6C8 was staining that lay to the right of the vertical line; specific LMP staining was staining that was obtained with anti-LMP antiserum and that lay above the horizontal line.

RESULTS

Bcl-2 expression in EBV-infected primary B cells. A possible relationship between the level of Bcl-2 expression and EBV infection (and LMP expression) in human B cells was investigated by comparing the time course of expression of Bcl-2 with that of LMP in B95-8-infected, peripheral blood B cells. Fractionated lymphocytes (80% sIg⁺) were infected with a supernatant from B95-8 cells for 2 h and then plated on growth medium for the duration of the experiment. Infected cells were harvested at various times and assayed for the expression of LMP and Bcl-2 by Western blot analysis. As shown in Fig. 1, infection of primary B cells with the B95-8 strain of EBV resulted in a time-dependent increase in the expression of LMP. LMP was detected by day 5 after infection, and its level increased dramatically between days 5 and 7. We also observed that the truncated "lytic" form of LMP was easily detected in infected cells at the earliest times assayed and remained detectable throughout the course of the experiment (day 3; Fig. 1A). This form of LMP was detectable in extracts from cells exposed to virus for only 1 h and appeared to be carried into the infected cells by the EBV virion (data not shown). The expression of Bcl-2 protein in infected versus uninfected B cells was analyzed by Western blot analysis with an anti-human Bcl-2 monoclonal antibody (Fig. 1B). Uninfected B cells expressed high levels of Bcl-2 (relative to the EBV-negative BL cell line DG75; Fig. 1B, compare lane 0 with lane DG75). We found that sIg-negative leukocytes, isolated and assayed throughout the purification of sIg⁺ cells, expressed levels of Bcl-2 similar to those expressed by sIg⁺ cells (data not shown). Cells infected with EBV and plated for 7 days expressed at most 1.8 times more Bcl-2 protein than did uninfected cells (this number ranged from 1.0 to 1.8 times, depending on the experiment). The expression of Bcl-2 did not change significantly between days 3 and 5 of infection, whereas LMP expression was observed to increase dramatically over the same period. During this period, the volume of each infected cell more than doubled, possibly causing or contributing to the slight increase in Bcl-2 expression. The course of the infection was monitored by [³H]thymidine

FIG. 1. Western blot analysis of LMP and Bcl-2 in EBV-infected primary B cells. Extracts prepared from 10^5 uninfected (day 0 postinfection) or infected (days 3 to 7 postinfection) B cells were fractionated by SDS-PAGE, transferred to nitrocellulose, and stained with an affinity-purified anti-LMP antiserum (A) or an anti-human Bcl-2 monoclonal antibody (B) as described in Materials and Methods. The days postinfection are indicated above the corresponding lanes. BALB/288, extract from an LMP-transformed BALB/c 3T3 cell line included as a marker for LMP; DG75, extract from an EBV-negative BL cell line included as a marker for Bcl-2 (10^5 cells). M, molecular mass markers (described in Materials and Methods). The arrows indicate the 62-kDa LMP protein and the 25-kDa Bcl-2 protein.

uptake, and as shown in Fig. 2, infected cells incorporated [³H]thymidine as early as 4 days after exposure to the virus (this was the earliest time tested). Fifty percent of the cells were infected, as shown by EBNA expression assayed 5 days after exposure to the virus (data not shown). In parallel

FIG. 2. Response of B cells to infection with EBV. The data in Fig. 1 were quantified by laser densitometry and plotted in arbitrary units relative to day 0 postinfection. The incorporation of [³H]thymidine is shown as the fold increase relative to incorporation on day 0; on day 0, cells incorporated 1,000 cpm/ 5×10^5 cells.

FIG. 3. The expression of Bcl-2 is unaffected by infection with EBV. sIg expression and Bcl-2 expression were measured simultaneously in permeabilized cells with a rabbit anti-human immunoglobulin antiserum and a hamster anti-human Bcl-2 monoclonal antibody (6C8) as described in Materials and Methods. Two-dimensional histograms representing the log fluorescence intensity of sIg staining versus the log fluorescence intensity of Bcl-2 staining are shown for cells stained before (A) and 6 days after (B) infection with EBV. The volume and shape of infected cells differed from those of uninfected cells and contributed to the larger, more diffuse distribution seen in panel B. The positions of the horizontal and vertical lines delineating specific versus nonspecific staining were established from histograms of cells stained with the appropriate irrelevant control antibodies (see Materials and Methods). Specific Bcl-2 staining obtained with 6C8 was staining that lay to the right of the vertical line; specific sIg staining was staining that lay above the horizontal line. In panel A, 22.9% of the stained cells were in the upper left quadrant, 60.8% were in the upper right quadrant, 16% were in the lower left quadrant, and 0.4% were in the lower right quadrant. In panel B, 11.0% of the stained cells were in the upper left quadrant, 35.8% were in the upper right quadrant, 44.9% were in the lower left quadrant, and 8.3% were in the lower right quadrant. On day 6, 50% of the cells were sIg⁺ and 50% of the cells were EBNA positive (infected). It is likely that all the sIg⁺ cells were infected. (C) Western blot of extracts (5×10^4 cells) of the same populations of cells as shown in panels A and B stained with 6C8. Lanes: 1, uninfected cells; 2, infected cells.

experiments, the expression of Bcl-2 in infected cells was independent of the multiplicity of infection used; cells infected with concentrations of virus ranging from that in a viral supernatant diluted 1:10 to that in the same viral supernatant concentrated 10-fold expressed LMP dose dependently and did not differ in their expression of Bcl-2 on each day (2 to 6 days postinfection) tested (data not shown). In addition, the time course of expression of Bcl-2 was independent of that of LMP. LMP was detected in infected cells as early as 2 days postinfection to as late as 5 days postinfection, depending on the multiplicity of infection used in the experiment; however, Bcl-2 expression did not vary temporally from experiment to experiment (data not shown).

The expression of Bcl-2 in sIg⁺ B cells at day 0 (uninfected cells) and 6 days after exposure to the virus was also analyzed at the single-cell level by double immunofluores-

FIG. 4. Expression of Bcl-2 in primary B cells and recently immortalized B-cell clones. Extracts prepared from 10^5 cells were fractionated by SDS-PAGE, transferred to nitrocellulose, and stained with an anti-human Bcl-2 monoclonal antibody as described in Materials and Methods. B-cells, uninfected primary B cells at day 0; B-cells; T=4d, primary B cells grown in B95-8 viral supernatant for 4 days; immort. B-cell clones, EBV-immortalized (with the B95-8 strain of EBV) B-cell clones picked from agarose and grown for 20 generations. M, molecular mass markers. The arrow indicates the 25-kDa Bcl-2 protein.

cence staining for Bcl-2 and sIg and then by flow cytometry (Fig. 3). The distribution and level of expression of Bcl-2 were similar in the two populations. Of the sIg⁺ cells at day 0, 78% were Bcl-2 positive; of the sIg⁺ cells at day 6, 76% were Bcl-2 positive. In particular, we detected no population of cells that at day 0 expressed low levels of sIg and high levels of Bcl-2 (Fig. 3A, lower right quadrant) and that by day 6 could have evolved to express low levels of sIg and low levels of Bcl-2. Had such a population been detected, it might have masked an induced increase in the expression of Bcl-2 in EBV-infected sIg⁺ B cells. The wide distribution of sIg-negative, Bcl-2-negative cells by day 6 is likely to reflect metabolically inactive, uninfected cells destined to die in culture. No substantial difference in the expression of Bcl-2 in the uninfected and infected cells was detected by Western blotting, as shown in Fig. 3C. In addition, Western blot analysis indicated that the level of Bcl-2 expression was not significantly different in uninfected cells, cells infected for 4 days, and infected cells (clonal populations) propagated for 20 generations (Fig. 4); Bcl-2 levels in these cell populations were indistinguishable from Bcl-2 levels in EBV-positive lymphoblastoid cell lines (LCLs) established in vitro (data not shown). We found that the levels of LMP expressed (as determined by Western blot analysis) differed dramatically in these populations; recently immortalized clones (grown for 20 generations) expressed levels of LMP identical to the levels expressed in LCLs, whereas B cells infected for 4 days with EBV expressed approximately 10 to 20% as much LMP as did LCLs (data not shown). These results support the conclusion drawn from the results of Fig. 1 and 3 that the expression of LMP in human B cells does not correlate with the expression of Bcl-2, nor does infection with EBV detectably alter the expression of Bcl-2 in primary human B cells.

Bcl-2 protein is undetectable in LMP-transformed BALB/c 3T3 cells. For determination of whether Bcl-2 protein contributes to the LMP-mediated induction of the anchorage-independent growth of BALB/c 3T3 cells, the levels of Bcl-2 protein were assayed in various derivatives of these cells. BALB/c 3T3 cells transfected with LMP expression vectors can form anchorage-independent colonies in soft agarose (3, 4, 10). The expression of LMP and the expression of Bcl-2 in

FIG. 5. Expression of Bcl-2 in BALB/c 3T3 cells transformed by LMP. BALB/c 3T3 cell lines transformed by LMP or expressing nonfunctional (NΔ43) or partially functional (NΔ9) LMP mutants were analyzed by Western blot analysis as described in Materials and Methods. (A) BALB/c 3T3 cell lines (3×10^4 cells per lane) stained for LMP with an affinity-purified anti-LMP antiserum. (B) BALB/c 3T3 cell lines (10^5 cells per lane) stained for Bcl-2 with an anti-mouse Bcl-2 monoclonal antibody (3F11). BALB/c, extract from the parental cell line; LMP, extracts from LMP-transformed cell lines; NΔ43, extract from an NΔ43-expressing cell line; NΔ9, extract from an NΔ9-expressing cell line; FL512, extract from a murine pro-B-cell line included as a marker for Bcl-2. M, molecular mass markers (see Materials and Methods). Horizontal bars, 80- and 46-kDa markers. The bands migrating faster than LMP and mutant LMP proteins were degradation products derived from these proteins, were inconsistently detected, and were generated during cell lysis or in cells (2).

cell lines derived from LMP-transformed, anchorage-independent colonies and cell lines stably expressing nonfunctional (NΔ43) or impaired (NΔ9) (3, 4, 10) LMP mutants were compared. Cell extracts were assayed by Western blot analysis with an affinity-purified anti-LMP antiserum (Fig. 5A) or an anti-mouse Bcl-2 monoclonal antibody (Fig. 5B). The cell lines expressed various amounts of wild-type or mutant LMP (Fig. 5A); neither parental BALB/c 3T3 cells nor derived cell lines expressed Bcl-2 detectably (Fig. 5B). FL5.12 is a Bcl-2-positive murine pro-B-cell line included as a positive control. That the Bcl-2 protein was undetectable in these endothelial cells transformed by LMP indicates that Bcl-2 expression cannot be correlated with the transformed phenotype.

Expression of LMP at high levels does not correlate with changes in the expression of Bcl-2 in lymphoblastoid cells. The effects of expression of LMP at high levels on Bcl-2 in HH514 cells were studied to determine whether LMP-induced cytotoxicity directly or inversely correlates with the expression of Bcl-2 in these clonal derivatives of BL cells. HH514 cells harbor a nonimmortalizing strain of EBV (21). When wild-type LMP or transforming mutant LMP is expressed at high levels in a variety of cells, including HH514, the cells are killed (10). This cytotoxicity can be measured

FIG. 6. The expression of LMP at high levels in HH514 cells does not detectably induce Bcl-2. HH514 cells transfected with the indicated expression vectors (Mock, no DNA; VC, vector control; LMP, wild-type LMP; NΔ43, nonfunctional LMP mutant; CΔ55, functional LMP mutant) were analyzed 48 h posttransfection by Western blotting and stained for LMP (A) or Bcl-2 (B) as described in Materials and Methods. Unless indicated otherwise, all lanes contained the equivalent of 5×10^4 cells. B95-8, extract from the LMP-expressing EBV-positive marmoset cell line B95-8; DG75, extract (10^5 cells) from the EBV-negative BL cell line DG75. M, molecular mass markers. The bands migrating faster than LMP and mutant LMP proteins were degradation products derived from these proteins, were inconsistently detected, and were generated during cell lysis or in cells (2).

by introducing an efficient expression vector for LMP containing a drug resistance gene or the vector with the resistance gene alone into cells, selecting for the resistance gene, and enumerating the surviving cells. Only 0.1 to 1% of HH514 cells transfected with LMP and the resistance gene survive relative to those transfected with the resistance gene alone (10). We asked whether the expression of Bcl-2 correlates with the expression of LMP at high levels either before or after the selection of recipient cells on hygromycin. Vectors carrying wild-type or mutant LMP expressed from the human CMV immediate-early promoter and enhancer, the EBV origin of plasmid replication, and the gene for hygromycin phosphotransferase were introduced into HH514 cells by electroporation, assayed for the expression of LMP and Bcl-2 48 h later, or plated at limiting dilutions on hygromycin. Figure 6 shows a Western blot analysis of LMP and Bcl-2 expression in transiently transfected HH514 cells. Although only 10% of these cells took up and expressed DNA (data not shown), the expression of LMP, nonfunctional mutant NΔ43, and functional mutant CΔ55 was easily detectable (Fig. 6A). A B95-8 cell extract served as a positive control for the expression of LMP (see Materials and Methods). An analysis of the same cells for Bcl-2 showed that the expression of LMP had no effect on Bcl-2 levels (Fig. 6B). Cells transfected with the parental vector expressed levels of Bcl-2 similar to those expressed by cells transfected with wild-type or mutant LMP.

TABLE 1. Cytotoxic expression of LMP at high levels in HH514 cells^a

Plasmid	No. of Hyg ^r wells/six wells plated at the following initial plating density (cells/ml):			
	10 ⁵	10 ⁴	10 ³	10 ²
No DNA	0	0	0	0
CMV-hyg	6	6	6	5
CMV-LMP	6	0	0	0
CMV-CA55	6	0	0	0
CMV-NΔ43	6	6	2	0

^a HH514 cells were electroporated with the following vectors: no DNA, the LMP gene (CMV-LMP), derivatives of the LMP gene (CMV-CA55 and CMV-NΔ43) expressed from the human CMV immediate-early promoter and enhancer, or the parental vector (CMV-hyg); these expression vectors contain the origin of plasmid replication of EBV and a selectable marker (the gene encoding hygromycin phosphotransferase). Two days after electroporation, cells were assayed for the expression of LMP and Bcl-2 (Fig. 6) or plated at the indicated densities on 300 μg of hygromycin per ml (volumes of 3 ml for 10⁵ cells per ml and 1 ml for all other densities). After 3 weeks of growth on hygromycin, the number of hygromycin-resistant wells per six wells plated at each density was scored. Approximately 1 cell in 10⁵ cells for CMV-LMP and CMV-CA55 gave rise to hyg^r colonies, compared with 1 cell in 10³ cells for CMV-NΔ43.

An effect of LMP on Bcl-2 expression may remain undetected because of the relative inefficiency of DNA uptake; therefore, transfected populations were also assayed for both LMP and Bcl-2 by two-color staining and flow cytometry. Bcl-2 expression in LMP-positive cells was compared with Bcl-2 expression in NΔ43 (nonfunctional)-positive cells. The NΔ43 mutant has been shown to be nonfunctional when assayed for the transformation of BALB/c 3T3 cells (4) and for the induction of cytotoxicity following high-level expression in LCL GG68 (10); importantly, the data in Table 1 demonstrate that the NΔ43 mutant was nonfunctional in HH514 cells as well. Thus, on the basis of these assays, the NΔ43 mutant was nonfunctional relative to wild-type LMP (the NΔ43 mutant has been shown to retain partial function when assayed in BL cell line BJAB [15]). We were unable to detect any difference in Bcl-2 expression in cells expressing wild-type versus mutant LMP (Fig. 7). Thus, we conclude that the expression of LMP in HH514 cells neither induces nor suppresses the expression of Bcl-2. We were also unable to detect any effects of the expression of LMP on Bcl-2 in EBV-negative BL cell line DG75 (data not shown).

Two days following electroporation, cells were plated at limiting dilutions on hygromycin. The number of drug-resistant colonies arising following selection for 3 weeks on hygromycin is shown in Table 1. Cells transfected with nonfunctional mutant NΔ43 gave rise to 100-fold more colonies than did cells transfected with either the wild type or functional mutant CA55. Surviving populations were harvested and assayed for LMP and Bcl-2 by Western blot analysis. The expression of LMP in six drug-resistant pools of LMP and CA55 transfectants and seven drug-resistant pools of NΔ43 transfectants is shown in Fig. 8. Each Western blot included for comparison an extract from an equivalent number of cells from the initially transfected population (Fig. 8, trans.). Populations transfected with the wild type or functional mutant CA55 expressed less LMP protein or CA55 protein after selection than before selection (only 10% of the population expressed LMP before selection). Populations transfected with nonfunctional mutant NΔ43 expressed more NΔ43 protein after selection than before. These results are characteristic of the cytotoxic effect of LMP (10). The corresponding analysis of Bcl-2

FIG. 7. LMP-positive HH514 cells do not express more Bcl-2 protein than NΔ43-positive HH514 cells. Cells were electroporated with LMP (A) or NΔ43 (B) expression vectors and analyzed 2 days following electroporation for LMP and Bcl-2 by two-color immunofluorescence staining as described in Materials and Methods. Two-dimensional histograms representing the log fluorescence intensity of LMP (or NΔ43) staining versus the log fluorescence intensity of Bcl-2 staining are shown. The positions of the horizontal and vertical lines delineating specific versus nonspecific staining were established from histograms of cells stained with the appropriate irrelevant control antibodies (see Materials and Methods). Specific Bcl-2 staining obtained with 6C8 was staining that lay to the right of the vertical line; specific LMP staining was staining that was obtained with anti-LMP antiserum and that lay above the horizontal line. Cells that were LMP positive did not express significantly higher levels of Bcl-2 than cells that were NΔ43 positive.

levels in the drug-resistant populations is shown in Fig. 9. Of the cells transfected with functional LMP, four of six LMP-transfected populations and two of six CA55-transfected populations expressed elevated levels of Bcl-2. None of the NΔ43-transfected populations (nonfunctional LMP) expressed Bcl-2 at levels in excess of those observed in cells before selection or in cells transfected with the parental vector. Thus, while Bcl-2 expression does not correlate with a high level of LMP expression before selection on hygromycin, a correlation between functional LMP and Bcl-2 is observed after selection for the expression of hygromycin. One interpretation of the data from the transient expression experiments (Fig. 6 and 7) and from the experiments with the selected populations (Fig. 8 and 9) is that although LMP does not induce the expression of Bcl-2 in HH514 cells, its cytotoxicity is mitigated in cells that spontaneously express Bcl-2 efficiently.

Infection of BJAB cells by EBV induces the expression of Bcl-2. Given the results obtained with HH514 cells (Fig. 6 to 9) and the increased expression of Bcl-2 in BJAB cells after selected expression of LMP (11), we sought to determine whether the expression of LMP induces Bcl-2 in BJAB cells. First, we infected BJAB cells with EBV to introduce LMP into them efficiently. Figure 10 shows the time course of expression of LMP and Bcl-2 in B95-8-infected BJAB cells, as determined by Western blot analysis. LMP was detectable as early as 1 day postinfection. Although not detectable in Fig. 10, the lytic form of LMP also has been detected in BJAB cells early after infection (within 1 h postinfection) (data not shown); the ability to detect this form of LMP in infected BJAB cells appears to be dependent upon the conditions of infection and subsequent analysis (data not shown). The expression of Bcl-2 was undetectable in uninfected BJAB cells (day 0) and increased in a time-dependent manner, with a time course that correlated with the time

FIG. 8. Expression of LMP and mutant LMP in hygromycin-resistant HH514 populations. HH514 cells electroporated with the expression vectors described in the legend to Fig. 6 were plated at limiting dilutions on hygromycin 2 days postelectroporation. Drug-resistant populations were assayed for LMP expression by Western blot analysis as described in Materials and Methods. (A) Lanes 1 to 6, extracts from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the LMP expression vector; trans., extract taken 2 days after transfection (prior to selection) from HH514 cells (5×10^4 cells) transfected with the LMP expression vector; VC, extract from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the parental vector. M, molecular mass markers. (B) Lanes 1 to 6, extracts from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the CA55 expression vector; trans., extract taken 2 days after transfection (prior to selection) from HH514 cells (5×10^4 cells) transfected with the CA55 expression vector; VC, extract from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the parental vector. M, molecular mass markers. (C) Lanes 1 to 7, extracts from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the NA43 expression vector; trans., extract taken 2 days after transfection (prior to selection) from HH514 cells (5×10^4 cells) transfected with the NA43 expression vector; VC, extract from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the parental vector. M, molecular mass markers. The bands migrating faster than LMP and mutant LMP proteins were degradation products derived from these proteins, were inconsistently detected, and were generated during cell lysis or in cells (2).

course of the expression of LMP. Infection of BJAB cells with the P3HR1 strain of EBV (lacking the EBNA-2 gene) resulted in a much lower level of expression of LMP than that seen following infection with the B95-8 strain and a small but detectable level of induction of Bcl-2 (data not shown). These results are consistent with the work of Henderson et al. (11) and indicate that LMP may directly affect the expression of Bcl-2 in BJAB cells.

For demonstration that the induction of Bcl-2 by EBV infection of BJAB cells results from the expression of LMP, LMP or mutant LMP expression vectors were introduced into BJAB cells, and cells surviving the electroporation were analyzed for the expression of LMP and Bcl-2 by Western blot analysis 2 days after electroporation. Populations of cells transfected with the LMP gene expressed detectable

FIG. 9. Expression of Bcl-2 in hygromycin-resistant HH514 populations correlates with LMP-induced cytotoxicity. Drug-resistant populations (described in the legend to Fig. 8) were assayed for Bcl-2 expression by Western blot analysis as described in Materials and Methods. (A) Lanes 1 to 6, extracts from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the LMP expression vector; trans., extract taken 2 days after transfection (prior to selection) from HH514 cells (5×10^4 cells) transfected with the LMP expression vector; VC#1 and VC#2, extracts from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the parental vector. M, molecular mass markers. (B) Lanes 1 to 6, extracts from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the CA55 expression vector; trans., extract taken 2 days after transfection (prior to selection) from HH514 cells (5×10^4 cells) transfected with the CA55 expression vector; VC#1 and VC#2, extracts from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the parental vector. M, molecular mass markers. (C) Lanes 1 to 7, extracts from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the NA43 expression vector; trans., extract taken 2 days after transfection (prior to selection) from HH514 cells (5×10^4 cells) transfected with the NA43 expression vector; VC#1, extract from Hyg^r pools of HH514 cells (5×10^4 cells) transfected with the parental vector. M, molecular mass markers. Some Hyg^r populations expressing LMP or CA55 expressed elevated levels of Bcl-2 relative to those expressed in vector controls; Hyg^r populations expressing NA43 did not.

Bcl-2, and populations transfected with the mutant LMP gene, encoding a nonfunctional LMP derivative, did not (Fig. 11). In addition, cells transfected with an EBNA-2 expression vector also expressed detectable Bcl-2, while cells transfected with an EBNA-2 gene encoding a carboxy terminally truncated EBNA-2 protein (nonfunctional [9]) did not. The coexpression of the LMP and EBNA-2 genes did not result in significantly higher levels of Bcl-2 expression than did the expression of either gene alone.

DISCUSSION

The stable expression of LMP in some BL cell lines correlates with an increased expression of Bcl-2. We asked whether cellular phenotypes induced by or associated with LMP correlate with alterations in the levels of Bcl-2 expres-

FIG. 10. The time course of Bcl-2 expression in EBV-infected BJAB cells correlates with the time course of LMP expression. Extracts from 5×10^4 uninfected (day 0 postinfection) or EBV-infected (days 1 to 6 postinfection) BJAB cells were fractionated by SDS-PAGE, transferred to nitrocellulose, and stained with an anti-LMP antiserum (A) or an anti-human Bcl-2 monoclonal antibody (B) as described in Materials and Methods. The days postinfection are indicated above the corresponding lanes. B95-8, extracts from the LMP-positive B95-8 cell lines included as a marker for LMP; 721, extracts from an EBV-positive LCL included as a marker for Bcl-2. M, molecular mass markers. The arrows indicate the LMP and Bcl-2 proteins.

sion. Fractionated B cells purified from peripheral blood expressed readily detectable levels of Bcl-2; infection of these cells with EBV had no significant effect on Bcl-2 expression assayed 2 to 7 days after infection (Fig. 2) or 20 cell generations after infection (Fig. 4). It is formally possible that the target for EBV infection is a subpopulation of sIg⁺ B cells that express low levels of Bcl-2, that EBV infection does induce Bcl-2 in these cells, and that this infected population replaces the uninfected population (Bcl-2 positive) that does not proliferate. We addressed this possibility by staining uninfected and infected cells (day 6 postinfection) for both Bcl-2 and sIg and then measuring (by flow cytometry) the distribution of Bcl-2 in cells that were sIg⁺ (Fig. 3). We found (i) no difference in Bcl-2 expression in sIg⁺ cells before or after infection with EBV and (ii) a range of specific Bcl-2 staining in uninfected or infected cells that varied less than 10-fold, with little detectable heterogeneity in the expression of Bcl-2. In other words, we found no evidence for distinct subpopulations of uninfected cells that expressed lower or higher levels of Bcl-2 than did the bulk population. The results shown in Fig. 4 demonstrate that the expression of Bcl-2 in EBV-infected B cells is independent of the length of time following infection; Bcl-2 levels are high at day 4 postinfection and do not change thereafter, whether assayed 20 cell generations later or assayed in established LCLs. It remains possible for LMP functions not to induce, but rather to maintain, the high constitutive level of Bcl-2 found to be expressed in uninfected B cells isolated from peripheral blood. However, the levels of Bcl-2 expressed in populations of fractionated sIg⁺ B cells did not detectably decrease over the course of 1 week following isolation from peripheral blood (data not shown). We conclude that infec-

FIG. 11. The expression of LMP in transiently transfected BJAB cells induces the expression of Bcl-2. BJAB cells transfected with expression vectors (described in Materials and Methods) encoding the indicated viral proteins (LMP, wild-type LMP protein; LMP*, $\Delta 25$ -132 deletion mutant of LMP [nonfunctional] [10]; LMP + EBNA-2*, wild-type LMP and carboxy terminally truncated EBNA-2 [nonfunctional] [9]; LMP + EBNA-2, wild-type LMP and wild-type EBNA-2; EBNA-2*, carboxy terminally truncated EBNA-2 [nonfunctional]; EBNA-2, wild-type EBNA-2; no DNA, mock transfection; LCL, extract from a human peripheral B-cell clone recently immortalized with the B95-8 strain of EBV) were analyzed 48 h postelectroporation by Western blotting and stained for LMP (A) or Bcl-2 (B) as described in Materials and Methods. All lanes contained the equivalent of 10^5 cells. M, molecular mass markers. The arrows indicate the LMP, mutant LMP, and Bcl-2 proteins.

tion of primary B cells by EBV yields readily detectable expression of LMP that does not correlate with the expression of Bcl-2; therefore, modulation of the expression of Bcl-2 by LMP is unlikely to contribute to the immortalization of B cells by EBV.

For determination of whether modified expression of Bcl-2 was involved in the transformation of BALB/c 3T3 cells by LMP, the Bcl-2 levels in LMP-transformed BALB/c 3T3 cells were compared with the Bcl-2 levels in cell lines expressing LMP mutants (Fig. 5). Bcl-2 protein was undetectable in the parental BALB/c 3T3 cell line as well as in its LMP- or LMP mutant-expressing derivatives. Either LMP does not induce the expression of Bcl-2 in these cells or it does so at a level below that detectable in our experiments. The latter possibility seems less likely given the ease with which Bcl-2 is detected in murine and lymphoid cells, in which it is known to function.

The expression of LMP at high levels in many cell lines is cytotoxic, and the cytotoxic activity of mutants of LMP correlates with their transforming activity (10). There was no correlation between the expression of LMP and the expression of Bcl-2 in HH514 cells transiently transfected with LMP expression vectors (Fig. 6). HH514 cells that survived

selection after having been transfected with efficient expression vectors for LMP or its functional mutants expressed reduced levels of LMP or its derivatives relative to the levels expressed 48 h postelectroporation (Fig. 8 and Table 1). Those that were transfected with nonfunctional mutants survived 100 times more frequently and expressed high levels of mutant proteins. The expression of Bcl-2 varied in the hygromycin-resistant populations transfected with LMP or the functional CA55 mutant and was consistently low in populations transfected with the nonfunctional NA43 mutant (Fig. 9). Thus, the expression of LMP had no direct effect on the levels of Bcl-2 in HH514 cells (Fig. 6), but stable LMP transfectants often expressed elevated levels of Bcl-2 (Fig. 8 and 9). One interpretation of these results consistent with the documented survival function of Bcl-2 is that LMP-induced cytotoxicity selects for cells in the original population that already express high levels of Bcl-2. It is not clear why 100% of the surviving hygromycin-resistant populations (LMP and CA55 transfectants) did not express elevated levels of Bcl-2. It is possible that the LMP or CA55 gene expressed in a fraction of the Hyg^r populations was altered, giving rise to a protein that was nonfunctional but migrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a manner indistinguishable from that of the wild type; the cytotoxic effect of LMP often results in populations of cells expressing deleted forms of LMP detectable by Western blot analysis (10).

The ability of LMP to alter the levels of cellular proteins upon expression in EBV-negative BL cell lines varies with the host cell (31, 32). This selectivity indicates that the effects of LMP are likely to depend on the differentiated state of the cell. That infection of EBV-negative BL cell line BJAB with EBV resulted in a dramatic induction of Bcl-2 (Fig. 9) underscores the need for caution when such established BL cell lines are used as models for studying the EBV-mediated immortalization of B cells.

The presence of EBV in certain BL cell lines correlates with the expression of Bcl-2 and with the ability of these cells to proliferate in the presence of low levels of serum and at low cell densities (8, 22). The expression of Bcl-2 in EBV-positive LCLs enhances their growth in the presence of low levels of serum and at low cell densities (26). We found that the expression of LMP induced Bcl-2 in EBV-negative BL cell line BJAB within 48 h after transfection of the LMP gene (Fig. 11). However, LMP did not detectably affect the expression of Bcl-2 in EBV-positive BL cell line HH514 or in primary B cells. It is possible that the pathway(s) leading to the upregulation of Bcl-2 differs among various cell types or that LMP has no further effect on Bcl-2 expression in cells that express high levels of Bcl-2 constitutively (e.g., primary B cells). The expression of LMP in transformed BALB/c 3T3 cells does not detectably affect the expression of Bcl-2, indicating that this LMP-induced transformed phenotype is unlikely to require the expression of Bcl-2.

Although the biochemical activity of LMP is not understood, it is reasonable to propose that early events in LMP signaling are common among cell lines in which LMP variously affects the expression of cell proteins but that the ultimate downstream effects are dependent upon the cells into which LMP is introduced. The identification of cellular proteins directly regulated by LMP provides a means by which LMP signaling can begin to be dissected.

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