JOURNAL OF VIROLOGY, Nov. 1988, p. 4173–4184
0022-538X/88/114173-12$02.00/0

Vol. 62, No. 11

Epstein-Barr Virus Latent Infection Membrane Protein Alters the Human B-Lymphocyte Phenotype: Deletion of the Amino Terminus Abolishes Activity

DAVID WANG,[1] DAVID LIEBOWITZ,[1] FRED WANG,[1] CHRIS GREGORY,[2] ALAN RICKINSON,[2] RICHARD LARSON,[3] TIMOTHY SPRINGER,[3] AND ELLIOTT KIEFF[1]*

Departments of Medicine and Microbiology and Molecular Genetics[1] and Department of Pathology,[3] Harvard University Medical School, 75 Francis Street, Boston, Massachusetts 02115, and Cancer Research Campaign Laboratories, Department of Cancer Studies, University of Birmingham, Birmingham, England B15 2TJ[2]

Received 18 April 1988/Accepted 5 August 1988

A latent infection membrane protein (LMP) encoded by the Epstein-Barr virus (EBV) genome in latently infected, growth-transformed lymphocytes alters the phenotype of a human EBV-negative B-lymphoma cell line (Louckes) when introduced by gene transfer. These LMP-expressing cells exhibit increased homotypic adhesion due to increased expression of the adhesion molecules LFA-1 and ICAM-1. Increased homotypic adhesion could foster B-cell growth by facilitating autocrine growth factor effects. LFA-3 expression is also induced. The induction of LFA-3 and ICAM-1 results in increased heterotypic adhesion to T lymphocytes. This could result in more effective T-cell immune surveillance. Since LMP is expressed in EBV-transformed lymphocytes and has been demonstrated to transform rodent fibroblasts in vitro, a wide range of possible effects on B-lymphoma cell growth were assayed. In the Louckes B-lymphoma cell line, EBV LMP causes increased cell size, acid production, plasma membrane ruffling, and villous projections. Although cell proliferation rate was not greatly affected, the steady-state intracellular free calcium level, transforming growth factor beta responsiveness, and expression of the lymphocyte activation markers (CD23 and transferrin receptor) were increased. Thus, LMP appears to be a mediator of EBV effects on B-cell transformation. In transfected lymphoma cells, LMP localizes to patches at the cell periphery and associates with the cytoskeleton as it does in EBV-transformed B lymphocytes or in rodent fibroblasts. A partially deleted form of LMP (D1LMP) does not aggregate in patches or associate with the cytoskeleton and had little effect on B-cell growth. Thus, cytoskeletal association may be integral to LMP activity.

Epstein-Barr Virus (EBV) is a prevalent human herpesvirus closely associated with the etiology of African Burkitt's lymphoma and nasopharyngeal cancer (for reviews, see references 9 and 41). EBV infection of normal B lymphocytes results not in virus replication but in rapid and efficient cell growth (47). This is evident from the rapid increase in cell size, increased expression of several B-cell activation markers, increased RNA and DNA synthesis, and increased immunoglobulin secretion soon after EBV infection (1, 17, 20, 31, 59, 60). In addition, EBV-infected proliferating lymphocytes are transformed to tumorigenicity, as indicated by the EBV-infected lymphocyte proliferative disorders which occur in immunosuppressed humans and in experimentally infected marmosets (53; for a review, see reference 7). The entire virus genome persists in infected, growth-transformed lymphocytes either as an episome or by integrating into cell DNA (36, 40). Despite the presence of the complete genome, which encodes over 50 proteins in productive virus infection, only a few genes, which encode several nuclear proteins and a membrane protein, are characteristically expressed in the latently infected, growth-transformed lymphocyte (for a review, see reference 9). These genes are being intensively investigated, since they are likely to be involved either in maintaining latent EBV infection or in its concomitant effects in inducing proliferative transformation.

The EBV latent infection membrane protein (LMP) is the only latent infection cycle-specific protein encoded by the R strand of the EBV genome (12, 21). At 60 copies per cell, it is the most abundant EBV mRNA in latent infection. From its DNA sequence, LMP is predicted to consist of a short hydrophilic amino terminus; six hydrophobic, alpha-helical, potential transmembrane domains; and a long hydrophilic acidic carboxy terminus. Live-cell protease cleavage experiments indicate a cytoplasmic orientation of the amino- and carboxy-terminal domains and an extrinsic plasma membrane orientation of the first outer reverse-turn domain (35). At least 30% of the LMP in EBV-transformed lymphocytes are in the plasma membrane. A substantial fraction localizes to a patch at the cell periphery, where it is closely associated with vimentin intermediate filaments (34). Shortly after being synthesized, LMP is phosphorylated (2, 34) and becomes tightly bound to the cell cytoskeleton (34). LMP is able to transform rodent fibroblasts to cause loss of contact inhibition, anchorage independence, and tumorigenicity in nude mice, providing evidence that LMP plays a key role in EBV-induced lymphoproliferation or transformation (63).

Many of the changes in cellular phenotype seen in EBV-induced growth transformation of normal B lymphocytes are reproduced by EBV infection of EBV-negative human Burkitt's lymphoma cell lines. The infected cells constitutively express the latent infection viral genes, grow in tight clumps, and exhibit increased surface expression of B-cell activation antigens (5, 51). Since EBV-negative Burkitt's lymphoma cells exhibit altered growth after EBV infection, they can be used to assay the effects of individual EBV genes.

Expression of each of the EBV-encoded latent-infection cycle nuclear proteins or LMP under the control of the

* Corresponding author.

Moloney murine leukemia virus (MoMuLV) promoter in EBV-negative Burkitt's lymphoma cells revealed an effect on B-cell growth activity only for the EBV nuclear protein 2 gene (65). The nuclear protein 2 gene specifically transactivated expression of the B-cell activation antigen CD23 and caused cells to grow in tight clumps. Surprisingly, in light of its transforming effects in continuous rodent fibroblasts, LMP expressed from the MoMuLV promoter produced no change in B-lymphoblast growth. However, while expression of the EBV nuclear protein genes in human B-lymphoma cells under the control of the MoMuLV promoter resulted in protein levels similar to those observed in latent EBV infection, LMP was expressed at a considerably lower level than that at which it is ordinarily expressed in latent infection. Impressed by the activity of LMP in rodent fibroblasts and by recent experiments which reveal a correlation between LMP expression level and rodent cell transformation parameters (64), we set out to investigate the hypothesis that LMP expression of an appropriate level would alter B-lymphocyte growth. We now report that under the control of the human metallothionein promoter, EBV LMP is expressed in EBV-negative Burkitt's lymphoma cells at a level similar to that at which it is expressed in EBV-infected B lymphocytes and that lymphocyte phenotype is affected. In parallel, we have investigated the effects of a naturally altered form of LMP (D1LMP). D1LMP lacks the amino terminus and first four transmembrane domains and is ordinarily expressed only in productive EBV infection (24).

MATERIALS AND METHODS

Cells. Louckes is an EBV-negative American Burkitt's lymphoma B-cell line. B95-8 is an EBV-infected marmoset cell line that is partially permissive of virus replication. IB4 is an EBV-infected cord blood B-lymphocyte line that is nonpermissive of virus replication. Cells were maintained in growth medium which consisted of RPMI 1640 supplemented with 10% fetal calf serum.

DNA transfection. Linearized plasmid DNA (10 μg) was electroporated into Louckes cells. Briefly, 5×10^6 log-phase growing cells were washed and suspended in 0.5 ml of cold phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+} in a 1-ml plastic cuvette lined with platinum plates. DNA (10 μg) was added for 5 min on ice, and an electric pulse of 2,000 V and 0.9 mA was delivered by an ISCO 494 power supply. The cells were held on ice for 10 min and then suspended in 15 ml of growth medium. Two days after the electroporation, the medium was replaced with guanine phosphoribosyltransferase selective medium with 0.75 μg of mycophenolic acid per ml. The cells were then counted and plated into 96-well plates at 1,000 or 2,000 cells and 0.25 ml of growth medium per well. Three to four weeks later, cell clones emerged in 1 of 10 wells. Clones were picked and expanded into lines and maintained in 0.5 μg of mycophenolic acid selective medium per ml.

Monoclonal antibodies. Monoclonal antibody S12 generated against the C-terminal portion of a LMP-β-galactosidase fusion protein was used to detect LMP or D1LMP (38). MHM6, from one of our laboratories (A.R.), and EBVCS 2, 4, and 5, from B. Sugden, Madison, Wisconsin, are monoclonal antibodies which react with the B-cell activation antigen CD23. Monoclonal antibody 60.3, from P. G. Beatty, Seattle, Washington, reacts with the common beta subunit of LFA-1, Mac-1, or p150,95 (3). Monoclonal antibodies against LFA-1 alpha (TS1/22 or MHM24), LFA-1 beta (MHM23), p150,95 (S-HCL-3), LFA-3 (TS2/9), and ICAM-1 (RR1/1) were from one of our laboratories (T.S.) or from A. J. McMichael, Nuffield Department of Medicine, Oxford University, Oxford, England. Monoclonal antibodies against transferrin receptor and interleukin-2 receptor were purchased from Interferon, Inc. (San Francisco, Calif.).

Immunoblot. Immediately after centrifugation, cell pellets were mixed with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis loading buffer. After being boiled for 5 min, equal amounts of protein (corresponding to 3×10^5 cells) were loaded into each lane of a 7.5% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose paper and visualized by Ponceau S staining. The nitrocellulose blot was blocked with 2.5% milk-PBS-Tween 20. The S12 antibody, rabbit anti-mouse antibody, and ^{125}I-labeled protein A were then used for immunostaining.

Cell surface labeling and immunoprecipitation. Cells were washed in PBS and then surface-iodinated with ^{125}I by using iodogen (Pierce Chemical Co.)-coated glass wells according to the procedures recommended by the manufacturers. After extensive washes, cells were lysed with 0.5% Nonidet P-40-PBS-iodoacetamide-phenylmethylsulfonyl fluoride and the lysate was passed over a G-50 column to remove unincorporated free iodine. Equal counts of ^{125}I-labeled protein were precipitated with the appropriate monoclonal antibody and with protein A-Sepharose.

Detergent extraction. Cells were extracted in nonionic detergent, and the proteins were analyzed as described previously (34).

Scanning electron microscopy. In normal medium, log-phase growing cells were incubated with poly-L-lysine-precoated cover slips overnight. The cover slips were rinsed with PBS and fixed in 2% glutaraldehyde-Collidine buffer at room temperature for 1 h. After being stained with osmic acid, the cells were dehydrated in a graded series of ethanol and amyl acetate and finally dried to the critical point with liquid nitrogen. Scanning electron microscopy was done with a Siemens EX instrument.

Immunofluorescence and flow cytofluorometry. Live-cell fluorescence staining was done by using saturating amounts of monoclonal antibodies and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin. Binding was quantitated by using a flow cytometer (FACS 4; Becton Dickinson and Co.). The results are expressed as mean fluorescence per volume ratio of 20,000 cells. For cell-cycle flow cytometry, one million log-phase growing cells were pelleted gently and suspended with vigorous pipetting in 0.25-ml of residual medium. One milliliter of propidium iodide solution (0.05 mg of propidium iodide per ml in 0.1% sodium citrate supplemented with 0.1% Triton X-100) was added and mixed gently, and the cells were analyzed after 15 min of incubation at 4°C (8). The fraction of cells in G_0-G_1, S, or G_2-M phase was estimated from analysis of area under the curve (14).

Intracellular free calcium measurement (46). Log-phase growing cells (about 5×10^5 cells) were placed in 2.5 ml of prewarmed RPMI medium lacking bicarbonate but supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) (pH 7.4) and 0.1% fetal calf serum. For fura-2AM loading, 6 μl of 1 mM fura-2AM in dimethyl sulfoxide-pheronic F127 was added to the 2.5-ml culture, which was then incubated for 1 h at 37°C in 5% CO_2. Cells were washed with prewarmed medium without fura-2AM for 30 to 60 min. The medium was replaced with prewarmed Hanks balanced salt solution, and intracellular

calcium was measured with a Toshiba spectrophotometer with an excitation of 340 or 380 nm and an emission of 510 nm. The absolute level of intracellular calcium was calculated from the emission ratio with 340 versus 380 nm excitation and plotted against a standard curve. The mean intracellular free calcium level of vector control Loukes cells was 108 ± 38 nM for 12 different experiments.

Phospholipid diacylglycerol and triglyceride analysis. The procedures used were adapted from previously reported methods (13, 25, 61, 67). One day after feeding, at 2×10^5 cells per ml, cells were either incubated with 30 μCi of $^{32}P_i$ per ml in phosphate-free RPMI medium supplemented with 1% of bovine serum albumin or 5% dialyzed fetal calf serum for 2 h, or incubated with 2 μCi of myo-[2-^{3}H]inositol in low inositol containing M199 supplemented with 5% fetal calf serum for 16 h. After being labeled, cells were spun down and extracted with 1.0 ml of $CHCl_3$-MeOH-12 M HCl (200:100:0.75 [vol/vol/vol]) at 0°C. After 187 μl of 0.6 M HCl was added to separate the two phases, the lower phase was removed and re-extracted twice more with 1.0 ml of $CHCl_3$-MeOH-0.6 M HCl (3:48:47 [vol/vol/vol]). The lower phase was dried under a stream of nitrogen, washed with 50 μl of CH_3Cl, and then dissolved in the proper volume of $CHCl_3$-MeOH-H_2O (75:25:2 [vol/vol/vol]). It was then loaded on the origins of silica gel plates. The genuine markers of phosphatidylcholine, phosphatidylinositol, and phosphatidylethanolamine were run side by side, and the plate was developed with $CHCl_3$-MeOH-methylamine (60:36:20 [vol/vol/vol]). For diacylglycerol measurements, 2.0×10^5 log-phase growing cells were labeled with 0.3 μCi of [^{14}C]arachidonic acid per ml at 37°C for 36 h. After the extraction (performed as described above), the organic phase was concentrated with Speedvac and loaded on a thin-layer chromatography plate. It was developed in a system of ether:hexane:acetic acid (70:30:1 [vol/vol/vol]). After autoradiography, the bends were scraped and counted at the appropriate channels.

Phosphatidyl inositol kinase and protein kinase C translocation. The procedures used were adapted from those reported previously (26, 57, 66).

Isolation of RNA and Northern (RNA) blotting. Cytoplasmic RNA was isolated from 2×10^8 cells by first washing the cells in PBS and then lysing the cells in 0.5% Nonidet P-40–10 mM Tris–10 mM NaCl–3 mM $MgCl_2$. Nuclei were pelleted at 500 × *g*. The supernatant was harvested, adjusted to 1% SDS and 0.2 M NaCl, extracted several times with phenol and chloroform, and ethanol precipitated. Cytoplasmic RNA was loaded in a 1% formaldehyde-agarose gel (10 μg per lane). The gel was transferred to Genescreen (Amersham Corp.) and hybridized as previously described to radioactive proves labeled with [^{32}P]dCTP by random priming (Amersham). The probes used were the CD23 cDNA (kindly provided by H. Kikutani and T. Kishimoto), the plasmid 3R1 containing 1.8 kilobases of the coding region for the alpha chain of LFA-1 (R. Larson and T. Springer, unpublished data), the plasmid 9.1.1 containing the cDNA of the beta chain of LFA-1 (32), and an actin probe containing a 284-base-pair fragment of the gamma actin gene (kindly donated by K. Robbins).

Conjugate formation assay. Conjugate formation between Loukes target cells and EBV-specific but HLA class I antigen-mismatched cytotoxic T cells was measured in a rapid in vitro assay described elsewhere (19). Such conjugation occurs within 5 min of effector-target cocentrifugation and is independent of antigen-specific (HLA class I-restricted) recognition (52). The assay was carried out either in culture medium alone or in medium containing saturating concentrations (usually a 1:1,000 dilution of ascitic fluid) of monoclonal antibodies to the adhesion molecules LFA-1, ICAM-1, and LFA-3. The percentage of effector cells forming conjugates was assessed by fluorescence-activated cell sorting analysis of 20,000 fluorescent events, the effector and target cells having been pre-labeled with fluorescein diacetate (green) and hydroethidine (red), respectively.

RESULTS

LMP (but not D1LMP) expression induces B-lymphoma cell aggregation. LMP or D1LMP, each under control of the human metallothionein type 2 promoter, were linked to a positive selection vector and transfected into an EBV-negative Burkitt's lymphoma line (Loukes) (65). Twelve transfected clones of each type and vector-transfected control clones were selected. Vector-transfected and D1LMP-transfected clones grew as suspensions of single cells similar to untransfected Loukes cells (Fig. 1B, L-V or L-D1LMP1). In contrast, some LMP-transfected clones grew as tight clumps (e.g., Fig. 1B, L-LMP6 or 8), while others grew partially clumped (e.g., Fig. 1B, L-LMP4) or were indistinguishable from vector-transfected controls (e.g., Fig. 1B, L-LMP7). The LMP-transfected clones also varied in LMP expression (Fig. 1A). The LMP expression level correlated with the extent of cell aggregation. Clones expressing high levels of LMP (L-LMP2, 5, 6, or 8) grew in large dense clumps. Clones expressing intermediate levels of LMP (L-LMP1 or 4) grew partially in clumps. LMP-transfected clones in which LMP was not detectable (L-LMP3 or 7) grew the same as D1LMP or vector-transfected controls.

Most D1LMP clones expressed an intermediate to high level of the truncated D1LMP (42; Fig. 1A, L-D1LMP1 or LD1LMP2; see Fig. 9, D1LMP versus LMP8 [note that the number of D1LMP1 or D1LMP2 cells in Fig. 1A is less than that in other lanes]), which in the case of LMP correlated with partial or full clumping. However, the D1LMP-expressing cells did not grow in clumps and continued to grow as dispersed cells even when a higher level of D1LMP expression was induced by growth in medium containing 75 μM zinc sulfate.

LMP affects lymphocyte adhesion. In phase, dark-field, or fluorescence microscopy, LMP-expressing cells were surrounded by prominent membranous projections, while D1LMP-expressing or control vector-transfected cells were not. In a scanning electron microscope more frequent, broader, and longer ruffled membrane projections of LMP-expressing cells were evident in comparison with control vector-transfected or D1LMP-expressing cells (Fig. 2). Multicellular clumps joined by long membranous ruffles were only seen in the LMP-expressing cell cultures (Fig. 2D). The long membranous ruffles appeared to extend from one cell to another, with tapering toward the midpoint but without discernible interruptions.

In view of these findings and the observation that LMP-expressing cells grow in clumps, we examined these cells for altered expression of surface proteins known to be involved in cell adhesion. LFA-1 (gp 180,95) is a member of a family of leukocyte surface proteins with a common beta chain and distinct alpha chains. Other family members include MAC-1 (gp170,95) and p150,95 (56). Monoclonal antibodies specific for either the common beta chain (60.3 [3]; MHM23 [23]) or for the LFA-1 alpha chain (MHM24 [23]) recognized these molecules on the surfaces of all of the lymphoblast cell clones but gave more intense staining of LMP-expressing cells than of D1LMP or vector control cells (Fig. 3 and Table

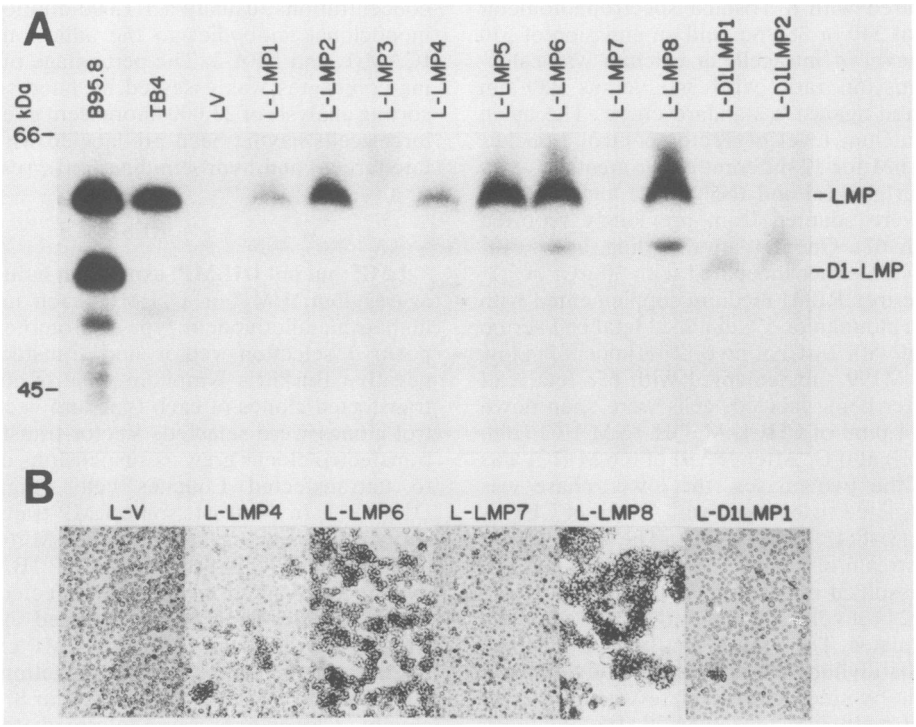

FIG. 1. LMP expression in Louckes cells correlates with cell clumping. Louckes cells (an EBV-negative Burkitt's lymphoma cell line) were transfected with pSV2gpt, pSV2gptLMP (carrying the intact LMP gene under control of the human metallothionein type 2 promoter [Hmt2]), or pSV2gptD1LMP (carrying the truncated LMP gene under control of the Hmt2 promoter). Mycophenolic acid-resistant clones were checked for LMP or D1LMP expression by Western blot analysis (A) by using anti-LMP, S12 monoclonal antibody, rabbit anti-mouse second antibody, and ^{125}I-labeled protein A. Cells lines are indicated above the lanes. B95-8 is a marmoset lymphocyte cell line which is partially permissive of EBV infection and therefore expresses LMP and D1LMP. IB4 is a tightly latent cell line which expresses only LMP. LV represents the vector control. Total protein loaded in D1LMP1 and D1LMP2 lanes in this gel was less than in other lanes. The light microscope photographs (magnification, ×25) (B) show that pSV2gpt-transfected LMP clones varied in clumping, while pSV2gpt D1LMP-transfected clones or control vector-transfected clones did not clump.

1). This increased LFA-1 expression was confirmed by immunoprecipitation with the 60.3 monoclonal antibody (Fig. 3, insert). The immunoprecipitation experiments also revealed a small increase in surface expression of p150,95 on the LMP-expressing cells (Fig. 3, insert). This was subsequently confirmed by using a p150 (alpha chain)-specific monoclonal antibody (data not shown).

The higher surface expression of LFA-1 is at least partially attributable to higher levels of alpha chain mRNA (Fig. 4C). Alpha chain mRNA levels were higher in LMP-expressing cells than in D1LMP- or vector control-transfected cells. The LFA-1 alpha chain mRNA level induced by LMP was similar to that induced by EBV transformation of normal B lymphocytes (L-LMP8 versus IB4; Fig. 4C). In contrast, the LFA-1 beta chain mRNA level (relative to the actin mRNA level) was unaffected by LMP expression. These data are compatible with LFA-1 regulation largely being at the level of alpha mRNA expression and beta chain being constitutively expressed in excess.

Homotypic cell adhesion leading to cell growth in clumps, such as is seen in EBV-transformed B-cell lines, is achieved through interactions between LFA-1 and its natural ligand ICAM-1 (39, 50). By surface immunofluorescence, ICAM-1 was higher on the LMP-transfected cells but not on D1LMP- or vector control-transfected cells (Table 1). This contrasts with the clumping induced in Louckes cells by EBNA-2 expression, which is achieved with no change in LFA-1 or ICAM-1 expression (Table 1) (65). LFA-1 and ICAM-1 were

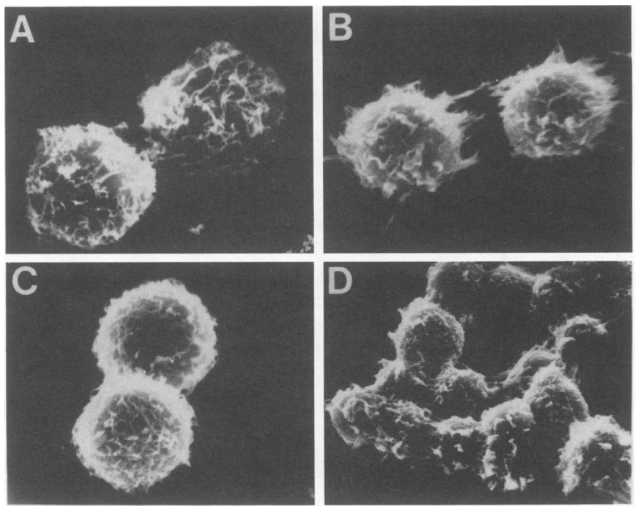

FIG. 2. LMP expression causes increased ruffling of cell membrane and cell clumping. Vector control cells (A), LMP-expressing cells (B), and D1LMP-expressing cells (C) were attached to cover slips by poly-L-lysine treatment. Scanning electron microscopy shows the different cell membrane morphologies. Panel D shows the cell clumping characteristics of LMP-expressing cells. Bars in panels A and D, 1 μm.

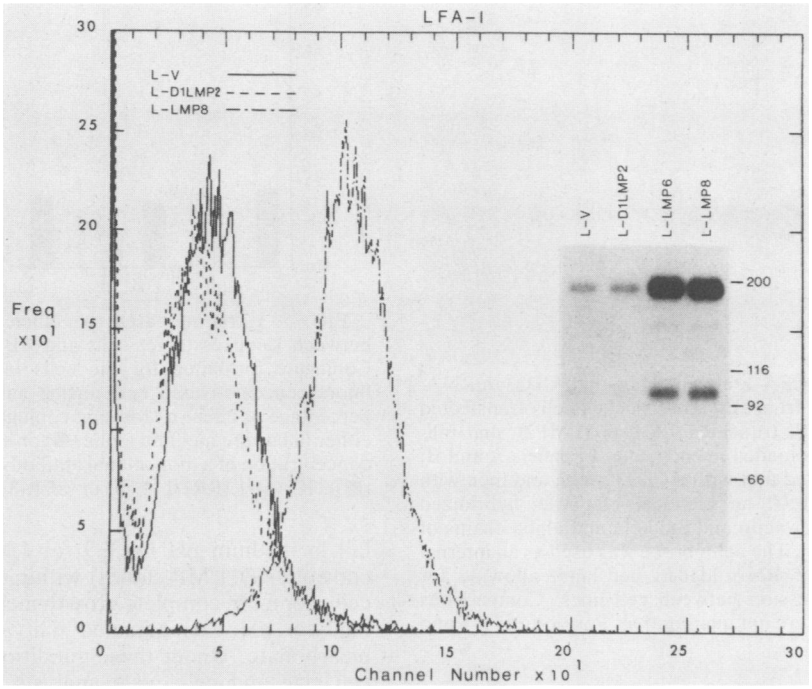

FIG. 3. LMP induces LFA-1 expression. Clones expressing D1LMP (L-D1LMP2) or LMP (L-LMP8) or vector-transfected control cells were stained with the anti-LFA-1 60.3 monoclonal antibody and FITC-conjugated goat anti-mouse Fab fragments. Profile of fluorescence intensity by fluorescence-activated cell sorter analysis is shown. The insert shows an autoradiogram of an immunoprecipitate (with 60.3 monoclonal antibody) of surface-iodinated Louckes clones expressing D1LMP (L-D1LMP2) or LMP (L-LMP6 and L-LMP8) or vector-transfected control cells which were immunoprecipitated with the 60.3 monoclonal antibody. Numbers indicate kilodaltons.

TABLE 1. Summary of cell adhesion molecule expression on Louckes transfectants

Clone[a]	Growth pattern	Control[b] (background)	Expression of			
			LFA-1		ICAM-1	LFA-3
			alpha	beta		
L-pZ-3	Single cells	0.6	8	9	24	0.5
L-E1-10	Single cells	0.2	10	11	21	2
L-E3-2	Single cells	0.5	9	9	14	1
L-ELP-8	Single cells	4	7	8	18	2
L-ELP-9	Single cells	0.3	12	11	27	3
L-E2-5	Large clumps	0.4	10	9	34	4
L-E2-7	Large clumps	0.5	11	9	27	2
L-gpt-1	Single cells	0.5	9	8	21	2
L-gpt-2	Single cells	0.2	6	6	24	2
L-D1LMP1	Single cells	0.4	5	5	20	0.9
L-D1LMP2	Single cells	0.5	4	4	34	2
L-D1LMP3	Single cells	0.7	9	9	30	3
L-D1LMP4	Single cells	0.4	8	8	28	3
L-LMP7	Small clumps	0.4	14	15	28	9
L-LMP1	Medium clumps	0.7	10	11	45	11
L-LMP4	Medium clumps	0.9	12	15	30	16
L-LMP5	Large clumps	0.7	14	16	57	27
L-LMP2	Large clumps	1	20	22	52	33
L-LMP6	Large clumps	1	23	22	47	34
L-LMP8	Large clumps	1	27	27	49	41

[a] Cells used were Louckes cells expressing MoMuLV vector control (L-pZ-3) or MoMuLV vector with EBNA-1 (L-E1-10), EBNA-3 (L-E3-2), EBNA-LP (L-ELP-8 and 9), or EBNA-2 (L-E2-5 and 7). Other cells included Louckes cells expressing the pSV_2gpt vector control (L-gpt-1 and 2), truncated-form D1LMP (L-D1LMP 1 to 4) or full-length LMP (L-LMP 1 to 8).

[b] Numbers represent mean fluorescence intensity (from fluorescent antibody staining) normalized for cell surface area. There were no significant differences between any of the groups of transfectants with regard to the HLA class I antigen expression when assayed in this experiment (data not shown).

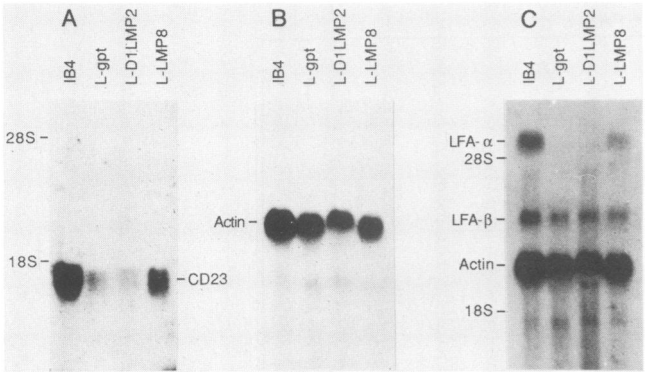

FIG. 4. LMP induces LFA-1 alpha chain and CD23 mRNA. Cytoplasmic RNA (10 μg) from IB4 and Louckes cells transfected with vector control (L-gpt), truncated LMP (D1LMP2), and full-length LMP (L-LMP8) was loaded in each lane. In panels A and B, the same blot was hybridized first with a CD23 probe and then with an actin probe. In panel C, an identical blot was hybridized sequentially with probes for actin and the beta and alpha chains of LFA-1 (LFA-α and LFA-β). The actin probe was used as an internal control for the amount of RNA loaded per lane, allowing for comparison of RNA expression between cell lines. Comparisons between probes, however, are not quantitative. Positions of 18S and 28S RNAs are also shown.

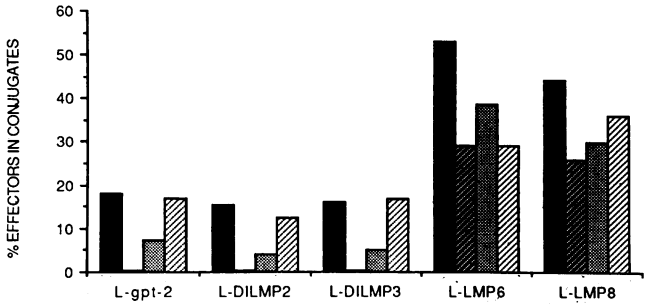

FIG. 5. LMP increases the efficiency of conjugate formation between Louckes target cells and EBV-specific cytotoxic T cells. Conjugate formation for the cells indicated was determined by fluorescence-activated cell sorting analysis and expressed as the percentage of effectors forming conjugates. Assays were conducted either in culture medium alone (■) or in the presence of a saturating concentration of a monoclonal antibody specific for LFA-1 (MHM23 [▨]), ICAM-1 (RR1/1 [▨]), or LFA-3 (TS2/9 [▨]).

shown to be responsible for the aggregation of LMP-expressing cells by monoclonal antibody inhibition of cell aggregation. Fab fragments of monoclonal antibodies specific for ICAM-1 (R6.5) (R. Rothlein, personal communication) or LFA-1 alpha chain (TS1/22) completely inhibited LMP-expressing cell aggregation as determined by a qualitative aggregation assay (50).

LFA-3, another cell adhesion molecule, is not involved in B-lymphoblast homotypic cell adhesion but is the natural ligand for the T-cell antigen CD2 (56). Interactions between B and T lymphocytes are facilitated through LFA-3-CD2 and ICAM-1-LFA-1 adhesion pathways (37, 52). Changes in LFA-3 expression are important because they could affect the susceptibility of EBV-infected cells to T-cell surveillance (19, 48). LFA-3 is very low on Louckes cells and was induced to very high levels by LMP expression but not by D1LMP expression (Table 1). Increased LFA-3 expression on LMP-expressing cells was indeed associated with a greater efficiency of conjugate formation with effector T cells (Fig. 5, solid bars). (Conjugate formation in this assay measures effector-target interactions which are independent of specific immune recognition [19, 52].) The relatively low numbers of conjugates formed between control or D1LMP-expressing cells and effector T cells only involved the LFA-1 pathway, since anti-LFA-1 monoclonal antibodies completely prevented conjugation whereas LFA-3 antibody had no effect (Fig. 5). In contrast, LFA-1, ICAM-1, or LFA-3 monoclonal antibodies each partially inhibited the conjugation of LMP-expressing cells with T cells (Fig. 5), and complete inhibition required both anti-LFA-1 and anti-LFA-3 reagents (data not shown). This conjugation is therefore supported by both LFA-1-ICAM-1 and CD2-LFA-3 adhesion pathways.

LMP (but not D1LMP) affects cell size, acid production, TGF-beta responsiveness, and markers of B-cell activation. In order to study the effects of LMP or D1LMP on cell growth, clones of LMP-, D1LMP-, or control vector-expressing cells were maintained in log-phase cell growth by daily feeding. LMP-expressing cell clones characteristically caused a rapid fall in medium pH (to 6.9 for LMP clones versus 7.2 for control or D1LMP clones) within 48 h of seeding at 2×10^5 cells per ml in complete growth medium, in which the culture medium pH was adjusted daily to pH 7.4 with sodium bicarbonate. Under these conditions of continuous growth, cell size and cell cycle analysis were performed by flow cytometry. The average diameter of LMP-expressing cells as determined by forward light scattering was 16% larger than the diameter of D1LMP-expressing or control vector-transfected cells (Fig. 6). Despite the increased acid production and increased cell volume, there was no change in cell cycle distribution as determined by propidium iodide staining (Fig. 7, upper panels) nor in the rate of cell growth as determined by overnight [^{3}H]thymidine incorporation (data not shown). Over a 5-day period after seeding at low density in fresh medium, the LMP-expressing clones grew 15% more slowly than did the D1LMP or vector-transfected clones (data not shown). The saturation densities of LMP-, D1LMP-, and vector control-transfected cells were similar.

Transforming growth factor (TGF) beta is known to have differential effects on the proliferation of transformed cells

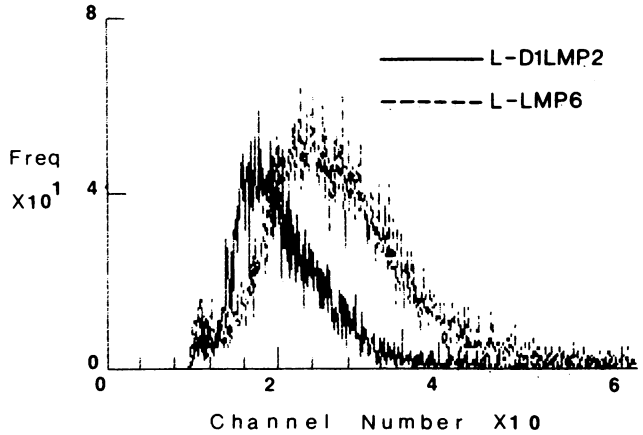

FIG. 6. LMP causes increase in lymphocyte cell volume. The cell size of LMP-expressing cells (L-LMP6) versus that of D1LMP-expressing cells (L-D1LMP2) was measured by forward-light-scattering flow cytometry. The channel numbers are on a logarithmic scale; 83 channels are equivalent to 1 log cycle. Control vector-transfected cells were identical in size to pSV2gptD1LMP-transfected cells.

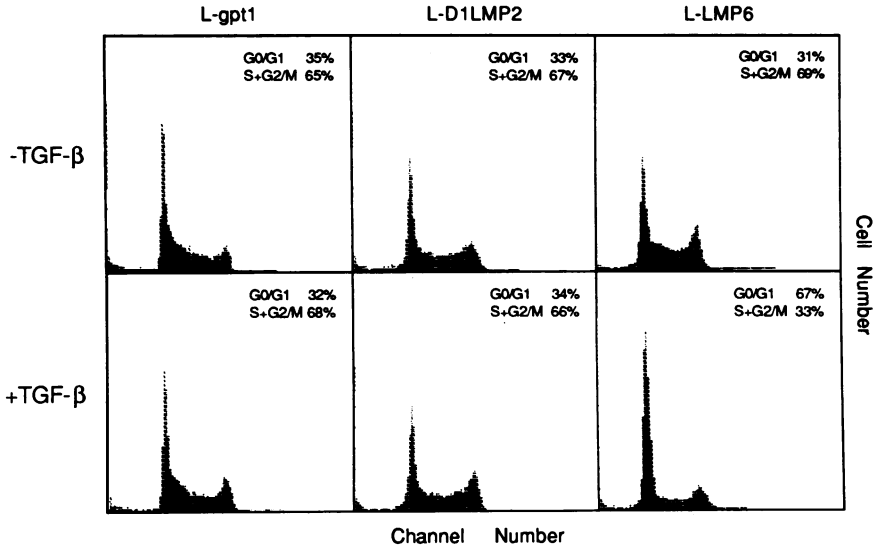

FIG. 7. Effect of TGF beta on the cell cycle of vector control-, D1LMP-, and LMP-transfected cells. Lgpt-1, L-D1LMP2, and L-LMP6 cells growing in log phase were initially plated at 2.5×10^5 cells per ml. After 48 h, cell cycle distribution was determined by propidium iodide staining and fluorescence-activated cell sorting analysis in medium containing 0 or 2 ng of TGF beta per ml (indicated at left).

(for a review, see reference 28) and has been noted to increase the growth rate of EBV-transformed cells (4, 27). We investigated whether LMP would be responsible for the increased response of EBV-transformed cells to transforming growth factor (TGF) beta. Surprisingly, the proliferation of the LMP-expressing Burkitt's lymphoma cells was markedly inhibited by TGF beta, as determined by [^{3}H]thymidine incorporation of LMP-expressing clones compared with D1LMP or vector control-transfected cells (Table 2). This was due at least in part to a marked arrest of the LMP-expressing cells in G_0-G_1 phase by TGF beta treatment (Fig. 7, lower panels). Actively growing LMP-expressing clones had consistently higher intracellular free calcium than did D1LMP- or control vector-expressing cells as measured by fura-2AM. The intracellular free calcium level in LMP-expressing cells was significantly higher than it was in vector control cells ($P < 0.01$ by Wilcoxon signed-rank test) and was on average 48% higher than it was in vector control cells in 12 different experiments. In contrast, the intracellular free calcium level in D1LMP-expressing cells was not different than that in vector control cells. Since the level of intracellular free calcium has sometimes been linked to the phosphatidylinositide turnover pathway, phosphatidylinositide (61) and diacylglycerol (33) levels were measured in LMP- or D1LMP-expressing cells or in control vector-transfected cells. With short-term (1 h) $^{32}PO_4$ labeling, ^{32}P incorporation into all organic phosphates was increased twofold in LMP-expressing versus D1LMP- or control vector-transfected cells; phosphatidylethanolamine, phosphatidylcholine, phosphatidic acid, phosphatidylinositol, phosphatidylinositol phosphate, and phosphatidylinositol biphosphate were all similarly increased. With overnight $^{32}PO_4$, [^{3}H]inositol, or [^{14}C]arachidonic acid labeling, LMP-expressing cells did not differ in phospholipid, phosphoinositol, diacylglycerol, or triglyceride levels per amount of cell protein. LMP-expressing cells also did not differ in phosphatidylinositol kinase activity (57, 66) or protein kinase C translocation (28).

TABLE 2. Effect of TGF beta on LMP or D1LMP-expressing or vector-transfected (Loukes) lymphoblast clones[a]

Clone	Thymidine incorporation (cpm [10^5])	
	Control (no TGF beta)	TGF beta (0.5 ng/ml)
gpt-1	1.2	1.4
gpt-2	1.6	1.5
D1LMP-1	1.5	1.5
D1LMP-2	1.6	1.5
LMP-6	1.4	0.44
LMP-8	1.5	0.77

[a] Cells in log-phase growth were seeded into 12-well plates at 2×10^5 cells per ml in medium with or without TGF beta. After 48 h at 37°C, [^{3}H]thymidine (1 μCi/ml) was added to the medium for 16 h. Cells were then harvested onto filters for determination of radioactivity.

CD23 and transferrin receptor surface expression, two markers of B-cell activation, are known to increase within 48 h of EBV infection of normal B cells (17). CD23 and transferrin receptor are both expressed at low levels on the Loukes cell and were induced to higher levels in the high-LMP-expressing clones 5, 6, and 8, but not in the D1LMP-expressing clones 1 and 2 or in the vector control-transfected cells (Fig. 8 and 9, respectively). LMP-expressing cells had more than twofold higher surface CD23 expression than did D1LMP- or control vector-transfected cells (Fig. 8). As determined by Northern blot analysis, increased surface CD23 expression is at least in part due to increased CD23 mRNA levels (Fig. 4A and B, respectively). The level of CD23 mRNA compared with that of actin mRNA was severalfold higher in LMP-expressing Burkitt's tumor cells and in the continuous EBV-infected lymphoblastoid cell line IB4 compared with D1LMP- or vector transfected-cells. In contrast to CD23 and transferrin receptor, human class I and class II HLA antigens and the interleukin-2 receptor were not expressed at higher levels in transfected clones compared with in D1LMP or vector control clones (data not shown).

LMP (but not D1LMP) patches and associates with cytoskeleton. In latently infected, growth-transformed lymphocytes, LMP localizes in a patch at the cell periphery and is diffusely distributed through the cytoplasm (35). In transfec-

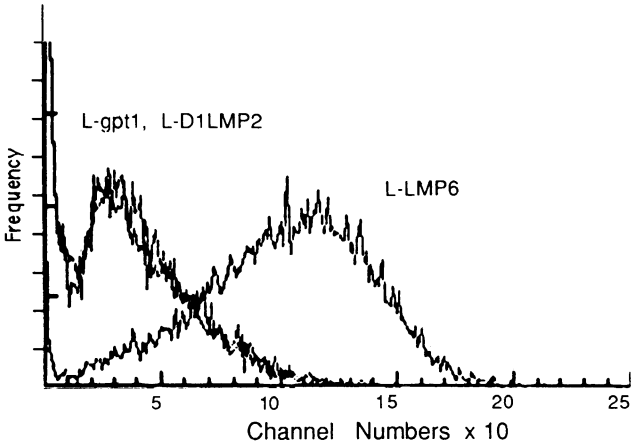

FIG. 8. LMP induces CD23 expression. CD23 expression was measured by flow cytometry with EBVCS 2, 4, and 5 antibodies and FITC-conjugated goat anti-mouse immunoglobulin. A representative high-LMP-expressing clone (L-LMP6) expressed more than twice as much CD23 than did D1LMP-expressing (L-D1LMP2) or vector control (L-gpt) cells.

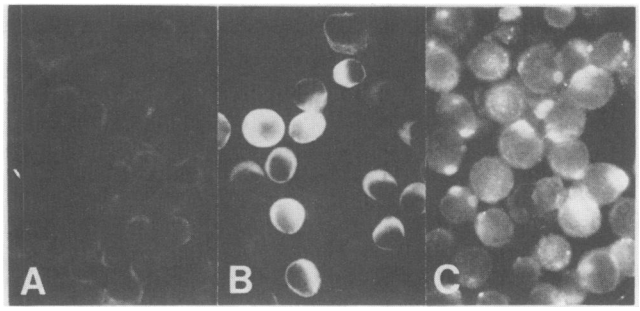

FIG. 10. Intracellular localization of LMP and D1LMP. Actively growing or control pSV2gpt vector-transfected clone (A), pSV2gpt D1LMP-transfected cells (B), or pSV2gpt LMP-expressing cells (C) were fixed in cold methanol. The S12 monoclonal antibody, biotinylated goat anti-mouse F(ab')2, and FITC-conjugated streptavidin were sequentially used for immunofluorescence staining. The pSV2gpt D1LMP-transfected clone was treated with 75 μM zinc sulfate overnight to induce a level of D1LMP similar to that of LMP in the pSV2gptLMP cells.

ted Louckes cells, LMP was also in a dense patch at the cell periphery and was diffusely distributed through the cytoplasm (Fig. 10C). In contrast, D1LMP-expressing cells exhibited only uniform, diffuse, cytoplasmic fluorescence. No localization to the cell periphery or to patches was observed (Fig. 10B).

Since in EBV growth-transformed B lymphocytes or in LMP-transformed rodent fibroblasts about 50% of the cell LMP is bound to the cytoskeletal network and resists persistent extraction with buffers containing nonionic detergent (34, 64), the extent to which LMP or D1LMP associates with the cytoskeleton of the transfected cells was examined. Louckes cells expressing LMP or D1LMP were extracted twice with PBS containing the nonionic detergent Triton X-100, and the level of LMP or D1LMP in the detergent-soluble and -insoluble cell fractions was analyzed by Western (immuno-) blotting (Fig. 11). Approximately 50% of the total LMP persistently resisted detergent extraction and was associated with the cytoskeletal residue (Fig. 11, lane 3). In contrast, D1LMP was completely extracted from cells by the

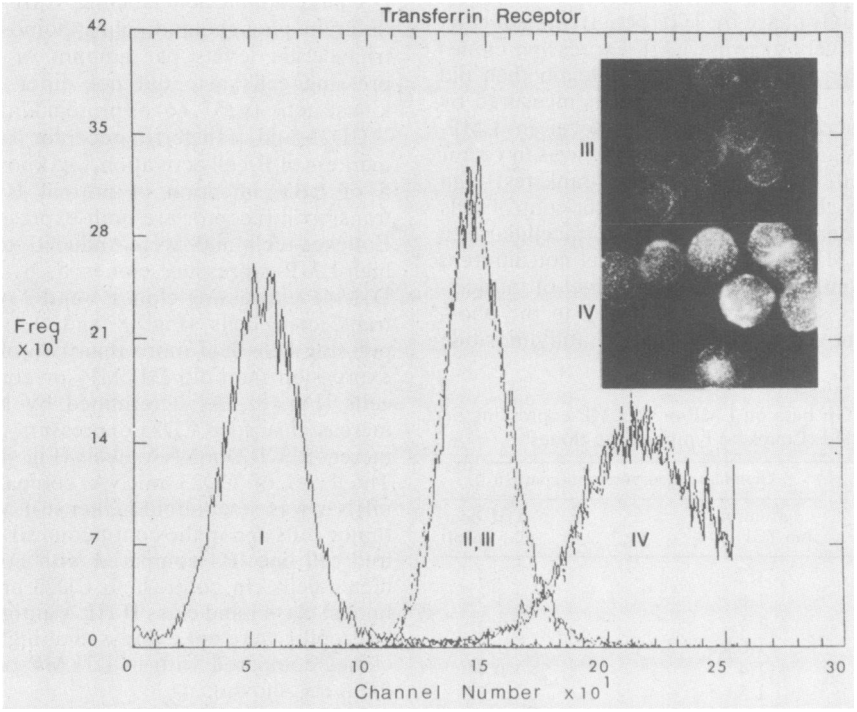

FIG. 9. LMP induces transferrin receptor expression. Shown are results of flow cytometry of representative clones of vector control cells (II), D1LMP-expressing cells (III [L-D1LMP2]), and high-LMP-expressing cells (IV [L-LMP5]) which were stained with anti-transferrin receptor antibody (Interferon, Inc.) and FITC-conjugated goat anti-mouse Fab fragments. Background cell staining (I) was done with the FITC-conjugated antibody only. Inset, immunofluorescent staining of L-D1LMP2 (III) and L-LMP5 (IV) cells.

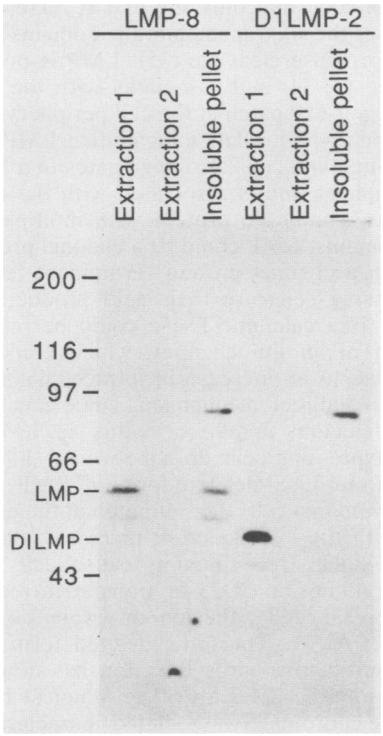

FIG. 11. Detergent extraction of LMP- and D1LMP-expressing cells. Cells (10^6) were extracted three times with PBS containing 0.5% Triton X-100. Each of the extractions and the insoluble pellet were analyzed by SDS polyacrylamide gel electrophoresis and Western blotting with an anti-LMP monoclonal antibody. Molecular masses (in kilodaltons) and locations of LMP and D1LMP are indicated to the left of the lanes.

first detergent treatment (Fig. 11, lane 4), indicating that it is not associated with the cytoskeleton. Thus, LMP patching in transfected lymphoma cells correlates with cytoskeletal association, as it does in EBV-transformed lymphocytes or LMP-transformed rodent fibroblasts, and D1LMP, which does not patch in these transfected cells, is not associated with the cytoskeleton.

DISCUSSION

These experiments demonstrate that EBV LMP expression in a continuous human B-lymphoblast cell line causes steady-state increases in intracellular free calcium, acid production, cell size, membrane ruffling, cell surface CD23 and transferrin receptor expression, and homotypic and heterotypic cell adhesion marked by increased LFA-1, LFA-3, and ICAM-1 expression. Each of these effects was known to be a prominent feature of EBV-, antigen-, or mitogen-induced B-lymphocyte proliferative activation. A transient increase in intracellular free calcium is a prominent immediate manifestation of B-lymphocyte activation (for a review, see reference 6). Within 24 h of EBV infection or mitogen stimulation, normal resting human B cells increase surface CD23 expression, and within 48 h they increase transferrin receptor expression (17, 59, 62). Both CD23 and transferrin receptor expression precede initiation of S phase. Several lines of evidence point to central roles for intracellular free calcium increase and increased CD23 and transferrin receptor in B-cell growth regulation (11, 18, 20, 29, 43). In EBV-infected B cells, CD23 expression is superinduced, and a soluble form of CD23 is present which has autocrine B-cell growth factor activity (31, 59). The specific induction of CD23 by EBNA-2 as shown in earlier work (65) and the increased expression of CD23, shown here to be associated with LMP, further support the working hypothesis that CD23 is central to EBV-induced B-lymphocyte growth transformation. Thus, LMP appears to have a role in EBV-induced B-lymphocyte proliferation and growth transformation based on its ability to modulate key points of B-cell growth regulation, namely increases in intracellular free calcium and in cell surface levels of CD23 and transferrin receptor.

LMP did not affect protein kinase C activation or phosphoinositol turnover, two other regular accompaniments of B-cell growth activation, and LMP-expressing Louckes B-lymphoma cells did not grow more rapidly than controls. However, it should be noted that protein kinase C activation and phosphatidylinositol turnover have not been shown to be directly affected by EBV infection. In addition, an increased rate of cell proliferation may not be an expected phenotype for an EBV transforming gene in an already established lymphoma cell line, since EBV infection of such cells does not increase their proliferation rate (44). In causing a steady-state increase in intracellular free calcium, LMP presumably provides constant relief of this stringent requirement for G_0-G_1 phase transition during logarithmic cell growth. A difference in G_0-G_1 versus S-phase cell cycle distribution might be anticipated and, in fact, a slight decrease in the G_0-G_1 phase cell fraction was seen in some experiments. However, attempts to bring out a G_0-G_1/S phase difference between LMP-expressing and control cultures by manipulating the conditions of cell growth (including analysis after growth to saturation density, after growth in medium supplemented with low serum, or shortly after release from growth inhibition) did not result in an increased difference between LMP-expressing and control cultures. Thus, LMP-expressing cells still exhibit substantial G_0-G_1/S transition block. In fact, TGF beta, which can inhibit G1-to-S transition and transferrin receptor expression on normal or neoplastic-stimulated B lymphocytes (55), inhibited the growth of LMP-expressing Burkitt's tumor cells but had no effect on the growth of nonexpressing control cells. LMP expression appears, therefore, to induce a susceptibility to TGF beta blockade of G_1 transition, which may counter some of the growth-stimulating effects of LMP. The TGF beta-induced G_1 blockade of LMP-expressing cells is somewhat surprising, since TGF beta was previously reported to increase the proliferation of EBV-infected normal or malignant B cells (4). However, growth factors can switch TGF beta inhibition of cell growth to cell growth stimulation (30). Thus, in EBV latent infection, another EBV gene product may directly or indirectly reverse the inhibitory effect of LMP-mediated TGF beta responsiveness.

Several lines of evidence indicate that the EBV EBNA-2 is at least one other EBV protein necessary for EBV-induced B-cell proliferation. EBV isolates deleted for EBNA-2 (22) lack growth-transforming activity, while transformation-competent recombinant isolates have regained EBNA-2 (54). Second, EBV isolates with a somewhat uncommon EBNA-2 allele differ from those with the more common EBNA-2 in their ability to maintain B-lymphocyte proliferation (49). Third, in Rat-1 cells, EBNA-2 caused partial relief from serum dependence (10). Fourth, in the same Burkitt's tumor cells used in these studies, EBNA-2 expression specifically induced CD23 mRNA, CD23 surface expression, and cell clumping (65). In Burkitt's tumor cells, EBNA-2 expression

was associated with a more restricted phenotype than LMP in that EBNA-2 did not cause cell enlargement, membrane ruffling, acid production, transferrin receptor expression, or induction of LFA-1, LFA-3, or ICAM-1. Thus, EBNA-2 and LMP probably act in concert in EBV induction of B-lymphocyte growth transformation.

A characteristic feature of all EBV-infected cell lines is the growth of cells in very tight clumps (44). Growth of EBV-infected cells in tight clumps may be advantageous to their growth because of their need for autocrine or paracrine growth factors (15, 16, 59). Previous data indicated that LFA-1 and ICAM-1 are involved in EBV-induced cell adhesion, since monoclonal antibodies to LFA-1 and ICAM-1 reversed the phorbol ester-induced adhesion of EBV-infected cells (45). Also, EBV-transformed B cells from individuals who are deficient in LFA-1 expression do not grow in tight clumps (56). Our data support a role for LFA-1 and ICAM-1 in EBV-induced cell adhesion and link their increased expression to LMP. Interestingly, LFA-1-ICAM-1-mediated cell clumping must not be stringently required for EBV to induce B-cell proliferation, since EBV does transform LFA-1-deficient B lymphocytes. Also, the EBNA-2-induced cell adhesion is not related to increased expression of these previously described lymphocyte adhesion molecules, suggesting that additional mechanisms may be operating in the EBNA-2-expressing or EBV-infected lymphocytes.

The most dramatic change in cell surface molecule expression induced by LMP was the change in LFA-3. This caused increased conjugate formation with human T lymphocytes, presumably through T lymphocyte CD2. Both LFA-3-CD2 and ICAM-1-LFA-1 are important in B- and T-lymphocyte conjugation; both appear to be necessary for efficient killing of EBV-infected B cells by virus-specific cytotoxic T cells (19). Although EBV-infected cell growth in clumps as a consequence of LFA-1 and ICAM-1 induction may enhance autocrine or paracrine growth factor effects, the induction of LFA-3 and ICAM-1 could have an adverse effect on EBV-infected B lymphocytes in vivo by enhancing the efficiency of immune T-cell surveillance. Prevention of EBV-induced lymphoproliferation in humans is believed to be due to virus-specific T-cell-mediated immunity (7, 48, 49). The levels of ICAM-1 and LFA-3 on target cells can influence the susceptibility of the cells to T-cell killing by determining the ease of effector-target conjugate formation (37, 56). Indeed, down-regulation of ICAM-1 and LFA-3 expression has been associated with EBV-positive Burkitt's tumor cell escape from immune T-cell surveillance (19). The induction of LMP of adhesion molecules increases the susceptibility of EBV-infected cells to immune surveillance. However, a strong T-cell response may, in the long run, be advantageous for EBV since it permits it to establish a long-term commensal relationship with its human hosts, which serve as a repository for infectious virus. In fact, recent studies indicate that LMP also provides some of the important target epitopes for virus-specific T-cell lysis (42).

The distinctly different intracellular localization of LMP and EBNA-2 and their different phenotypic effects (other than on CD23 expression) indicate that these two EBV growth-transforming proteins act through different mechanisms. The nuclear localization and likely DNA-binding properties of EBNA-2 suggest a direct role in inducing CD23 transcription. In EBV growth-transformed, latently infected lymphocytes, LMP localizes to the cytoplasmic membranes and to a patch at the cell periphery, presumably in the plasma membrane (34, 35). Plasma membrane LMP is in a configuration such that only the first reverse turn between the first two of the six transmembrane domains is susceptible to live-cell protease cleavage (35). LMP is posttranslationally modified (2, 34) and associates with the cytoskeleton (34). The large LMP patch at the cell periphery is associated with vimentin (34). Our data indicate that LMP expressed in Burkitt's lymphoma cells also aggregates in a large patch at the cell periphery and is associated with the cytoskeleton. As a plasma membrane protein with multiple membrane-spanning domains, LMP could be a channel protein or could interact with a channel protein. Prominent features of the LMP-expressing cells are their acid production and high intracellular free calcium. These could be related through modification of an ion channel, which could directly or indirectly lead to an increase in intracellular free calcium. We favor an indirect mechanism, since measurements of radioactive calcium uptake or efflux in LMP-expressing versus nonexpressing cells do not reveal a difference.

The amino-terminal-deleted form of LMP expressed in Burkitt's lymphoma cells did not patch at the cell periphery; associate with the cytoskeleton; increase acid production; raise intracellular free calcium; cause cell enlargement, membrane ruffling, or CD23 or transferrin receptor induction; or increase cell adhesion or expression of LFA-1, LFA-3, or ICAM-1. Thus, the deleted form of LMP expressed in productive virus infection has none of the cell activation properties of LMP. The simplest hypothesis to explain these coordinately associated characteristics of LMP and D1LMP is that the short, charged amino terminus or the first four transmembrane LMP domains are important in plasma membrane translocation and cytoskeletal association and that all of the other phenotypic manifestations of LMP require plasma membrane or cytoskeletal association.

ACKNOWLEDGMENTS

P. Spear, B. Roizman and K. Kishimoto provided advice and assistance. Flow cytometer analysis of cell cycle was performed with the assistance of Isabel Wood.

D. Wang is a predoctoral trainee supported by the Institute for International Studies grant IIE 15822181. D. Liebowitz is a predoctoral trainee supported by Public Health Service grant GM 07281 from the National Institutes of Health. This research was supported by grant CA47006 from the National Cancer Institute and the Cancer Research Campaign, London.

LITERATURE CITED

1. **Amen, P., N. Lewin, M. Nordstrom, and G. Klein.** 1986. EBV-activation of human B-lymphocytes. Curr. Top. Microbiol. Immunol. **132**:266–271.
2. **Baichwal, V. R., and B. Sugden.** 1987. Posttranslational processing of an Epstein-Barr virus-encoded membrane protein expressed in cells transformed by Epstein-Barr virus. J. Virol. **61**:866–875.
3. **Beatty, P. G., J. A. Ledbetter, P. J. Martin, T. H. Price, and J. A. Hansen.** 1983. Definition of a common leukocyte cell-surface antigen (Lp95-150) associated with diverse cell-mediated immune functions. J. Immunol. **131**:2913–2918.
4. **Blomhoff, H. K., E. Smeland, A. S. Mustafa, T. Godal, and R. Ohlsson.** 1987. Epstein-Barr virus mediates a switch in responsiveness to transforming growth factor, type beta, in cells in the B cell lineage. Eur. J. Immunol. **17**:299–301.
5. **Calender, A., M. Billaud, J.-P. Aubry, J. Banchereau, M. Vuillaume, and G. M. Lenoir.** 1987. Epstein-Barr virus (EBV) induces expression of B-cell activation markers on *in vitro* infection of EBV-negative B-lymphomà cells. Proc. Natl. Acad. Sci. USA **84**:8060–8064.
6. **Cambier, J. C., L. B. Justement, M. K. Newell, Z. Z. Chen, L. K. Harris, V. M. Sandoval, M. J. Klemsz, and J. T. Ransom.**

1987. Transmembrane signals and intracellular "second messengers" in the regulation of quiescent B-lymphocyte activation. Immunol. Rev. **95**:37–57.
7. **Cleary, M., R. Dorfman, and J. Sklar.** 1986. Failure in immunological control of the virus infection: post transplant lymphomas, p. 164–181. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus: recent advances. William Heinemann Medical Books Ltd., London.
8. **Crissman, H. A., and J. A. Steinkamp.** 1973. Rapid simultaneous measurement of DNA, protein, and cell volume in single cells from large mammalian cell populations. J. Cell Biol. **59**:766–771.
9. **Dambaugh, T., K. Hennessy, S. Fennewald, and E. Kieff.** 1986. The virus genome and its expression in latent infection, p. 13–45. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus: recent advances. William Heinemann Medical Books Ltd., London.
10. **Dambaugh, T., F. Wang, K. Hennessy, E. Woodland-Bushman, A. Rickinson, and E. Kieff.** 1986. Expression of the Epstein-Barr virus nuclear protein 2 in rodent cells. J. Virol. **59**:453–462.
11. **Dugas, B., A. Vazquez, J.-F. Delfraissy, J.-P. Gerard, M.-T. Rannou, and P. Galanaud.** 1986. Human B cell activation: selective sensitivity of the early stages to calcium channel-blocking drugs. Eur. J. Immunol. **16**:162–167.
12. **Fennewald, S., V. van Santen, and E. Kieff.** 1984. Nucleotide sequence of an mRNA transcribed in latent growth transforming virus infection indicates that it may encode a membrane protein. J. Virol. **51**:411–419.
13. **Fleischman, L. F., S. B. Chawaler, and L. Cantley.** 1986. *Ras*-transformed cells: altered levels of phosphatidylinositol-4,5-biphosphate and catabolites. Science **231**:407–410.
14. **Fried, J.** 1976. Method for the quantitative evaluation of data from flow microfluorometry. Comput. Biomed. Res. **9**:263–276.
15. **Gordon, J., G. Guy, and L. Walker.** 1985. Autocrine models of B-lymphocyte growth. I. Role of cell contact and soluble factors in T-independent B-cell responses. Immunology **56**:329–335.
16. **Gordon, J., S. C. Ley, M. D. Melamed, L. S. English, and N. C. Hughes-Jones.** 1984. Immortalized B lymphocytes produce B-cell growth factor. Nature (London) **310**:145–147.
17. **Gordon, J., L. Walker, G. Guy, G. Brown, M. Rowe, and A. Rickinson.** 1986. Control of human B-lymphocyte replication. II. transforming EBV exploits three distinct viral signals to undermine three separate control points in B cell growth. Immunology **58**:591–595.
18. **Gordon, J., A. Webb, L. Walker, G. Guy, and M. Rowe.** 1986. Evidence for an association between CD23 and the receptor for a low molecular weight B cell growth factor. Eur. J. Immunol. **16**:1627–1630.
19. **Gregory, C. D., R. J. Murray, C. F. Edwards, and A. B. Rickinson.** 1988. Down-regulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlies tumour cell escape from virus-specific T cell surveillance. J. Exp. Med. **167**:1811–1824.
20. **Guy, G., and J. Gordon.** 1987. Coordinated action of IgE and a B-cell-stimulatory factor on the CD23 receptor molecule upregulates B lymphocyte growth. Proc. Natl. Acad. Sci. USA **84**: 6239–6243.
21. **Hennessy, K., S. Fennewald, M. Hummel, T. Cole, and E. Kieff.** 1985. A membrane protein encoded by Epstein-Barr virus in latent growth-transforming infection. Proc. Natl. Acad. Sci. USA **81**:7207–7211.
22. **Hennessy, K., and E. Kieff.** 1985. A second nuclear protein is encoded by Epstein-Barr virus in latent infection. Science **227**: 1238–1240.
23. **Hildreth, J. E. K., F. M. Gotch, P. D. K. Hildreth, and A. J. McMichael.** 1983. A human lymphocyte-associated antigen involved in cell mediated lympholysis. Eur. J. Immunol. **13**:202–208.
24. **Hudson, G. S., P. J. Farrell, and B. G. Barrell.** 1985. Two related but differentially expressed potential membrane proteins encoded by the *Eco*RI Dhet region of Epstein-Barr virus B95-8. J. Virol. **53**:528–535.
25. **Jackowski, S., C. W. Rettenmier, C. J. Sherr, and C. O. Rock.** 1986. A guanine nucleotide-dependent phosphatidylinositol 4,5-diphosphate phospholipase C in cells transformed by the v-fms and v-fes oncogenes. J. Biol. Chem. **261**:4978–4985.
26. **Justement, L., Z. Chen, L. Harris, J. Ransom, V. Sandoval, C. Smith, D. Rennick, N. Roehm, and J. Cambrier.** 1986. BSF-1 induces membrane protein phosphorylation but not phosphoinositide metabolism, Ca^{2+} mobilization, protein kinase C translocation, or membrane depolarization in resting murine B lymphocytes. J. Immunol. **137**:3664–3670.
27. **Kehrl, J. H., A. B. Roberts, L. M. Wakefield, S. Jakowlew, M. B. Sporn, and A. S. Fauci.** 1986. Transforming growth factor beta is an important immunomodulatory protein for human B lymphocytes. J. Immunol. **137**:3855–3860.
28. **Keski-Oja, J., E. B. Leof, R. M. Lyons, R. J. Coffey, and H. L. Moses.** 1987. Transforming growth factors and control of neoplastic cell growth. J. Cell. Biochem. **33**:95–107.
29. **Kikutani, H., S. Inui, R. Sato, E. L. Barsumian, H. Owaki, K. Yamasaki, T. Kaisho, N. Uchibayashi, R. R. Hardy, T. Hirano, S. Tsunasawa, F. Sakiyama, M. Suemura, and T. Kishimoto.** 1986. Molecular structure of human lymphocyte receptor for immunoglobulin E. Cell **47**:657–665.
30. **Kimelman, D., and M. Kirschner.** 1987. Synergistic induction of mesoderm by FGF and TGF-B and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell **51**: 869–877.
31. **Kintner, C., and B. Sugden.** 1981. Identification of antigenic determinants unique to the surfaces of cells transformed by Epstein-Barr virus. Nature (London) **294**:458–460.
32. **Kishimoto, T. K., K. O'Connor, A. Lee, T. M. Roberts, and T. A. Springer.** 1987. Cloning of the β subunit of the leukocyte adhesion proteins: homology to an extracellular matrix receptor defines a novel supergene family. Cell **48**:681–690.
33. **Lacal, J. C., J. Moscat, and S. A. Aaronson.** 1987. Novel source of 1,2-diacylglycerol elevated in cells transformed by Ha-ras oncogene. Nature (London) **330**:269–272.
34. **Liebowitz, D., R. Kopan, E. Fuchs, J. Sample, and E. Kieff.** 1987. An Epstein-Barr virus transforming protein associates with vimentin in lymphocytes. Mol. Cell. Biol. **7**:2299–2308.
35. **Liebowitz, D., D. Wang, and E. Kieff.** 1986. Orientation and patching of the latent infection membrane protein encoded by Epstein-Barr virus. J. Virol. **58**:233–237.
36. **Lindahl, T., A. Adams, G. Bjursell, G. W. Bornkamm, G. Kaschka-Dierch, and U. Jehn.** 1976. Covalently closed circular duplex DNA of Epstein-Barr virus in a human lymphoid cell line. J. Mol. Biol. **102**:511–530.
37. **Makgoba, M., M. Sanders, C. Luce, M. Dustin, T. Springer, E. Clark, P. Mannoni, and S. Shaw.** 1988. ICAM-1 ligand for LFA-1 dependent adhesion of B, T, and myeloid cells. Nature (London) **331**:86–88.
38. **Mann, K., D. Staunton, and D. A. Thorley-Lawson.** 1985. An Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. J. Virol. **55**:710–720.
39. **Marlin, S., and T. Springer.** 1987. Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function associated antigen 1 (LFA-1). Cell **51**:813–819.
40. **Matsuo, T., M. Heller, L. Petti, E. O'Shiro, and E. Kieff.** 1984. The entire Epstein-Barr virus genome integrates into human lymphocyte DNA. Science **226**:1322–1325.
41. **Miller, G.** 1985. Epstein-Barr Virus, p. 563–589. *In* B. N. Fields, (ed.), Virology. Raven Press, New York.
42. **Murray, R., D. Wang, L. Young, F. Wang, M. Rowe, E. Kieff, and A. Rickinson.** 1988. Epstein-Barr virus-specific cytotoxic T-cell recognition of transfectants expressing the virus-coded latent membrane protein LMP. J. Virol. **62**:3747–3755.
43. **Neckers, L. M., G. Yenokida, and J. B. Trepel.** 1984. The role of the transferrin receptor in human B lymphocyte activation. J. Immunol. **133**:2437–2441.
44. **Nilsson, K., and G. Klein.** 1982. Phenotypic and cytogenetic characteristics of human B lymphoid cell lines and their relevance for the etiology of Burkitt's lymphoma. Adv. Cancer Res. **37**:319–380.
45. **Patarroyo, M., P. G. Beatty, K. Nilsson, and C. G. Gahmberg.** 1986. Identification of a cell-surface glycoprotein mediating cell adhesion in EBV-immortalized normal B cells. Int. J. Cancer

38:539–547.
46. **Poenie, M., J. Alderton, R. Steinhardt, and R. Tsien.** 1986. Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. Science **233**:886–889.
47. **Pope, J., M. Horne, and W. Scott.** 1968. Transformation of fetal human leukocytes in vitro by filtrates of a human leukemic cell line containing herpes-like virus. Int. J. Cancer **3**:857–866.
48. **Rickinson, A.** 1986. Cellular immunological responses to the virus infection, p. 75–126. *In* M. A. Epstein and B. G. Achong, (ed.), The Epstein-Barr virus: recent advances. William Heinemann Medical Books Ltd., London.
49. **Rickinson, A., L. Young, and M. Rowe.** 1987. Influence of the EBV nuclear antigen EBNA-2 in the growth phenotype of virus-transformed B cells. J. Virol. **61**:1310–1317.
50. **Rothlein, R., M. L. Dustin, S. D. Marlin, and T. A. Springer.** 1986. A human intracellular adhesion molecule (ICAM-1) distinct from LFA-1. J. Immunol. **137**:1270–1274.
51. **Rowe, M., C. M. Rooney, C. F. Edwards, G. M. Lenoir, and A. B. Rickinson.** 1986. Epstein-Barr virus status and tumor cell phenotype in sporadic Burkitt's lymphoma. Int. J. Cancer **37**: 367–373.
52. **Shaw, S., G. E. G. Luce, R. Quinones, R. E. Gress, T. A. Springer, and M. E. Sanders.** 1986. Two antigen-independent adhesion pathways used by human cytotoxic T-cell clones. Nature (London) **323**:262–264.
53. **Shope, T. C., D. Dechairo, and G. Miller.** 1973. Malignant lymphoma in cotton-top marmosets after inoculation with Epstein-Barr virus. Proc. Natl. Acad. Sci. USA **70**:2487–2491.
54. **Skare, J., J. Farley, J. Strominger, K. Fresen, M. Cho, and H. Zur Hausen.** 1985. Transformation by Epstein-Barr virus requires DNA sequences in the region of *Bam*HI fragments Y and H. J. Virol. **55**:286–297.
55. **Smeland, E. B., H. K. Blomhoff, H. Holte, E. Ruud, K. Beiske, S. Funderud, R. Godal, and R. Ohlsson.** 1987. Transforming growth factor beta inhibits G1 to S transition, but not activation of human B lymphocytes. Exp. Cell Res. **171**:213–222.
56. **Springer, T. A., M. L. Dustin, K. Takashi, T. K. Kishimoto, and S. D. Marlin.** 1987. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. Annu. Rev. Immunol. **5**:223–252.
57. **Sugano, S., and H. Hanafusa.** 1985. Phosphatidylinositol kinase activity in virus-transformed and nontransformed cells. Mol. Cell. Biol. **5**:2399–2404.
58. **Swendeman, S., and D. A. Thorley-Lawson.** 1987. The activation antigen BLAST-2, when shed, is an autocrine BCGF for normal and transformed B cells. EMBO J. **6**:1637–1642.
59. **Thorley-Lawson, D. A., and K. P. Mann.** 1985. Early events in Epstein-Barr virus infection provide a model for B cell activation. J. Exp. Med. **162**:45–49.
60. **Tosato, G., and R. Blaese.** 1985. EBV infection and immunoregulation in man. Adv. Immunol. **37**:99–149.
61. **Volpi, M., R. Yassin, P. H. Naccache, and R. I. Shaafi.** 1983. Chemotactic factor causes rapid decrease in phosphatidylinositol, 4,5-biphosphate and phosphatidylinositol 4-monophosphate in rabbit neutrophils. Biochem. Biophys. Res. Commun. **112**: 957–964.
62. **Walker, L., G. Guy, G. Brown, M. Rowe, A. E. Milner, and J. Gordon.** 1986. Control of human B-lymphocyte replication. I. Characterization of novel activation states that precede the entry of G0 B cells into cycle. Immunology **58**:583–587.
63. **Wang, D., D. Liebowitz, and E. Kieff.** 1985. An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells. Cell **43**:831–840.
64. **Wang, D., D. Liebowitz, and E. Kieff.** 1988. The truncated form of the Epstein-Barr virus latent infection membrane protein expressed in virus replication does not transform rodent fibroblasts. J. Virol. **62**:2337–2346.
65. **Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, H. Kikutani, T. Kishimoto, and E. Kieff.** 1987. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B-cell activation antigen CD23. Proc. Natl. Acad. Sci. USA **84**:3452–3456.
66. **Whitman, M., D. R. Kaplan, B. Schaffhausen, L. Cantley, and T. M. Roberts.** 1985. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature (London) **315**:239–242.
67. **Wolfman, A., and I. G. Macara.** 1987. Elevated levels of diacylglycerol and decreased phorbol ester sensitivity in ras-transformed fibroblasts. Nature (London) **325**:359–361.