Monoclonal Antibodies to the Latent Membrane Protein of Epstein–Barr Virus Reveal Heterogeneity of the Protein and Inducible Expression in Virus-transformed Cells

By MARTIN ROWE, HELEN S. EVANS, LAWRENCE S. YOUNG, KEVIN HENNESSY, ELLIOT KIEFF AND ALAN B. RICKINSON.

1CRC Laboratories, Department of Cancer Studies, University of Birmingham, Medical School, Birmingham B15 2TJ, U.K. and 2Kovler Viral Oncology Laboratories, University of Chicago, Illinois 60637, U.S.A.

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SUMMARY

Monoclonal antibodies specific for the 'latent membrane protein' (LMP) of Epstein–Barr virus (EBV), one of the effector proteins of EBV-induced B cell transformation, have been generated from mice immunized with a β-galactosidase fusion protein containing the carboxyl half of the B95.8 strain LMP sequence. Four monoclonal IgG1 antibodies, designated CS.1, CS.2, CS.3 and CS.4, which together recognized at least three different epitopes on the molecule, were used to examine various aspects of LMP expression in B cell lines transformed in vitro. The pooled CS.1 to 4 reagent detected the LMPs encoded by each of 20 geographically distinct EBV isolates, despite a degree of inter-isolate heterogeneity in the size and antigenicity of the protein. In cell lines carrying the prototype B95.8 virus strain, particularly if these were virus producers, an additional lower molecular weight LMP was also detected; this appeared to correspond to the truncated form of the protein already predicted to exist from the analysis of B95.8 lytic cycle mRNAs. Attempts were made to identify an analogous truncated form of LMP in cell lines carrying other virus isolates after treatment with phorbol ester and/or sodium butyrate to induce virus production. Surprisingly these experiments showed that expression of the full length LMP molecule was itself strongly inducible by these agents; when monitored at the single cell level, this was a generalized response and was not restricted to cells entering a lytic cycle. Expression of LMP in EBV-transformed B cells therefore appears to be subject to a distinct type of regulation.

INTRODUCTION

Epstein–Barr virus (EBV) is an ubiquitous human herpes virus which readily infects resting B lymphocytes in vitro and transforms them into immortal lymphoblastoid cell lines (LCLs). Every cell within these LCLs carries multiple copies of the EBV genome but viral gene expression is restricted to a small number of so-called 'latent' proteins. The latent gene products so far identified consist of several nuclear antigens, EBNA-1 encoded within the BamHI K fragment of the viral genome, EBNA-2 and EBNA-LP encoded within BamHI WYH, and the EBNA-3 family of related proteins largely encoded within BamHI E, plus a 'latent membrane protein' (LMP) encoded within BamHI N (for review, see Dambaugh et al., 1986). The precise functions of these latent proteins are unclear, although EBNA-1 appears to play a role in the maintenance of the episomal form of EBV DNA (Yates et al., 1985), and EBNA-2 is probably involved in the initiation of B cell transformation (Skare et al., 1985) as well as in influencing the growth phenotype of transformed cells (Rickinson et al., 1987; Wang et al., 1987).

The existence of LMP was first postulated from the sequence of the 1158 nucleotide first open reading frame (BNRF1) in the BamHI N region of the EBV genome (B95.8 strain); this was predicted to encode a protein whose short hydrophilic amino terminus followed by six
hydrophobic domains and a 210 amino acid hydrophilic carboxyl terminus suggested a membrane location (Fennewald et al., 1984; Hudson et al., 1985). Naturally occurring antibodies to such a protein have never been detected in human sera and successful identification of the molecule was first achieved using a polyclonal rabbit antiserum raised against a β-galactosidase fusion protein incorporating the carboxy half of the BNRF1 gene product. Thus LMP was detected as a 63K band in immunoblots of cell and membrane extracts prepared from EBV-transformed cells (Hennessy et al., 1984; Mann et al., 1985; Liebowitz et al., 1986). One or more additional lower molecular weight bands were also detected in immunoblots from certain cell lines including the virus producer line B95.8 (Mann et al., 1985; Wang et al., 1985) but it was not clear whether these were simply breakdown products or corresponded to the truncated form of LMP predicted from mRNA studies to be expressed in lytically infected B95.8 cells (Hudson et al., 1985).

Interest in LMP has been stimulated by two recent observations. First, experiments in which the LMP gene was transfected and expressed in cells of the Rat-1 established cell line showed that this viral protein induces morphological transformation and conversion to a tumorigenic phenotype (Wang et al., 1985). The apparent oncogenic potential of LMP is particularly relevant since EBV is strongly implicated in the pathogenesis of two human tumours, Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (for review, see de Thé, 1982). Second, the location of the protein in the plasma membrane has led to speculation (Hennessy et al., 1984; Mann et al., 1985) that it may serve as the target structure for virus-specific cytotoxic T lymphocytes, which recognize EBV-transformed B cells in vitro and which are thought to play a crucial role in controlling EBV infection in vivo (for review, see Rickinson, 1986).

In the present work we have used the previously described β-galactosidase–LMP fusion protein (Hennessy et al., 1984) to generate a series of monoclonal antibodies (MAbs) specific for different epitopes on the viral protein. These new reagents have been used to study LMP expression in virus-transformed cells carrying either the B95.8 prototype virus strain or one of a range of new geographically distinct virus isolates.

**METHODS**

**Fusion protein.** Construction of the pKH548 plasmid, which encodes a β-galactosidase fusion protein containing 189 amino acids of the carboxy terminus of LMP, i.e. 49% of the LMP molecule, has been described elsewhere (Hennessy et al., 1984). MC1061 bacteria transformed with pKH548 were grown in L broth containing 50 μg/ml ampicillin; cells from 4 litres of culture were harvested by centrifugation and washed once in Dulbecco's phosphate-buffered saline (PBS). The cell pellet was resuspended in 45 ml lysing buffer (0.2 M-Tris–HCl pH 7.6, 0.25 M-NaCl, 0.01 M-magnesium acetate, 0.01 M-2-mercaptoethanol, 5% glycerol), mixed with 5 ml 20 mg/ml lysozyme at 0 °C, and briefly sonicated in the presence of freshly added 1 ml phenylmethylsulphonyl fluoride. After the addition of 5 ml 30% (w/v) streptomycin sulphate, the lysed cell mixture was clarified by centrifugation, washed once in 33% saturated ammonium sulphate, redissolved and dialysed extensively in phosphate-buffered saline (PBS) against 0.1 M-NTM (0.1 M-NaCl, 0.02 M-Tris–HCl pH 7.6, 0.01 M-2-mercaptoethanol, 5% glycerol), mixed with 5 ml 20 mg/ml lysozyme at 0 °C, and briefly sonicated in the presence of freshly added 1 mm-phenylmethylsulphonyl fluoride. The precipitate was collected by centrifugation at 20000 g, for 1 h at 4 °C to remove DNA and other insoluble material. The fusion protein was selectively precipitated from the supernatant by adding ammonium sulphate to 33% saturation. The precipitate was collected by centrifugation, washed once in 33% saturated ammonium sulphate, redissolved and dialysed extensively against 0.1 M-NTM (0.1 M-NaCl, 0.02 M-Tris–HCl pH 7.6, 0.01 M-2-mercaptoethanol) and applied to a Whatman DE-52 column pre-equilibrated with the same buffer. The fusion protein was eluted from this column by applying a 0.1 to 0.5 M-NaCl gradient in NTM buffer. Throughout the purification procedure the fusion protein was monitored enzymically using a scaled-down version of the β-galactosidase colorimetric assay described by Miller (1972).

Control preparations of β-galactosidase were isolated exactly as above from pUR291 (Rüther & Müller-Hill, 1983) plasmid-containing cells induced with 0.5 mM-isopropyl-β-D-thiogalactopyranoside.

**Generation of monoclonal antibodies.** BALB/c mice were primed intraperitoneally (i.p.) with 100 μg pKH548 fusion protein in complete Freund's adjuvant, and hyperimmunized by two further i.p. injections of 50 μg fusion protein with alum adjuvant at 3 week intervals. Spleen cells were collected on the third day after the final antigen injection, and fused with the non-secreting mouse myeloma line X63-Ag8.653, as described elsewhere (Rowe et al., 1982). The initial screening of the hybridoma culture supernatants for relevant antibodies was performed using an ELISA with the pKH548 fusion protein as the positive target antigen and with β-galactosidase as an irrelevant target antigen. Those hybridoma cultures which produced apparently LMP-specific antibodies in the ELISAs were subsequently tested for reactivity with EBV-positive and EBV-negative cell lines in an indirect immuno-
protein-binding sites were blocked with a 1% solution of bovine serum albumin in PBS for 1 h at room temperature. The plates were then washed with PBS containing 0.1% Tween-20 detergent (PBS-Tween), incubated with test hybridoma supernatants for 2 h at room temperature, and again washed with PBS-Tween. Specifically bound antibody was detected by incubation for 2 h with horseradish peroxidase-conjugated goat antibodies to mouse Ig (Sigma) diluted 1:1500 in PBS-Tween, washing with PBS-Tween, and developing with o-phenylenediamine substrate.

**Indirect immunofluorescence upon fixed cell preparations.** Cytocentrifuge cell preparations were fixed in methanol at −20°C for 5 min and dried under a fan at room temperature. For visualization of LMP alone, hybridoma culture supernatants were diluted 1:3 with PBS containing 40% normal goat serum, and 40 μl was incubated on each cytocentrifuge preparation for 1.5 h at room temperature in a humid box. After two 10 min washes in PBS, the cell spots were incubated for 1 h with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig (Sigma) diluted 1:100 in PBS containing 10% normal goat serum and 10% normal human serum (EBV seronegative). The slides were then washed twice for 10 min in PBS, mounted in a 1:4-diazabicyclo[2.2.2]octane-based anti-fading mountant (Johnson et al., 1982), and observed with a fluorescence microscope. Simultaneous visualization of LMP-positive cells and of cells in the virus productive cycle (positive for early antigen, EA, and for capsid antigen, VCA) was performed by two-colour immunofluorescence. Cells were incubated with CS. 1 to 4 as above, and then for a further 1 h with a mixture of the following conjugates: a 1:25 dilution of tetramethylrhodamine isothiocyanate (TRITC)-conjugated sheep anti-mouse Ig (Serotec) and a 1:100 dilution of FITC-conjugated IgG purified from a human serum (EE) with exceptionally high anti-EBV (1:20000) and anti-VCA (1:100000) antibody titres.

**SDS-PAGE and immunoblotting.** For LMP-specific detection, the relevant fusion proteins or whole cell pellets were solubilized by sonication in sample buffer (0.05 M-Tris–HCl pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 10% glycerol, 0.01% bromophenol blue), and boiled for 3 min. Samples (0.1 μg fusion protein per lane or 10⁶ cells per lane) were separated by the discontinuous gel electrophoresis technique of Laemmli (1970), using a stacking gel of 5% acrylamide and a resolving gel of either 10% or 7.5% acrylamide, then blotted onto nitrocellulose for 4 h at 150 mA prior to immunodetection with antibody and radiolabelled Protein A by the following procedure based on the method of Burnette (1981). The blotted filters were incubated in PBS containing 5% skimmed milk (PBS-milk) for 2 h to block non-specific binding sites before incubation with antibody, diluted in PBS-milk, for 16 h at 4°C. After washing in PBS-Tween, the filters were incubated for 1 h at room temperature with a rabbit anti-mouse Ig (Dakopatts) diluted 1:5000 in PBS-milk, and washed again in PBS-Tween. Specifically bound antibody was detected by incubation for 2 h with 125I-labelled staphylococcal Protein A (Amersham) diluted to 0.1 μCi/ml in PBS-milk. Following a final wash in PBS-Tween, the filters were dried and subjected to autoradiography for 1 to 2 days with an intensifying screen. Molecular weight determinations were made using protein standards (Sigma) which had been prestained with remazol brilliant blue according to the method of Griffith (1972). For detection of virus productive cycle antigens, cell extracts were separated by gel electrophoresis and blotted as above, then probed with a 1:100 dilution of human serum EE as already described (Rowe et al., 1986).

**Cell lines.** EBV-negative BL lines used included BL2, BL30, BL31, BL40 and BL41 (provided by Dr G. M. Lenoir, IARC, Lyon, France), D. J. BL (from Dr D. Moss, QIMR, Brisbane, Australia) and Louckes (van Santen et al., 1987). The various LCLs containing different isolates of EBV were designated so as to indicate the BL cell line from which the EBV was derived; thus, B + KYU-BL (X) indicates that normal B cells were co-cultivated with X-irradiated cells of the EBV-positive BL cell lines (Rickinson et al., 1987). The various LCLs containing different isolates of EBV were designated so as to indicate the BL cell line from which the EBV was derived; thus, B + KYU-BL (X) indicates that normal B cells were co-cultivated with X-irradiated cells of the KYU-BL cell line. The BL cell lines that served as sources of the EBV isolates have been described in detail elsewhere (Rowe et al., 1985; Rooney et al., 1986) and were derived from patients in Algeria, Kenya, La Reunion, New Guinea and Western Europe. A further series of LCLs was established from foetal bone marrow (FBM lines) and foetal liver (FL lines) by infection in vitro of marmoset B cells with an EBV isolate derived from a North American infectious mononucleosis patient (Miller & Lipman, 1973).

Several lymphoblastoid cell lines (LCLs) were established from normal resting B cells, isolated from the blood of EBV seronegative adults, by co-culturing in vitro with X-irradiated cells of EBV-positive BL cell lines (Rickinson et al., 1987). The various LCLs containing different isolates of EBV were designated so as to indicate the BL cell line from which the EBV was derived; thus, B + KYU-BL (X) indicates that normal B cells were co-cultivated with X-irradiated cells of the KYU-BL cell line. The BL cell lines that served as sources of the EBV isolates have been described in detail elsewhere (Rowe et al., 1985; Rooney et al., 1986) and were derived from patients in Algeria, Kenya, La Reunion, New Guinea and Western Europe. A further series of LCLs was established from foetal bone marrow (FBM lines) and foetal liver (FL lines) by infection with the B95.8 EBV isolate (C. G. Gregory, unpublished).

All cultures were grown in RPMI 1640 medium, supplemented with 10% foetal calf serum and penicillin/streptomycin antibiotics, and maintained at 37°C in humidified 5% CO₂. In some experiments, cell lines were chemically induced for 3 days with 20 ng/ml phorbol-12-myristate-13-acetate (TPA) and/or 1 mm-sodium butyrate.

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fluorescence test. At this stage, hybridomas of interest were recloned by limiting dilution and characterized further by immunoblotting experiments.

**ELISA.** Microtest plates were coated either with pKH548 LMP fusion protein or with pUR291 β-galactosidase by incubating each well with 250 ng protein in 50 μl 0.1 M-maleate buffer pH 9.5, for 16 h at 4°C. Excess protein-binding sites were blocked with a 1% solution of bovine serum albumin in PBS for 1 h at room temperature. The plates were then washed with PBS containing 0.1% Tween-20 detergent (PBS-Tween), incubated with test hybridoma supernatants for 2 h at room temperature, and again washed with PBS-Tween. Specifically bound antibody was detected by incubation for 2 h with horseradish peroxidase-conjugated goat antibodies to mouse Ig (Sigma) diluted 1:1500 in PBS-Tween, washing with PBS-Tween, and developing with o-phenylenediamine substrate.
RESULTS

Generation of monoclonal antibodies

HAT-resistant hybridoma cell colonies grew in 168/480 (35%) of the fusion cultures set up from 16 × 10⁶ immune spleen cells. The preliminary screen for antibody production, using ELISAs, showed that 40 of the hybridomas produced antibody reactive with the pKH548 fusion protein; of these, 15 were potentially LMP-specific since they did not react with β-galactosidase prepared from pUR291. These 15 hybridomas were subsequently tested in an immunofluorescence assay with methanol-fixed EBV-positive (Raji and Ag876) and EBV-negative (Louckes and BL41) BL cell lines; 3/15 did not react at all in immunofluorescence, 1/15 reacted both with the EBV-positive and with the EBV-negative cells, and 11/15 reacted with the two EBV-positive cell lines but not with either of the EBV-negative cell lines. Four of the latter 11 hybridomas (designated CS.1, CS.2, CS.3 and CS.4) were recloned, and further characterized. All four CS MAbs were shown by Ouchterloney tests to be IgG1 subclass antibodies, and they were only weakly reactive, if at all, with staphylococcal Protein A in ELISA tests.

Specificity for LMP fusion protein in immunoblots

The pKH548 LMP fusion protein and β-galactosidase were separated by SDS–PAGE and transferred to nitrocellulose filters for probing with antibodies (Fig. 1). When a polyclonal antibody raised against the LMP fusion protein (Hennessy et al., 1984) was used to probe these blots, a single band of 116K mol. wt. was seen in the β-galactosidase lane and three bands, two major (150K and 127K) and one minor (72K), were seen in the lane containing the LMP fusion protein. The multiple bands detected with the fusion protein were a characteristic of different batches of LMP fusion protein isolates, and they probably arose from specific proteolytic degradation of the complete fusion protein.

Each of the MAbs CS.1, CS.2, CS.3 and CS.4 reacted with at least one component of the pKH548 fusion protein preparation, but none showed any reactivity with β-galactosidase. CS.1 reacted strongly with both of the major pKH548 bands, whereas CS.2, CS.3 and CS.4 reacted only with the higher (150K) band. These data suggest that CS.1 recognizes an epitope on the LMP fusion protein that is distinct from those recognized by the other three MAbs.
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![Image](a)

![Image](b)

Fig. 2. Specificity of pooled CS. 1 to 4 MAbs for EBV-positive cells by indirect immunofluorescence upon methanol-fixed cytocentrifuge cell preparations. (a) BL2 cell line, established from a rare EBV-negative sporadic BL patient, shows no specific immunofluorescence above the background dull yellow staining of the occasional cell. (b) A normal EBV-positive LCL [B + B95-8(X)], established by infection *in vitro* of normal resting B cells with B95.8 virus, shows specific positive immunofluorescence whose intensity varies greatly between individual cells.

**Specificity of CS MAbs for EBV-infected cells by indirect immunofluorescence**

Fig. 2 illustrates the pattern and specificity of staining observed when using a pool of CS.1, CS.2, CS.3 and CS.4 MAbs (pool CS.1 to 4) in indirect immunofluorescence tests upon methanol-fixed cells. No specific reactivity was seen with EBV-negative cells (BL2; Fig. 2a), but LCL cells, derived by infection of normal resting B cells with the B95.8 strain of EBV *in vitro*, showed a characteristic speckled membrane staining in about 30 to 60% of the cells and often an intense cytoplasmic staining in less than 5% of the cells [B + B95.8(X); Fig. 2b]. On further testing either individually or as a pool, the four MAbs produced a similar pattern of staining on LCLs transformed by any of the following six virus isolates: from B95.8, CHEP-BL, ELI-BL, BL29, BL36 and WW1-BL cells. However one particular isolate (from BL18) gave unusual results; CS.1 and CS.2 gave typical staining of about 30% of B + BL18(X) cells, but both CS.3 and CS.4 gave barely detectable staining in less than 5% of the cells (data not shown).

**Specificity of CS MAbs for LMP in immunoblots of EBV-infected cells**

On the basis of this preliminary screening of the four CS MAbs in immunofluorescence assays, three cell lines were selected to provide extracts for subsequent immunoblotting studies; these were B95.8, B + BL18(X), and B + WW1-BL(X). Cell extracts from an EBV-negative BL cell line, BL31, were used as a negative control. As shown in Fig. 3, MAbs CS.1 and CS.2 readily detected a 57K LMP band in the B + WW1-BL(X) cells (a to c, lanes 4). When these two MAbs were pooled (c), additional weak reactivities were observed against 64K LMP bands in B + BL18(X) cells (lane 3) and B95.8 cells (lane 2). The control cell extract (lanes 1) gave no band. In contrast, MAbs CS.3 and CS.4 readily detected the LMPs in B + WW1-BL(X) cells and in B95.8 cells, but did not detect the corresponding protein in B + BL18(X) cells whether the antibodies were used separately (d and e) or pooled (data not shown). MAbs CS.3 and CS.4 also cross-reacted in immunoblots with two uncharacterized cellular proteins of 41K and 44K apparent molecular weights. When all four antibodies were pooled (Fig. 3f) there was additional cooperative binding such that the LMPs of all three virus isolates were readily detected.
Fig. 3. Immunoblots showing the reactivity of (a) CS.1, (b) CS.2, (c) pooled CS.1 and 2, (d) CS.3, (e) CS.4 and (f) pooled CS.1 to 4 MAbs with protein extracts from cells carrying one of three geographically distinct isolates of EBV. Lanes 1, BL31, an EBV-negative control BL cell line; lanes 2, B95.8 virus-producer cell line; lanes 3, LCL carrying BL18-derived EBV; lanes 4, LCL carrying the WW1-BL-derived EBV. The cell extracts were separated on a 7.5% acrylamide gel and probed with the individual CS MAbs as in Fig. 1. Pooled reagents CS.1, 2 and CS.1 to 4 were prepared by mixing equal parts of the diluted constituent MAbs. Molecular weight markers (×10⁻³) are indicated on the right.

Fig. 4. Immunoblots showing the specificity of the pooled CS.1 to 4 reagent with EBV-positive and -negative B cell lines. Molecular weight markers (×10⁻³) are indicated on the right.
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In further tests, this pooled reagent reacted with a major LMP band of 57K to 66K in immunoblots from each of 20 LCLs carrying geographically distinct virus isolates (Table 1). No equivalent band was seen in cell extracts from seven EBV-negative B cell lines, from tonsillar B and T cells, from foetal liver and thymus, from cultured foetal fibroblasts or from human leukaemic cell lines such as HSB2 and K562 (data not shown), though all such preparations contained the cross-reactive doublet of cellular proteins. Representative results in Fig. 4 illustrate the different patterns of LMP bands obtained with different virus isolates and show the extent of molecular weight variation of the protein.

LMP expression and the productive cycle in B95.8 cells

It was clear from Fig. 3 and 4 that B95.8 cells themselves or cell lines carrying the B95.8 strain of EBV displayed a major LMP band and three LMP-related lower molecular weight species. We sought to determine whether such species were merely breakdown products of the major band, or whether any corresponded to the truncated form of LMP which Hudson et al. (1985) had predicted to be expressed in B95.8 cells on entering the virus productive cycle.

For this purpose, immunoblots were prepared from a panel of foetal LCLs, all carrying the B95.8 virus strain but differing markedly in virus producer status; B95.8 cells themselves, harvested from a culture producing high virus yields, were also included for comparison. As shown in Fig. 5, there was a striking correlation between producer status (as reflected by immunoblotting for EA; see panel a) and the abundance of one particular LMP species of apparent molecular weight 55K (Tr-LMP, see panel b). The other two low molecular weight forms showed no such correlation. This strongly suggested that the 55K LMP band represented the truncated form of the protein expressed late in the B95.8 virus productive cycle. Interestingly, when the B95.8 cell line was examined at the single cell level by two-colour immunofluorescence, the 5 to 10% cells spontaneously activated into virus production and recognized by EA/VCA staining (Fig. 6a) were also among the most intensely labelled for LMP (Fig. 6b). This is consistent with enhanced expression of the truncated form of LMP being an accompaniment of virus replication in B95.8 cells.
Fig. 5. Immunoblots showing cell extracts from several B95.8 virus-transformed foetal cell lines and from the virus producer B95.8 cell line itself, probed (a) for expression of productive cycle antigens (particularly the EA complex) using a suitable human serum, and (b) for LMP expression using the pooled CS. 1 to 4 reagent as in Fig. 3. The position of the major LMP band and of the relevant truncated LMP (Tr-LMP) are indicated by arrows. Molecular weight markers ($\times 10^{-3}$) are indicated on the right.

Fig. 6. B95.8 cells stained by two-colour immunofluorescence (a) for EA/VCA expression using directly FITC-conjugated IgG human serum EE, and (b) for LMP expression using pooled CS.1 to 4 reagent and a TRITC-conjugated anti-mouse Ig second antibody. Arrows identify individual cells showing both FITC and TRITC labelling.

LMP expression in chemically induced cells

These results prompted us to examine whether a truncated form of LMP was associated with lytic infection by other EBV strains. As already apparent from Fig. 4, lower molecular weight species were detectable in immunoblots of at least some of the cell lines carrying other virus isolates, although their presence was not obviously related to virus producer status (data not shown); in fact, it correlated much more closely with the presence of an intense major LMP band (see Fig. 4).
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To examine this further, a number of low- or non-producer LCLs carrying different virus isolates were treated for 3 days with TPA and/or sodium butyrate at concentrations that optimally induce EA/VCA expression. Unexpectedly, it was found that such treatment regularly increased expression of the major LMP band; there was no selective amplification of any of the lower molecular weight species. For example, Fig. 7(a) shows the effect of a 3 day induction of B + BL59(X) cells and of B + OBA-BL(X) cells with TPA plus butyrate: in both cases, the major LMP band was considerably stronger after induction. Although lower molecular weight bands became detectable in the induced B + OBA-BL(X) cells, longer exposure of the gel revealed that

Fig. 7. (a) Immunoblots illustrating the induction of LMP in B + BL59(X) and B + OBS-BL(X) LCLs cultured in the presence of 20 ng/ml TPA and 1 mM sodium butyrate for 3 days. Cell extracts, prepared from non-induced control cultures (-) and from parallel cultures containing TPA and sodium butyrate (+), were probed with the pooled CS. 1 to 4 reagent as in Fig. 3. Molecular weight markers (×10⁻²) are indicated on the right. (b, c) B + OBA-BL(X) cells were induced as above, and then stained by two-colour immunofluorescence (b) for EA/VCA, and (c) for LMP as in Fig. 6. Arrows identify individual cells showing either FITC or TRITC labelling.
these were indeed present in trace amounts even before induction, again arguing against any selective effect upon one particular species of LMP.

In the above example, chemical induction of the cell lines increased the fraction of EA/VCA-positive cells from a very low background (0.1%) to between 1 and 2%. In view of the earlier results with B95.8 cells (see Fig. 6), we questioned whether the chemically induced increase in LMP expression selectively involved cells entering the lytic cycle. Two-colour immunofluorescence testing indicated that this was not the case. As shown in Fig. 7(b), induced cultures contained a subpopulation of lytically infected cells staining for EA/VCA. However, such cells did not overlap with the subpopulation showing the most intense staining for LMP (Fig. 7c). This was representative of results observed with each of eight different LCLs examined in this way, each carrying an individual EBV isolate. Even in non-induced cultures of these LCLs, cells spontaneously entering the lytic cycle were not part of the subpopulation strongly stained for LMP. Direct comparison of control and treated cultures for LMP expression, using the standard fluorescein-based assays, revealed that chemical induction had in fact increased the general intensity of LMP staining throughout the cell population (data not shown).

**DISCUSSION**

This report describes the generation of four MAbs, designated CS.1 to CS.4, which are specific for the LMP encoded by the BNLF1 reading frame of the EBV genome. The particular reactivities of the individual MAbs suggested that at least three different epitopes on the LMP protein were being recognized by (i) CS.1, (ii) CS.2 and (iii) CS.3 and CS.4. Thus, the reactivity of CS.1 was distinguished from that of CS.2, CS.3 and CS.4 by its binding pattern to partially degraded LMP fusion protein in immunoblots. Both CS.1 and CS.2 differed from CS.3 and CS.4 in their ability to detect LMP in three selected LCLs (each carrying a distinct isolate of EBV) in immunoblots and in immunofluorescence tests. Furthermore, in immunoblots CS.3 and CS.4 showed an unexpected cross-reactivity with normal cell proteins. The cross-reactive antigenic sites appear to be selectively exposed on the cellular proteins by the denaturing conditions of the immunoblotting technique, since no such cross-reactivity was seen by immunofluorescence with CS.3 and CS.4 on methanol-fixed cytocentrifuge cell preparations. The identity of these cellular proteins is not known.

There is only one other report of MAbs to LMP (Mann et al., 1985) and, of the three MAbs generated by these authors, only one (designated S12) was fully characterized. The S12 antibody appears to recognize an epitope distinct from those recognized by the CS MAbs, since (i) it reacts with both the 127K and the 150K components of the LMP fusion protein, like CS.1, (ii) it does not react with B + BL18(X) in immunoblots, like CS.3 and CS.4, yet does not cross-react with the normal cellular proteins which these antibodies detect and (iii) it works equally well in immunofluorescence on all LCLs, including B + BL18(X), as do CS.1 and CS.2 (M. Rowe, unpublished data).

There is clearly heterogeneity among the LMPs encoded by different EBV isolates, whether in terms of apparent size in SDS gels or in terms of reactivity with individual MAbs. The four antibodies described here, CS.1 to 4, provide a useful pooled reagent which is capable of detecting the LMPs encoded by each of 20 geographically distinct isolates. In this context, it is interesting to compare CS.1 to 4 with a recently described LMP-specific rabbit antiserum (Rowe et al., 1986) raised against a synthetic 10-mer oligopeptide corresponding to part of a direct repeat sequence in the C-terminal region of the B95.8 LMP. This antiserum recognized the LMPs encoded by most EBV isolates tested but not that present in B + WW1-BL(X) cells (Rowe et al., 1986). Since the WW1-BL EBV encodes the smallest LMP yet reported, it is quite possible that this particular product lacks the domain of reiterated amino acids which form the epitope for the anti-peptide antibodies.

Hudson et al. (1985) detected two mRNA species arising from the LMP gene in B95.8 cells. The most abundant transcript was predicted to encode the full length protein whose postulated structure, with six hydrophobic transmembrane domains near the N terminus and a large intracytoplasmic C-terminal region (Fennewald et al., 1984), has now been experimentally confirmed (Hennessy et al., 1984; Liebowitz et al., 1986). A second transcript, found only in
association with lytic virus replication, was predicted to encode a truncated product lacking the
original N terminus and four of the N-terminal transmembrane domains. In the present report,
we provide evidence to suggest that one of the lower molecular weight forms of LMP, seen in
immunoblots at an apparent size of 55K, is indeed the product of this second mRNA. There is a
clear correlation between the abundance of this particular LMP band and the virus producer
status of cell lines carrying the B95.8 virus isolate; such a correlation is not seen for the other
smaller LMP bands, which may well be breakdown products of the full length protein. Two
LMPs, apparently corresponding to the full length and truncated forms, have in fact been found
in purified B95.8 virion preparations, raising the possibility of some structural role in virus
assembly (Mann et al., 1985). Even in the highly productive B95.8 cell line, only 5 to 10% of the
cells are in a lytic cycle and recognizable by EA/VCA staining. It was therefore striking that in
two-colour immunofluorescence, this EA/VCA-positive population contained within it all the
cells staining most intensely for LMP.

When such analysis was extended to cell lines carrying other EBV isolates, lower molecular
weight forms of LMP were detectable in some but not all cases. Circumstantial evidence
suggests that these were breakdown products, rather than analogues of the B95.8 truncated
LMP, since their detectability correlated better with the intensity of the accompanying major
band than with the virus producer status of the particular cell line. In fact, most LCLs in the
study were either non-productive or contained small numbers of EA/VCA-positive cells so that
any lytic cycle form of LMP could have gone undetected. For this reason, such lines were
induced towards virus production by treatment with TPA and/or butyrate, but there was still no
selective amplification of a lower molecular weight species. On the contrary, chemical induction
causd a marked increase in expression of the major LMP band (as well as of any lower
molecular weight forms which had been initially present). When analysed at the single cell level,
this increased expression was reflected generally throughout the culture and it was not restricted
to those cells induced into lytic cycle; indeed, in contrast to the results with the B95.8 cell line,
lytically infected cells in the above LCLs were not brightly stained for LMP.

Clearly the regulation of LMP expression in EBV-transformed cells is more complex than has
been hitherto imagined. Of the results presented here, one must be careful to distinguish
between findings which may be unique to the B95.8 virus strain, or even to the B95.8 cell line
itself, and findings which may be more generally relevant. In this regard, we view the
inducibility of LMP expression by TPA/butyrate as being of particular significance. The
phenomenon has been observed with many LCLs transformed in vitro carrying different EBV
isolates, and more recently in several lines of BL origin with no concomitant change in the
expression of other EBV latent gene products such as EBNA-1 and EBNA-2 (M. Rowe,
unpublished observations). Moreover, the up-regulation of LMP occurs in many cells within a
culture and is not restricted to cells entering the lytic cycle. This unexpected response to
TPA/butyrate suggests that the protein may be under a regulatory control distinct from those
governing other known EBV latent gene products. The heterogeneous distribution of LMP
among the individual cells in any transformed line, perhaps reflecting some marked cell cycle
dependence of expression, may be another manifestation of this same distinct regulatory
control.

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