Different Epstein-Barr virus–B cell interactions in phenotypically distinct clones of a Burkitt’s lymphoma cell line

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Epstein-Barr virus (EBV)-positive Burkitt’s lymphoma (BL) biopsy cells and early passage BL cell lines have been reported as showing an unusual type of virus–cell interaction; at least two EBV latent proteins appear not to be expressed. Serial passage of such lines is often accompanied by a broadening of virus latent gene expression and a corresponding change in the cell surface/growth phenotype. The sequence of events, both viral and cellular, involved in this transition needs to be defined properly. In the present work, phenotypically distinct cell clones have been derived from early passage cultures of a BL cell line in phenotypic transition, thereby giving access to relatively stable cell populations through which the different EBV–B cell interactions within the parental line can be studied. Clones retaining the original BL biopsy cell phenotype (CD10/CD77-positive, activation antigen/adhesion molecule-negative) expressed the virus-encoded nuclear antigen EBNA 1 but not any of the other known latent proteins, EBNAs 2, 3a, 3b, 3c, -LP and latent membrane protein (LMP). Other clones which had developed an LCL-like phenotype (CD10/CD77-negative, activation antigen/adhesion molecule-positive) now expressed all the above latent proteins and also contained significant numbers of cells in lytic cycle. Phenotypic change occurring within the parental BL cell line itself was initiated in a small subpopulation of cells in which the virus-encoded proteins EBNA 2 and LMP were transiently induced to an unusually high level of expression; this was accompanied by the first detectable changes in cell surface phenotype, namely the increase of cellular adhesion molecules. Some control over EBNA 2/LMP expression then appeared to be re-imposed since the presumed clonal descendents of these cells stably expressed EBNA 2 and LMP at much reduced levels typical of those seen in conventional LCLs.

Introduction

There are at least two types of non-productive infection which Epstein–Barr virus (EBV) can establish in human B cells. By far the best characterized is that produced by experimental infection in vitro of normal resting B cells, where the cells are activated into cycle and grow out to give permanent virus genome-positive lymphoblastoid cell lines (LCLs). Such growth transformation is initiated and maintained through the expression of a limited number of viral gene products, the EBV ‘latent proteins’ (reviewed by Dambaugh et al., 1986; Knutson & Sugden, 1989). These include the nuclear antigens, EBNAs 1, 2, 3a, 3b, 3c and -LP, whose expression is controlled from one of two transcriptional promoters in the BamHI CW region of the virus genome (Speck & Strominger, 1989), and a latent membrane protein (LMP) whose expression is under separate transcriptional control. These seven virus-encoded latent proteins can now be individually identified using monospecific or monoclonal antibody (MAb) preparations; an eighth latent gene product, terminal protein or LMP2, has been predicted to exist from cDNA cloning work but has not yet been identified unequivocally (Laux et al., 1988; Sample et al., 1989).

The consistent pattern of EBV gene expression seen in LCLs is matched by an equally consistent pattern of cellular gene expression. Thus, whilst target cells at a number of different stages of B cell differentiation can be transformed by EBV in vitro, the cell lines so produced all display the same ‘lymphoblastoid’ phenotype as defined by cell surface marker analysis and growth in cell aggregates (Katamine et al., 1984; Ernberg et al., 1987; Gregory et al., 1987a, 1988a). In particular, such in vitro transformed lines reproducibly express high levels of the B cell activation antigens CD23, CD30, CD39 and CD70 (Thorley-Lawson & Mann, 1985; Rowe et al., 1985), and
of the cellular adhesion molecules LFA-1 (CD11a/18), ICAM-1 (CD54), LFA-3 (CD58) (Gregory et al., 1988b) and the lymphocyte homing receptor CD44 (C. D. Gregory, unpublished observations). It is significant that these same markers, which are either absent or found at very low levels on resting B cells, are also induced transiently to high levels when such cells are activated into short-term proliferation by antigenic or mitogenic stimulation (Thorley-Lawson & Mann, 1985; Gordon, 1989). Thus EBV appears to immortalize human B cells by constitutively driving a particular programme of cellular gene expression which is induced only transiently during physiological B cell activation. This is supported by the observation that EBV latent proteins, notably EBNA 2 and LMP, when expressed individually in human B cells can activate the expression of particular sets of cellular genes and thereby reproduce particular facets of the LCL phenotype (Wang et al., 1987a; Wang et al., 1988).

A second type of non-productive EBV infection in human B cells, distinct from that seen in LCLs, has recently been identified from studies on EBV-positive Burkitt's lymphoma (BL), a B cell malignancy which is most common in equatorial regions of Africa and New Guinea. Here the tumour biopsy cells and early passage BL cell lines were clearly EBNA 1-positive but did not express detectable levels of two other viral latent proteins, EBNA 2 and LMP (Rowe et al., 1987a). Moreover, the EBV-positive tumour cells displayed two cell surface markers, CD10 (CALLA) and CD77 (BLA), not usually seen on LCLs and were negative for several of the cellular activation antigens and adhesion molecules which form an integral part of the LCL phenotype (Rooney et al., 1986). Interestingly this unusual form of EBV-B cell interaction was not always stably retained in vitro. Thus continued passage of the BL cell lines was often accompanied by a broadening of viral gene expression such that EBNA 2 and LMP became detectable, and by a coincident change in cell surface/growth phenotype towards that seen in LCLs (Rowe et al., 1987a).

The speed with which these changes occurred on culturing many EBV-positive BL biopsies, and the paucity of good serological reagents then available against EBV latent proteins, made it difficult to define in any detail the different virus–cell interactions involved. In the present work we have established individual clones of cells from an early passage BL cell line at a time when the line was changing towards an LCL phenotype. Phenotypically distinct clones are compared here for EBV latent gene expression and for cell surface marker profile, and the process of phenotypic change occurring within the parental BL cell line is monitored at the single cell level.

Methods

Cell line establishment and cloning. Using published methods (Rooney et al., 1986), the MUTU-BL cell line was established in culture from an EBV genome-positive BL biopsy obtained via Dr H. Rupani, ENT Department, Kenyatta National Hospital, Nairobi, from a high BL incidence area of Kenya. Proliferating cells were observed within 7 days of explanting biopsy cells in culture and the first passage was made on day 22. At this stage the cultures were growing as a carpet of small round cells, no multicellular aggregates being present. The cells were mononclonal for surface immunoglobulin expression (μκ-) and carried a characteristic t(8:14) chromosomal translocation indicative of BL origin. As described for certain other recently established EBV genome-positive BL cell lines (Rooney et al., 1986), the MUTU-BL line showed rapid transition from a group I (BL-like) cell surface/growth phenotype to a group III (LCL-like) phenotype within its first 20 passages in vitro. The phenotypically altered cells which dominate late passage cultures retained the immunoglobulin and chromosomal markers characteristic of the original tumour, and so were clearly derived from the malignant cell population and not from contaminating normal B cells which might have been present in the biopsy initially.

For cloning at passage 10, a cell suspension of the MUTU-BL line containing single cells and small clumps of aggregated cells was carefully layered onto a cushion of foetal calf serum (FCS) and fractions enriched for single cells or for small clumps were obtained after brief gravity sedimentation. 'Single cell' and 'clumped cell' fractions were washed, then each dispersed to give a single cell suspension in culture medium (RPMI 1640, 2 mM-glutamine, 100 μg/ml streptomycin, 100 international units/ml penicillin and 10% selected FCS) and seeded onto an underlay of human embryo fibroblasts in microtest plate wells at an average of 1 or 0.3 cells/well. The cultures were maintained by regular refeeding for 4 to 5 weeks and observed for clonal outgrowth of BL cells.

Cell surface immunofluorescence and phenotypic grouping of clones. Indirect immunofluorescence staining of viable cells was carried out using MAbs as described (Gregory et al., 1988a). Surface immunofluorescence was either assessed by microscopy or measured on a fluorescence-activated cell sorter (FACS 440, Becton-Dickinson). FACS analyses are summarized either as mean fluorescence intensities or as ratios of fluorescence intensity: cell volume as described (Gregory et al., 1988a).

The phenotypic groups were defined according to criteria established in earlier work (Rowe et al., 1985). Briefly, group I lines were designated as those growing as dispersed cell suspensions and co-expressing the BL markers CD10 (CALLA) and CD77 (BLA); group II cultures contained small multicellular aggregates of cells and expressed, in addition to CD10 and CD77, one or more of the LCL-associated 'activation' markers CD23, CD30, CD39 and CD70; group III lines grew as large, multicellular clumps and no longer expressed CD10 or CD70, but expressed high levels of all the LCL-associated markers. The CD nomenclature is based on the 4th Workshop on Leukocyte Differentiation Antigens, Vienna (1989). Surface immunofluorescence analysis of representative MUTU-BL clones was extended to include the pan-B markers (CD19, CD20 and CD40), additional 'activation' markers (CD21, B1 and G28.8), and cellular adhesion molecules (LFA-1, ICAM-1, LFA-3 and CD44). Details of the individual MAbs used in this work are included in Table 1.

Dichromatic cell surface immunofluorescence was performed using the FACS 440 and fluorescein isothiocyanate (FITC) and phycoerythrin (PE) labels. Viable cells were labelled for CD77 using the rat IgM MAbs 38.13 and FITC-conjugated goat anti-rat IgM (Nordic Pharmaceutical). A variety of other antigens (ICAM-1, LFA-3, CD10 and CD39) were simultaneously detected by subsequent labelling of the
cells with appropriate mouse IgG1 MAbs which were visualized using biotinylated goat anti-mouse IgG1 and streptavidin–PE (Southern Biotechnology Associates; 1070-08 and 7100-09).

Conjugate formation assay. The capacity of BL clones to bind to T cells through the LFA-1/ICAM-1 and CD2/LFA-3 conjugation pathways was determined as described (Gregory et al., 1988a). Briefly, target BL cells labelled with hydroxidine were cocentrifuged with FITC-labelled T cells, incubated for 6 min at 37 °C and then dispersed by vortexing. T cells were taken from polyclonal cytotoxic T lymphocyte (CTL) lines of normal EBV-seropositive individuals raised against autologous LCLs. The percentage of T cells forming conjugates with target cells was calculated from FACS analysis of free and bound cells.

Detection of EBV proteins by Western blotting. Separation of cell extracts on Laemmli discontinuous polyacrylamide gels, transfer onto nitrocellulose sheets, and subsequent detection of specific EBV-encoded proteins by antibodies and 121-labelled Protein A was carried out as described previously (Rowe et al., 1987a). For each sample lane 10⁶ cells were solubilized in electrophoresis buffer and separated on a 7-5% polyacrylamide resolving gel. The nitrocellulose blots were probed either with murine MAbs followed by rabbit anti-mouse IgG polyclonal antibodies, or with human antibodies. The human sera LGL and RS22 were used as polyspecific anti-EBNA sera. Serum LGL reacts with all of the EBNAs with the exception of EBNA-LP, whereas serum RS22 reacts with all six EBNA serotypes and also with some early lytic cycle antigens. RS22 was also used as a source of monospecific antibodies (anti-EBNA 3a, anti-EBNA 3b or anti-EBNA 3c) which were isolated by affinity chromatography on columns of immobilized β-galactoside fusion proteins containing sequences of either EBNA 3a, EBNA 3b or EBNA 3c; these reagents have been fully characterized (Rowe et al., 1989). A β-galactosidase-EBNA-LP fusion protein (Wang et al., 1987a) was similarly used to isolate antibodies to EBNA-LP from another human serum, LF. The MAbs CS. 1 to 4 (Rowe et al., 1987b) and PE2 (Young et al., 1989) were used to detect LMP and EBNA 2 respectively.

Immunofluorescence on fixed cells. Cell smears for the detection of viral and cellular antigens by indirect immunofluorescence were prepared as follows: cells were washed once in phosphate-buffered saline (PBS), air-dried on microscope slides, fixed in methanol at -20 °C for 10 min, and air-dried before storing at -20 °C until required.

For performing the three-step double FITC labelling for sensitive analysis of LMP and EBNA 2 expression (using MAbs CS. 1 to 4 and PE2 respectively), the slides were rehydrated for 15 min in PBS containing 10% normal rabbit serum (PBS-NRS), and then incubated for 2 h at 37 °C with MAb-containing culture supernatant diluted 1:1 in PBS-NRS, followed by 1 h with FITC-conjugated goat anti-mouse IgG (Sigma F0257) diluted 1:50 in PBS-NRS containing 10% normal EBV-seropositive human serum (PBS-NRS/NHS). The slides were then incubated for a further 1 h at 37 °C with FITC-conjugated rabbit anti-goat Ig (Sigma F2016), also diluted 1:50 in PBS-NRS/NHS. Finally they were mounted in DABCO-based anti-fading mountant (Johnson et al., 1982) and visualized by fluorescence microscopy.

For dichromatic fluorescence of double-labelled cells, slides were rehydrated in PBS containing 10% normal goat serum (PBS-NGS) before incubation with mixtures of IgG1 and IgG2a subclass MAbs for 2 h at 37 °C. In these experiments, the Sl2 anti-LMP MAb (at a 1:200 dilution of ascitic fluid in PBS-NGS) was chosen for the visualization of LMP since it is an IgG2a antibody (Mann et al., 1985). The IgG1 MAbs PE2 (at a 1:1 dilution of culture supernatant) or RR/1 (Rothlein et al., 1986; at a 1:2000 dilution of ammonium sulphate precipitation of ascites) were used to detect EBNA 2 or the intercellular adhesion molecule ICAM-1 respectively. After washing in PBS, the slides were incubated for 2 h at 37 °C with a mixture of FITC-conjugated goat anti-mouse IgG1 and rhodamine-conjugated goat anti-mouse IgG2a antibodies (Southern Biotechnology Associates; 1070-02, 1080-03), both diluted 1:20 in PBS-NGS/NHS.

Results

Establishment of phenotypically distinct clones of BL cells

Cloning of the MUTU-BL cell line was carried out at the tenth in vitro passage, at a time when the drift towards an LCL-like phenotype had begun. Lymphoblastoid cells showing cytoplasmic projections and a tendency to grow in small clumps were apparent (Fig. 1a) against a background of smaller round cells morphologically typical of the original biopsy population. Prior to cloning, sedimentation of the MUTU-BL cell suspension under gravity afforded a crude separation into one fraction containing mostly single cells (i.e. the bulk of the cell population) and another which was enriched for the cells growing in small clumps. Seeding of the ‘single cell’ fraction onto feeder layers at 1 and 0.3 cells/well gave a cloning efficiency of approximately 50%; some of these clones (nine out of 32 at 1 cell/well and five out of 10 at 0.3 cells/well) grew as a carpet of small round cells (Fig. 1b), whereas the rest grew out as tight clumps of cells with a more lymphoblastoid morphology (Fig. 1c). Parallel seeding of the ‘clumped’ fraction, after vigorous pipetting to produce a single cell suspension, gave a cloning efficiency of greater than 80% and virtually every clone grew as tight clumps (52 out of 53 at 1 cell/well and 21 out of 21 at 0.3 cell/well). Six representative clones of each type were expanded for more detailed analysis.

Phenotypic analysis of BL clones

The two sets of clones with different growth patterns also showed markedly different cell surface phenotypes. Representative data on three clones of each type are shown in Table 1 alongside data obtained in parallel on three reference LCLs established by EBV-induced transformation of peripheral blood B cells from healthy adult donors. The results are derived from immunofluorescence staining with specific MAbs against defined surface markers and are expressed as mean fluorescence intensity values obtained by FACS analysis.

Clones growing as single cells displayed a cell surface profile essentially like that described for BL biopsy cells and for certain BL cell lines which retain the group I (i.e. biopsy cell) phenotype in vitro. These clones were positive for the tumour markers CD10 and CD77 but lacked detectable expression of a wide range of B cell activation antigens (CD21, CD23, CD30, CD39, CD70, BB1 and G28.10) and cellular adhesion molecules.
Fig. 1. Cell growth phenotype of (a) the parental MUTU-BL cell line at the time of cloning (passage 10) showing the presence of a few lymphoblastoid cells, with cytoplasmic projections (arrowed) and usually growing in small clumps, against a background of smaller round cells: (b) a group I MUTU-BL cell clone with a single-cell pattern of growth; (c) a group III MUTU-BL cell clone with a clumpy pattern of growth. Bar marker represents 40 μm.

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with clumpy growth still expressed some CD10 and CD77 as well as the activation antigen/adhesion molecule markers (i.e. showed the intermediate group II BL cell phenotype) but on serial passage these clones soon lost the two tumour-associated markers and assumed a group III phenotype (data not shown). Note that all clones expressed the pan-B cell markers CD19, CD20 and CD40, although it was interesting to note that CD40 levels were consistently higher on the group III than on the group I cells (Table 1). Expression of other markers such as HLA class I and class II antigens detected by MAbs W6.32 and H143 respectively (both obtained from the 4th Workshop on Leukocyte Differentiation Antigens) was high on the two types of clone (data not shown).

Two additional findings indicated that the cellular adhesion molecules which were expressed on group III clones were functionally active. Firstly all group III clones grew in tight clumps and this clumping could be inhibited by saturating concentrations of MAbs against either ICAM-1 or LFA-1, confirming that such homotypic cell adhesion was indeed mediated via the ICAM-1/LFA-1 pathway described for other B cell lines (Rothlein & Spinger, 1986). Secondly, when tested for their ability to form conjugates with activated T cells in quantitative dichromatic FACS assays, group III clones gave yields of conjugates which were within 75 to 100% of those shown by reference LCLs and such conjugate formation was blocked in the presence of pooled MAbs to LFA-1 and LFA-3; in the same assays group I clones gave very few if any conjugates (<5% of LCL values). The conjugate-forming capacity of group III cell clones therefore reflects the increased availability on the group III cell surface of ICAM-1 (the ligand for LFA-1 on T cells) and LFA-3 (the ligand for CD2 on T cells), both pathways being involved in this form of heterotypic cell adhesion (Shaw et al., 1986).

**EBV protein expression in BL clones**

Immunoblots of protein extracts from the two types of phenotypically distinct clones were probed either with selected polyclonal human sera, with monospecific antibody preparations or with relevant MAbs in order to characterize the pattern of EBV gene expression. Fig. 2 illustrates the results obtained using a human serum RS22 with reactivity against the EBNA 1, EBNA 2 (type A) and EBNA 3a, 3b and 3c (type A) family of latent proteins, as well as reactivity against the EA-D antigen complex of the virus lytic cycle. Whereas only EBNA 1 was detectable in group I clones, the group III clones consistently expressed all five EBNA species and also the lytic cycle antigens. Conventional EA and VCA immunofluorescence staining in fact showed that group I

(ICAM-1, LFA-3 and CD44), the only exception being trace expression of LFA-1. In contrast the vast majority of MUTU-BL clones showing clumpy growth, as illustrated in Table 1, were negative for CD10 and CD77 but displayed LCL-like levels of the various activation antigens and cell adhesion molecules. Such clones therefore showed the group III (LCL-like) phenotype to which the parental MUTU-BL cell line itself moved within 20 passages in culture. A few MUTU-BL clones
Table 1. Cell surface phenotype of MUTU-BL cell clones assayed by FACS analysis

<table>
<thead>
<tr>
<th>Category</th>
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<th>Antigen†</th>
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<td>CD44</td>
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* References to MAbs are as follows: B4 (Nadler et al., 1983); B1 (Stashenko et al., 1980); G28.5 (Clark & Ledbetter, 1986); 55 (Gregory et al., 1987b); 38.13 (Wiels et al., 1981); MHM6 and AC2 (Rowe et al., 1982); Ki-1 and Ki-24 (Schwab et al., 1982); MHM24 and MHM23 (Hildreth et al., 1983); RR1/1 (Rothlein et al., 1986); TS2/9 (Sanchez-Madrid et al., 1982); Hermes-1 (Jalkanen et al., 1986); Bu-34, BB1 and G28.10 were from the 4th Workshop on Leukocyte Differentiation Antigens, Vienna (1989).
† Cluster of differentiation (CD) nomenclature in accordance with the 4th Workshop on Leukocyte Differentiation Antigens, Vienna (1989).
‡ NC, Not clustered.

clones were virtually devoid of lytically infected cells (<0-1% positive) whereas 1 to 10% of the cells in group III clones were EA/VCA positive.

These clear differences between group I and group III clones were confirmed with respect to EBNA 2 expression both by immunoblotting and by immunofluorescence using the specific MAb PE2 (data not shown, but see Fig. 4) and with respect to EBNA 3a, 3b and 3c expression by immunoblotting with monospecific affinity chromatography-purified human antibodies (Fig. 3a). As the sixth latent nuclear antigen, EBNA-LP, runs in the same position as the EA-D antigen complex on SDS-PAGE gels, it was necessary to use affinity chromatography-purified EBNA-LP antibodies from human serum to probe for expression of this protein. As shown in Fig. 3(b), all group I clones were negative for EBNA-LP whereas all group III clones were positive. Finally, probing immunoblots with the specific MAbs CS. 1 to 4 (Fig. 3c) indicated that detectable LMP expression was similarly confined to the group III clones.

Each of the above group I clones was examined for retention of the capacity to encode the various EBV latent proteins by treatment with the DNA demethylating agent 5'-azacytidine. The use of the drug in this context was first suggested by the work of Masucci et al. (1989). Within 5 days of exposure, all group I clones showed easily detectable levels of EBNA 2 and LMP; this is illustrated in Fig. 4 by an immunoblot of drug-treated compared to untreated clones which has been probed with a mixture of the PE2 and CS. 1 to 4 MAbs. Expression of EBNA 3a, 3b, 3c and -LP was likewise induced in group I clones by 5'-azacytidine (data not shown).

Analysis of phenotypic change in the MUTU-BL cell line

Subsequent experiments sought to examine the process of phenotypic change as it occurred with serial passage of the parental MUTU-BL cell line itself. First we were interested to ask whether a culture in the intermediate stage of transition and classified as phenotype group II (i.e. positive for CD10/CD77 and for activation antigens) actually contained individual cells which co-expressed both sets of markers or was composed of separate subpopulations of group I (CD10/CD77-positive) and group III (activation antigen-positive) cells.

Dichromatic immunofluorescence studies were conducted using FITC labelling for CD77, defined by the
Fig. 2. Immunoblot probed for the EBV latent proteins EBNAs 1, 2, 3a, 3b and 3c and for the EA(D) complex of the virus lytic cycle in whole cell protein extracts of five group I and five group III MUTU-BL cell clones. Control tracks include cell extracts from the EBV-negative human B lymphoma cell line BJAB and from an LCL generated by in vitro transformation of normal human B cells using EBV rescued from the parental MUTU-BL cell line. The blot was probed with human serum RS22. The relative migration of standard Mr marker proteins is indicated to the left of the blot.

rat MAb 38.13, and PE labelling for any one of a series of second markers defined by mouse MAbs. Fig. 5 shows a representative set of FACS displays obtained when a MUTU-BL cell culture at intermediate passage was analysed in this way, levels of FITC and PE labelling being shown on a logarithmic scale along the vertical and horizontal axes respectively. Compared to background signals (Fig. 5a), double labelling for CD77 and ICAM-1 (Fig. 5b) showed that most cells in the culture were CD77-positive but that the CD77-negative subpopulation could be divided further into ICAM-1 negative and weakly positive fractions; in addition there was a separate subpopulation of CD77-negative ICAM-1-positive cells. Double labelling for CD77 and LFA-3 (Fig. 5c) gave a very similar picture with CD77-positive/LFA-3-negative, CD77-positive/LFA-3-positive (weakly), and CD77-negative/LFA-3-positive subpopulations. In contrast, double labelling for CD77 and a B cell activation antigen such as CD39 (Fig. 5d) showed virtually no overlap between the CD77-positive and the CD39-positive subpopulations. Additional experiments confirmed that the CD77-positive population above was indeed positive for the other BL marker CD10 (data not shown). Up-regulation of adhesion molecule expression on MUTU-BL cells therefore represents an early feature of phenotypic change, which can occur without loss of the original BL markers; subsequently the cells appear to switch to a group III phenotype such that the BL markers are lost either before or simultaneously with the appearance of B cell activation antigens.
Fig. 3. Immunoblots similar to that shown in Fig. 2 but probed with (a) antibodies to EBNA 3a (top panel), EBNA 3b (centre panel) and EBNA 3c (bottom panel) affinity chromatography-purified from human serum RS22; (b) antibodies to EBNA-LP affinity chromatography-purified from human serum LF; (c) MAbs CS.1 to 4 to LMP. The cross-reactivity of CS.1 to 4 in immunoblots with a doublet of low $M_r$ cellular proteins confirms protein loading in each lane.
The process of phenotypic change in the MUTU-BL cell line was similarly analysed in terms of EBV latent gene expression. Fig. 6 shows the results obtained when successive protein samples taken from the biopsy cell stage through to passage 195 of the parental cell line were probed either with human serum LGL to detect expression of the various EBNA proteins (Fig. 6a) or with the LMP-specific MAbs CS.1 to 4 (Fig. 6b). Whereas the biopsy cells expressed only EBNA 1, low levels of EBNA 2 and LMP were detectable in the derived cell line as early as passage 3. We noted that the relative amounts of EBNA 2 and LMP rose quickly thereafter such that protein extracts made at passage 10 or 13 contained as much EBNA 2 and LMP as the long-established line (passage 195).

This was an unexpected finding in view of the fact that the passage 10 and 13 cultures were still dominated by cells with a group I phenotype (Fig. 1 and data not shown), whereas the late-passage cultures were exclusively made up of group III cells. Immunofluorescence staining for EBNA 2 and LMP was performed on cell preparations from the same successive subcultures of MUTU-BL as used above, and the percentage of positive cells observed on each occasion is recorded beneath the relevant tracks in Fig. 6. This confirmed that expression of these viral proteins was confined to a small subpopulation (<8%) of the cells up to at least passage 13. However, as shown in Fig. 7, the intensity of the fluorescence staining for EBNA 2 and for LMP was very much greater on the antigen-positive cells in early passage cultures compared to later passage cultures where essentially all cells were EBNA 2-positive and at least the majority LMP-positive; indeed the EBNA 2 and LMP staining patterns in later passage cultures were indistinguishable from those seen in conventional LCLs.

Dichromatic immunofluorescence staining of the MUTU-BL cell line at passage 6 revealed that these high levels of EBNA 2 and LMP were being expressed by the same small subpopulation of cells (Fig. 8a). Furthermore, using ICAM-1 staining as an early marker of...
phenotypic change, it was apparent that the above LMP-positive cells coincided with the ICAM-1-positive subpopulation (Fig. 8b).

Discussion

The derivation of phenotypically distinct clones (Table 1, Fig. 1) from an early passage BL cell line during its transition from a group I (biopsy-like) to a group III (LCL-like) phenotype has allowed a detailed study of the different EBV–B cell interactions involved. Our earlier study using a panel of different BL cell lines at different stages of phenotypic transition indicated that expression of at least two viral latent gene products, EBNA 2 and LMP, was phenotype-dependent (Rowe et al., 1978a). The present work using MUTU-BL cell clones and an improved panel of serological reagents, makes it clear that six latent gene products, EBNA's 2, 3a, 3b, 3c, -LP, and LMP, are down-regulated in BL cells with a biopsy-like group I phenotype; only EBNA 1, the viral protein known to be involved in maintenance/replication of the episomal viral genome (Yates et al., 1984), is detectable in such cells (Fig. 2 and 3). Furthermore group I cells showed no spontaneous movement of cells into the viral lytic cycle (Fig. 2). We are confident that this type of virus–cell interaction is generally true of group I BL cells and is not peculiar to MUTU-BL; thus re-analysis of

Fig. 5. Dichromatic flow cytometric analysis of an intermediate passage culture of the parental MUTU-BL cell line following staining for CD77 visualized with FITC and a second antigen visualized with PE. Samples were analysed on a FACS 440 and the data shown are log/log contour plots from 20000 events. (a) Control FITC versus control PE; (b) CD77 versus ICAM-1; (c) CD77 versus LFA-3; (d) CD77 versus CD39. Quadrants (dotted lines) indicate: I, single-labelled CD77-positive cells; II, double-labelled cells; III, background labelling; IV, single-labelled ICAM-1-positive, LFA-3-positive or CD39-positive cells.
Fig. 6. Immunoblots of wholecell protein extracts from MUTU-BL biopsy cells and from serial passages (e.g. pass. 10) of the derived MUTU-BL cell line as shown; control tracks contained extracts of the EBV-negative human B lymphoma cell line BL30 and of the EBV-transformed LCL X50-7. Blot (a) was probed with human serum LGL which reacts with EBNA 1, 2, 3a, 3b and 3c; (b) was probed with the LMP-specific MAbs CS.1 to 4. For each of the above cell preparations, the percentage of cells expressing EBNA 2 (data beneath blot a) or LMP (data beneath blot b) was determined using the sensitive three-step immunofluorescence assay. Note that the cross-reactivity of CS.1 to 4 with a doublet of low Mr cellular proteins in the immunoblots (blot b) does not interfere with the detection of LMP with these MAbs in immunofluorescence tests on methanol-fixed cells: the immunofluorescence has previously been shown to be exclusively due to LMP reactivity.
several BL cell lines identified in earlier work as retaining a group I phenotype (Rowe et al., 1987a) has similarly shown down-regulation of all the known EBV latent proteins except EBNA 1 and little if any lytic cycle antigen expression (M. Rowe, unpublished results). Our earlier suggestion that one or more proteins of the EBNA 3 family might be expressed in group I cells (Rowe et al., 1986) was based on results obtained with one poorly characterized human serum; the present affinity chromatography-purified antibody preparations monospecific for EBNA 3a, 3b or 3c (Fig. 3a) clearly show that expression of all three of these proteins is phenotype-dependent.

Earlier observations have suggested that the unusually restricted pattern of viral latent protein expression in group I BL cells is reflected, at least for LMP, at the level of gene transcription (Rowe et al., 1987a). If this is also true of the EBNA transcripts, then it implies the

Fig. 7. Representative immunofluorescence (upper) and corresponding phase-contrast (lower) photomicrographs of the parental MUTU-BL cell line at passages 13 (a and c) and 196 (b and d) after staining for EBNA 2 with the MAb PE2 (a and b) or LMP with the MAbs CS.1 to 4 (c and d) using the sensitive three-step immunofluorescence assay.
existence of transcriptional controls permitting selective expression of EBNA 1. Although this might be achieved by selective splicing, it also raises the possibility of a second transcriptional promoter for the EBNA 1-encoding BKRF1 reading frame which is not shared with the other EBNAs. The fact that 5'-azacytidine, a DNA demethylating agent, can rapidly induce the full spectrum of EBV latent gene expression in group I BL cells (Masucci et al., 1989 and Fig. 4), suggests that hypermethylation at critical areas of the viral and/or cellular genome may be associated with the unusually restricted form of EBV infection in such cells.

The present findings reinforce the view that EBV-positive BL cells in vivo, and group I BL cell lines in vitro, display a form of virus–cell interaction which is quite distinct from that seen in in vitro transformed LCLs. Thus in the absence of virus-encoded effector proteins such as EBNA 2 and LMP which are known to be capable of inducing cellular change towards the LCL phenotype (Wang et al., 1987a; Wang et al., 1988), group I cells maintain a surface profile characterized by the absence of activation antigen/adhesion molecule expression. In many BL cell lines, however, this form of virus–cell interaction appears to be inherently unstable and on
serial passage there is a broadening of viral gene expression accompanied by changes in cellular phenotype. The development of EBNA 2-specific and LMP-specific MAbs which work in immunofluorescence as well as immunoblotting has allowed the process of phenotypic change in BL cell lines to be analysed at the single cell as well as at the whole culture level. This proved important in view of the surprising finding that early passage cultures of the MUTU-BL cell line (passages 10 and 13) containing relatively few phenotypically altered cells appeared to show as much EBNA 2 and LMP in immunoblots as did late passage cultures where essentially all the cells had assumed a group III (LCL-like) phenotype.

Immunofluorescence staining showed that individual cells in early passage cultures expressed unusually high levels both of EBNA 2 and of LMP, far greater than those seen in late passage cultures where virtually all cells were antigen-positive but where the level of expression per individual cell had fallen to that seen in standard LCLs. Release of the blockade on EBNA 2 and LMP expression which exists in group 1 BL cells therefore appears to be associated with a period of unusually high expression of these viral proteins. From the data in Fig. 6 relating total amounts of detectable EBNA 2 and LMP to the percentage of antigen-positive cells, it seems that the cellular content of EBNA 2 and LMP at this time can exceed typical LCL levels by at least 10-fold. It is interesting to note that such high expression is not accompanied by any inhibitory effect on cell growth, even though in other situations high LMP expression has been shown to be toxic to cells (Hammerschmidt et al., 1989). Thus these high EBNA 2/LMP-expressing cells were segregated in the 'clumped' fraction (i.e. ICAM-1-positive) of early passage MUTU-BL cultures (Fig. 7 and 8) and in the limiting dilution experiments this fraction showed very efficient outgrowth to group III clones. The fact that the derived group III clones showed LCL-like levels of EBNA 2 and LMP suggests that some measure of control over EBNA 2 and LMP expression has to be re-imposed in order to achieve long-term cell outgrowth in vitro.

Virus-induced growth transformation of normal resting B cells in culture is associated with an ordered appearance of EBNA 2 and -LP, then the EBNA 1 and 3 proteins, followed 2 or 3 days later by LMP (Moss et al., 1986; Allday et al., 1989; Rooney et al., 1989; M. Rowe & C. D. Gregory, unpublished observations). We were unable to identify a similarly precise sequence of events during movement of BL cells from the EBNA 1-positive to the EBNA 1-, 2-, 3a-, 3b-, 3c-, -LP, LMP-positive state. Immunoblotting and immunofluorescence analysis of the early passage MUTU-BL cell lines suggested that EBNA 2 and LMP appear at about the same time and in the same cells (Fig. 6 and 8). Although this is contrary to the delayed appearance of LMP following experimental EBV infection of normal resting B cells, it is indeed consistent with our recent finding that EBNA 2 expression can activate the expression of LMP in EBV genome-positive B cells (Abbot et al., 1990). The rapidity of such activation in BL cells might explain why we were unable to identify cells in early passage cultures which were unequivocally EBNA 2-positive but still LMP-negative. The late appearance of the EBNA 3 proteins, compared to that of EBNA 2 and LMP, on serial passage of the MUTU-BL line (Fig. 6) may be more apparent than real because detection of the EBNA 3 family by immunoblotting is currently less efficient than for other viral latent proteins. The kinetics of EBNA 3 and EBNA-LP expression must remain sub judice until immunofluorescence staining with specific MAbs can be carried out at the single cell level in the same way as that for EBNA 2 and LMP.

The present study of the MUTU-BL cell line and of derived clones suggests that the process of phenotypic change within the parent cell line is initiated in a small subpopulation of cells where EBNA 2 and LMP expression is transiently activated to high levels. Phenotypic change is first reflected by up-regulation of adhesion molecules, followed by loss of the BL markers CD10 and CD77 and the up-regulation of B cell activation antigens. Subsequently, the cell population becomes dominated by cells with an LCL phenotype expressing the full spectrum of EBV latent proteins now at LCL-like levels. Further work will be required to understand in what way group III clones came to dominate the parental BL cell line, whether by simple overgrowth of the coresident group I clones, or by an inductive effect upon the group I clones which accelerates their own phenotypic change.

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