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Expression of Epstein–Barr virus latent membrane protein influences self-renewal and differentiation in a multipotential murine haemopoietic 'stem cell' line

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The product encoded by the latent membrane protein (LMP) gene of Epstein–Barr virus (EBV) has been implicated as a transforming protein by a number of studies. We have examined the effects of LMP expression in FDCP-mix cells, a growth factor-dependent multipotential murine 'stem cell' line. Our studies show that LMP reduces the generation of clonogenic cells and leads to the production of cells expressing a marker (lysozyme M) characteristic of mature monocytes and macrophages. Furthermore, cells expressing LMP are compromised in their ability to produce mature neutrophils. These data suggest that expression of LMP in primitive cells can modulate their self-renewal and differentiation potential and provide evidence in support of the suggestion that EBV may be involved in some of the maturation defects of haemopoiesis.

Introduction

Epstein–Barr Virus (EBV) is a human herpesvirus associated with a number of human malignancies, including African Burkitt's lymphoma, nasopharyngeal carcinoma (NPC) and lymphoproliferative disorders in the immune-suppressed (Epstein & Achong, 1986). *In vitro* infection of B lymphocytes with EBV induces increased proliferation and immortalization. Following immortalization, only a restricted set of EBV genes is expressed (the so-called latent genes), the products of which include several nuclear proteins, EBNA-1, -2, -3A, -3B, -3C and -LP (Dillner & Kallin, 1985), and two membrane proteins, the latent membrane protein (LMP; Fennewald *et al.*, 1984; Hennessy *et al.*, 1984) and the terminal protein (Laux *et al.*, 1988; Rowe *et al.*, 1990).

At least two of these latent genes, encoding EBNA-2 and LMP, have been implicated as contributing to the transforming ability of EBV. P3HR1, a deletion mutant variant of EBV that lacks the EBNA-2-encoding sequence (Bornkamm *et al.*, 1982; Cordier *et al.*, 1990; King *et al.*, 1982), is unable to induce cycling of resting B cells (Gordon *et al.*, 1986) and lacks any immortalizing activity (Miller *et al.*, 1974). Expression of EBNA-2 in B cells containing this mutant leads to the expression of activation markers (Cordier *et al.*, 1990) and rescue of immortalizing activity (Hammerschmidt & Sugden, 1989). In addition, gene transfer of EBNA-2 (in the absence of other EBV-encoded gene sequences) into B cells causes an increase in the expression of activation markers (Wang *et al.*, 1987, 1990) and expression of EBNA-2 in rodent fibroblasts reduces their serum dependence (Dambaugh *et al.*, 1986).

Expression of LMP in 3T3 and RAT1 cells is associated with their transformation to anchorage-independent growth and their tumorigenicity in nude mice (Baichwal & Sugden, 1988, 1989; Wang *et al.*, 1985, 1988*a*). LMP has also been shown to inhibit differentiation of human epithelial cells (Dawson *et al.*, 1990) and to cause morphological transformation of immortalized human keratinocytes (Fahraeus *et al.*, 1990). With the observation that LMP expression can be detected in 70% of NPC biopsies (Fahraeus *et al.*, 1988; Young *et al.*, 1988), these data suggest a role for LMP in NPC. In addition to its effects on fibroblasts and epithelial cells, expression of LMP in a human B cell line is associated with phenotypic changes consistent with B cell activation (Wang *et al.*, 1988*b*, 1990), implying that LMP may be important in EBV-induced B lymphocyte immortalization.

Recent evidence suggests that EBV may also have a role to play in some myelopoietic disorders, in that the EBV genome and some EBV-encoded proteins have been detected in cell lines of the monocyte/macrophage lineage isolated from children with maturation defects in myelopoiesis (Revoltella *et al.*, 1989). Although no causal relationship has been established between EBV and these various defects, it is tempting to speculate that the same genes which are primary candidates for mediating the effects of EBV on B lymphocytes and

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epithelial cells might also contribute to the pathology of these maturation defects.

To examine the effect of one of these EBV genes (encoding LMP) on myelopoiesis, we have used retroviral gene transfer to obtain expression of LMP in an interleukin-3 (IL-3)-dependent murine multipotential haemopoietic cell line, FDCP-mix (Spooncer *et al.*, 1986). When maintained in high concentrations of IL-3, these cells proliferate indefinitely and undergo self-renewal to produce daughter cells which are phenotypically indistinguishable from themselves, a characteristic which is extremely important for the maintenance of the stem cell pool. In the presence of limiting amounts of IL-3, however, these cells can be induced either by bone marrow stroma (Spooncer *et al.*, 1986) or by haemopoietic growth factors (Heyworth *et al.*, 1990) to undergo differentiation along a number of haemopoietic pathways, and this occurs concomitantly with a loss of self-renewal ability and the depletion of the stem cell pool. We report here that expression of LMP in these cells reduces their self-renewal capacity and their ability to generate mature granulocytes.

Methods

Cell culture. The isolation and characterization of IL-3-dependent, multipotential FDCP-mix cells have been described previously (Spooncer *et al.*, 1986). In these studies, FDCP-mix clone A4 cells were grown at 37 °C in Iscove's medium (Difco) supplemented with 20% donor horse serum (Medical & Veterinary Supplies Ltd), 2% (v/v) of conditioned medium from X63Ag8-653 cells (which had been transfected with a murine IL-3 cDNA and constitutively secreted large amounts of IL-3) as a source of growth factor (Karasuyama & Melchers, 1988), 50 μg/ml streptomycin sulphate and 5×10^6 units/ml penicillin. In these conditions, the cells maintain a primitive phenotype and grow indefinitely. To determine the number of clonogenic cells in FDCP-mix cell cultures, cells were inoculated at 5×10^3/ml or 10^4/ml in the above medium supplemented with 0·3% Noble agar (Difco), in 1 ml aliquots in 35 cm^2 tissue culture dishes. After 7 days at 37 °C in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2, colonies composed of more than 50 cells were counted. To induce differentiation of the FDCP-mix cells, cells were inoculated at 5×10^4/ml in 1 ml aliquots and grown at 37 °C in Iscove's medium supplemented with 20% foetal calf serum (Gibco), 10% lung conditioned medium (as a source of granulocyte and granulocyte/macrophage colony-stimulating factors; G-CSF and GM-CSF) and 1·5 units/ml recombinant murine IL-3 for 7 days in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 (Heyworth *et al.*, 1990). After this time, cells (10^4 to 10^5 in 0·2 ml) were spun onto glass slides using a Cytospin (Shandon), air-dried and stained with May–Grünwald/Giemsa. Retrovirus producer cells were grown in Dulbecco's MEM (Difco) supplemented with 10% foetal calf serum (Flow Laboratories), 4 mM-glutamine, 50 μg/ml streptomycin sulphate and 5×10^6 units/l penicillin.

Production of retroviruses and infection of haemopoietic cells. An LMP-encoding sequence, consisting of chimeric Raji/B95-8 sequences (Stewart *et al.*, 1989), was used in these studies. This chimera lacks the initiation ATG for truncated LMP found at position 168936 in B95-8 and instead contains the Raji sequence, ATT. The LMP-encoding sequence was inserted into pXT1, a retroviral vector which allows expression of an exogenous gene from a herpes simplex virus thymidine kinase (TK) promoter, and also incorporates the neomycin phosphotransferase gene (*neo*) allowing selection of transfected cells with G418 (Boulter & Wagner, 1987; Fig. 2*a*). The resultant construct, pXTLMP, was used to derive the retroviral producer cell line PAXTLMP using standard methodology (Brown & Scott, 1987). A control retroviral producer cell line, PAXT1, was derived in a similar manner.

FDCP-mix cells were infected by cocultivation with the retroviral producers as follows. Retroviral producer cells were seeded at 2×10^5 in a 25 cm^2 tissue culture flask and grown at 37 °C for 3 days with daily medium changes by which time they were almost confluent. The producer cells were then irradiated with 30 Gy at a dose rate of 3·6 Gy/min using a ^{137}Cs source and the flasks inoculated with 5×10^6 FDCP-mix cells in 10 ml medium containing polybrene at a final concentration of 2 μg/ml. After 24 h, the FDCP-mix cells were removed from the flasks and selected with 900 μg/ml G418 in fresh medium (without polybrene). Cultures were typically ready for use in experiments after 14 days selection, after which they were maintained in 600 μg/ml G418.

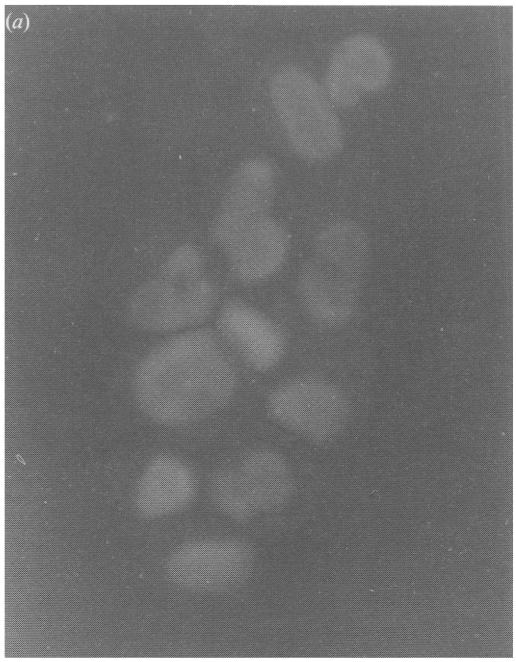
Immunocytochemistry. Cells for analysis (10^4 to 10^5 in 0·2 ml) were spun onto glass slides using a Cytospin (Shandon). After drying of slides, cells were fixed in methanol at −20 °C and stained with the monoclonal anti-LMP antibody pool, CS1-4 (Rowe *et al.*, 1987) using the alkaline phosphatase monoclonal anti-alkaline phosphatase technique (Cordell *et al.*, 1984). Antibody-stained cells were then counterstained with Harris' haematoxylin.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated by the guanidinium isothiocyanate method of Glisin *et al.* (1974). RNA for Northern blot analysis (5 μg per gel track) was ethanol-precipitated, then resuspended in 10 μl 7% formaldehyde, 50% formamide, 20 mM-MOPS pH 7·0 and denatured by incubation for 2 min at 68 °C. This was then electrophoresed at 10 V/cm through a 1% agarose gel containing 7·5% formaldehyde, 20 mM-MOPS pH 7·0, blotted onto a Hybond-N (Amersham) nylon membrane and u.v. cross-linked to the membrane. The membrane was then hybridized overnight to a ^{32}P-labelled LMP cDNA in 7% SDS, 1% BSA, 0·25 M-NaCl and 0·25 M-sodium phosphate as described by Church & Gilbert (1984), washed twice at 68 °C in 2 × SSC (1 × SSC is 0·15 M-NaCl, 0·015 M-sodium citrate, pH 7·2), 1% SDS (15 min per wash) and twice at 68 °C in 0·1 × SSC, 0·1% SDS (15 min per wash) before autoradiography.

In situ *hybridization. In situ* hybridizations with both anti-sense and sense riboprobes were performed, the latter as a control for non-specific hybridization. Cells were spun onto glass slides and air-dried as described above, then fixed in methanol for 20 min at room temperature. Slides were then washed twice in 2 × SSC (5 min per wash at room temperature), acetylated by incubation for 5 min at room temperature in 0·165 M-NaCl, 13% triethanolamine, 0·25% acetic anhydride, pH 8·0, then washed twice in 2 × SSC as before. After air drying, slides were hybridized overnight with a ^{32}P-labelled riboprobe at 35 °C in 6 × SSC, 50% deionized formamide, 10% dextran sulphate, 1 × Denhardt's solution (0·02% Ficoll, 0·02% polyvinylpyrrolidone, 0·02% BSA), 1 mg/ml tRNA, 0·1 mg/ml salmon testes DNA, and washed next day in 2 × SSC (60 min at room temperature), 1 × SSC (60 min at room temperature) and 0·5 × SSC (60 min at 45 °C). Slides were then incubated with 10 μg/ml RNase A in 2 × SSC for 15 min at 35 °C before a final rinse in 2 × SSC. After dehydration by sequential 2 min washes in 50%, 75%, 90% and 95% ethanol, slides were dipped in emulsion and incubated at −70 °C for 5 days before developing.

Results

The retroviral producers, PAXT1-3 and PAXTLMP-4, which produce vector control and LMP-expressing



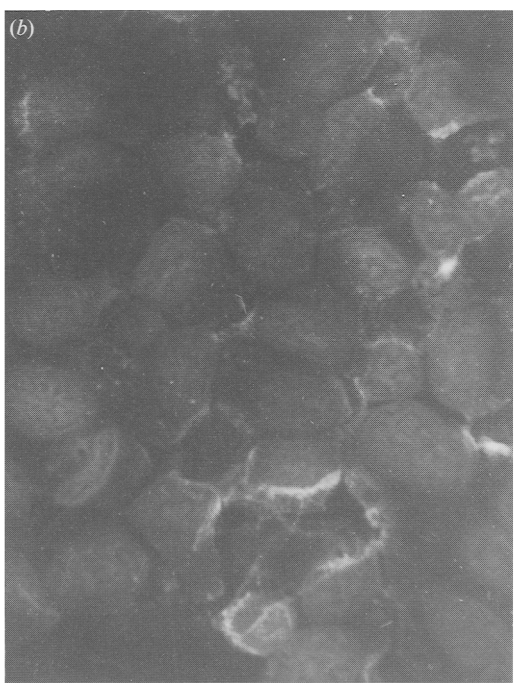


Fig. 1. Immunocytochemical analysis of vector control (*a*) and LMP-expressing (*b*) FDCP-mix cells using the CS1-4 monoclonal antibody pool.

retroviruses, respectively, were used to infect FDCP-mix cells by cocultivation. A number of separate control and experimental infections were performed and G418-resistant cells isolated from each. These cells retained their dependence upon IL-3. Control- and LMP virus-

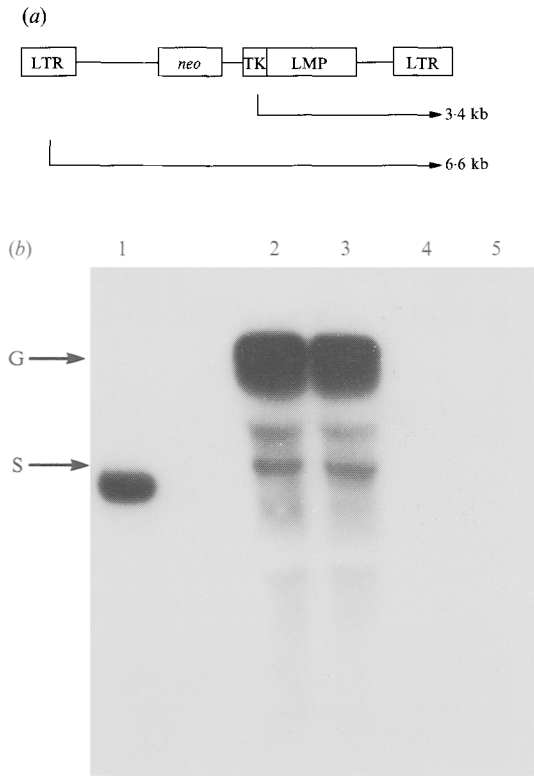


Fig. 2. (*a*) Construction of pXTLMP and expected transcript sizes from the viral LTR and internal TK promoter, respectively. (*b*) Northern blot analysis of LMP transcripts. RNA from: lane 1, Raji cells; lanes 2 and 3, LMP-expressing lines A4XTLMP-S and A4XTLMP-X; lanes 4 and 5, vector control lines A4XTneo-M and A4XTneo-N. Genomic (G) and subgenomic (S) retroviral transcripts are indicated.

infected cells were tested for expression of LMP by immunocytochemistry using the CS1-4 monoclonal antibody pool (Rowe *et al.*, 1987). Control virus-infected cells in common with uninfected cells showed no evidence of staining with CS1-4 (Fig. 1*a*). In contrast, six separately derived LMP virus-infected lines were tested and all were found to be stained by the CS1-4 pool. Fig. 1(*b*) shows the results obtained with one of these lines. Approximately 50% of G418-resistant FDCP-mix cells derived by infection with the XTLMP retrovirus showed staining by CS1-4 at the cell surface. This staining exhibited the characteristic patched appearance reported by other workers (Rowe *et al.*, 1987). The remaining cells do not express LMP or at least do not express it at levels detectable by immunocytochemistry. Northern blot analysis of cells infected with the XTLMP virus was carried out to determine the levels of viral mRNA (Fig. 2*b*). As expected (Fig. 2*a*), two distinct transcripts can be seen in XTLMP-infected cells: one large genomic RNA, transcribed from the retroviral long terminal repeat (LTR), from which the neomycin phosphotransferase is produced, and one smaller subgenomic RNA transcribed

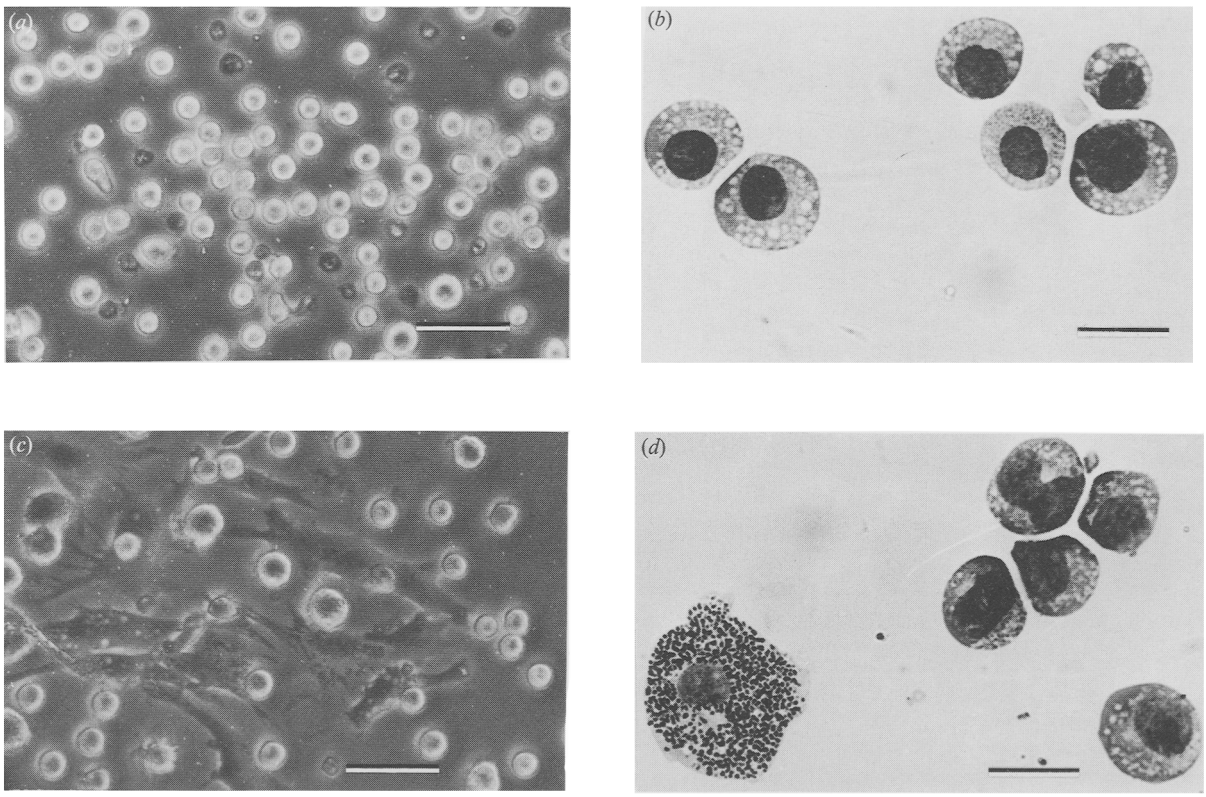


Fig. 3. Morphology of control (*a*, *b*) and LMP-expressing (*c*, *d*) FDCP-mix cells. Cells were analysed by phase-contrast microscopy (*a*, *c*) or after cytospinning and staining with May–Grünwald/Giemsa (*b*, *d*). Adherent cells are evident as phase-dark in (*c*). In (*d*), the larger cell is a mature macrophage with dark particles which have been phagocytosed from the culture medium. Bars represent 200 μm (*a*, *c*) and 100 μm (*b*, *d*).

from the internal TK promoter, from which LMP is produced. The levels of these transcripts differ considerably, the genomic RNA being present at much higher levels than the subgenomic RNA, reflecting the respective strengths of the promoters from which they are transcribed. Comparison with RNA from Raji cells indicates that the levels of LMP-expressing transcripts in the FDCP-mix cells are much lower than that found in this EBV-transformed cell line. The TK promoter is relatively weak and the low levels of transcript produced from this promoter probably account for the low levels of LMP protein detected in XTLMP cells.

Phenotypic effects of LMP expression

LMP-infected FDCP-mix cells showed obvious phenotypic differences compared with control virus-infected cultures. Control-infected FDCP-mix, in common with uninfected FDCP-mix cells, maintained an almost wholly exclusive non-adherent phenotype, comprising small round 'phase bright' cells (Fig. 3*a*), and a blast cell morphology (Fig. 3*b*). In contrast, cultures of LMP virus-infected cells contained a number (up to 50%) of adherent cells, resembling the phenotype adopted by cells of the monocyte/macrophage lineage (Fig. 3*c*). In order to exclude the possibility that these adherent cells were derived from the retroviral producers, LMP virus-infected FDCP-mix cells were analysed for the production of amphotropic virus. If the adherent cells were indeed attributable to the retrovirus producer (PAXTLMP) cells, they would produce amphotropic retrovirus carrying the *neo* and LMP genes. No amphotropic virus production from these cells could be detected (data not shown). Thus the adherent cells are not derived from the PAXTLMP cells and must therefore be LMP virus-infected FDCP-mix cells. Of the LMP virus-infected FDCP-mix cells, approximately 10% exhibited a monocyte/macrophage morphology as determined by May–Grünwald/Giemsa staining (large cells with a low nucleus/cytoplasm ratio and a lighter staining cytoplasm) and showed evidence of phagocytosis (Fig. 3*d*); such cells were only rarely seen in control cultures. Thus expression of LMP in the FDCP-mix cells leads to an increased incidence of cells exhibiting a more mature morphology.

A characteristic of FDCP-mix cells is their ability to form colonies in agar culture which can be further expanded by culture in either a liquid or semi-solid agar

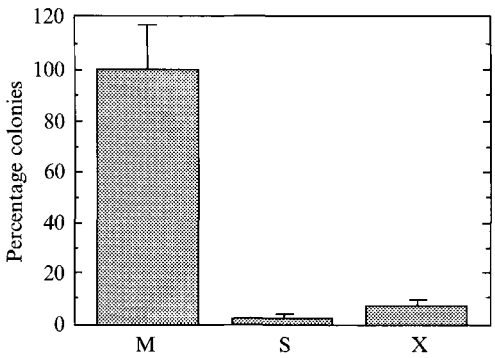


Fig. 4. Colony production in agar culture of one control [A4XTneo-M (M)] and two separately derived LMP-expressing [A4XTLMP-S (S) and -X (X)] lines. Data are expressed relative to a neomycin control cell line and represent means of three separate experiments ± S.E.M.

medium. This clonogenicity is a reflection of the primitive state of these cells and is lost as they are induced to differentiate (Heyworth *et al.*, 1990; Spooncer *et al.*, 1986). LMP virus-infected cells showed a sharp decrease in clonogenicity compared with control virus-infected cells, producing fewer than 10% of control colony numbers (Fig. 4).

All the above data are consistent with LMP expression in FDCP-mix cells causing them to lose their self-renewal capacity (i.e. the ability to produce daughter cells with the same phenotypic characteristics as the parental FDCP-mix cells) and consequently to attain a more differentiated phenotype. In addition to loss of clonogenicity, a characteristic of myeloid differentiation of FDCP-mix cells is a marked increase in expression of the lysozyme M gene (Cross *et al.*, 1988; I. Hampson & M. Cross, unpublished data). *In situ* hybridization of control and LMP virus-infected cells was carried out using a 425 bp riboprobe corresponding to nucleotides 352 to 777 of the lysozyme M gene. Both sense and anti-sense probes to this region of the lysozyme sequence were used in order to distinguish between specific hybridization to lysozyme RNA and non-specific hybridization. As can be seen in Fig. 5, the anti-sense probe hybridized to LMP but not to control virus-infected cells. The sense probe, however, failed to hybridize to either control or LMP virus-infected cells, confirming that the signal seen with the anti-sense probe in LMP virus-infected cells was due to detection of RNA encoding the myeloid maturation marker, lysozyme M. A number of the LMP virus-infected cells that hybridized to the anti-sense probe exhibited an immature morphology. Thus even those cells which appear morphologically immature show evidence of maturation or lineage commitment.

In response to either soluble growth factors (Heyworth *et al.*, 1990) or a stromal feeder layer (Spooncer *et al.*, 1986), FDCP-mix cells are induced to undergo differ-

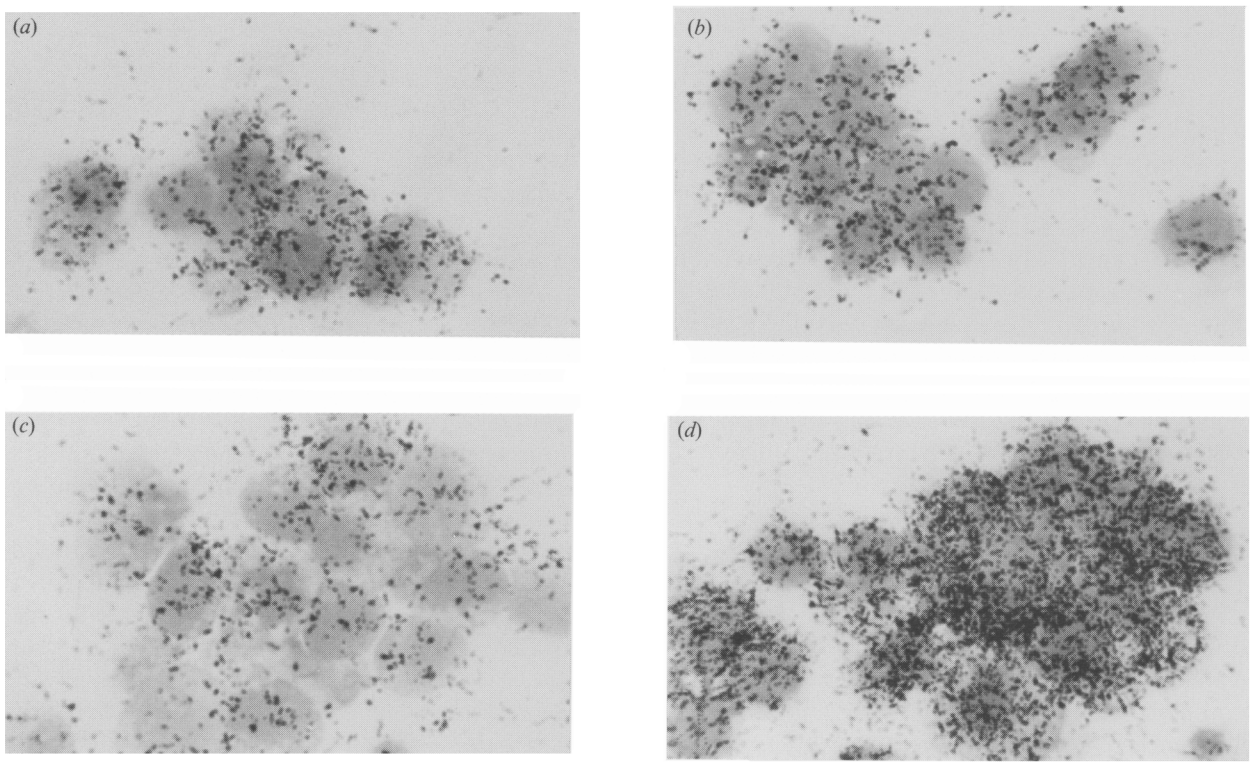


Fig. 5. Expression of lysozyme M by FDCP-mix cells. *In situ* hybridization of vector control (*a*, *b*) and LMP-expressing (*c*, *d*) FDCP-mix cells with sense (*a*, *c*) and anti-sense (*b*, *d*) ^{35}S-labelled lysozyme M riboprobes.

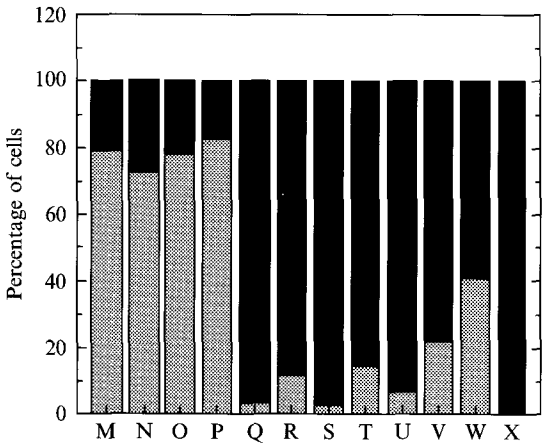


Fig. 6. Differentiation profiles of FDCP-mix cells cultured in granulocyte/macrophage differentiation conditions; amounts of granulocytes (shaded) and macrophages (filled) are expressed as a percentage of the total number of cells in culture. M to P, separately derived vector control lines A4XTneo-M to -P, respectively; Q to X, separately derived LMP-expressing lines A4XTLMP-Q to -X, respectively.

entiation along the various myeloid cell lineages. Unlike a number of haemopoietic differentiation systems (e.g. HL60, K562; Anderson *et al.*, 1978; Collins *et al.*, 1978), this differentiation process is accompanied by a considerable expansion of the cell population leading to the production of a large number of mature myeloid cells. This is analogous to the production of mature cells *in vivo*, supporting the view that FDCP-mix cells represent a reasonable *in vitro* model for myeloid cell development. The results of a number of differentiation assays optimized for the promotion of granulocyte and macrophage development according to the method of Heyworth *et al.* (1990) are summarized in Fig. 6. After 7 days in these assays, non-infected FDCP-mix cells and those infected with control virus generated a cell population comprising 90% mature granulocytes and 10% mature macrophages. In LMP virus-infected cells, the resulting mature population largely (> 80%) comprised mature macrophages. Thus, not only do LMP virus-infected FDCP-mix cells exhibit a more mature phenotype, their pattern of differentiation has been radically altered.

Discussion

The effects of EBV infection and LMP expression in epithelial cells and lymphoid cells are well documented, but little is known regarding possible effects on other cell types. Recently it has been proposed that expression of EBV proteins in myeloid cells may be associated with defects in their maturation (Revoltella *et al.*, 1989), and this led us to examine the effects of LMP expression in a murine myeloid progenitor cell line.

By retroviral gene transfer, we have been able to demonstrate expression of LMP in FDCP-mix, a multipotential haemopoietic stem cell line. Associated with this expression, we have observed a number of phenotypic effects. LMP virus-infected cells have a reduced clonogenic capacity, a characteristic often associated with commitment of cells to a differentiation programme. In addition, LMP expression also leads to the production of adherent cells that are morphologically monocytic and phagocytic in nature. This suggests that the FDCP-mix cells have acquired some characteristics of mature cells as a result of LMP expression. Indeed, the *in situ* hybridization data clearly show that these cells express lysozyme M, a maturation marker for cells of the monocyte/macrophage and neutrophil lineages (Cross *et al.*, 1988). Cells with a similar morphology, and also expressing lysozyme M, are seen when non-infected and control virus-infected FDCP-mix cells are induced to undergo differentiation in response to GM-CSF and G-CSF. In this case, however, neutrophils are also found and form the major cell population (Heyworth *et al.*, 1990). When the LMP virus-infected FDCP-mix cells were cultured under similar differentiation-inducing conditions, few neutrophils were produced and mainly cells of the monocyte/macrophage lineage were found. The LMP virus-infected FDCP-mix cells used in these experiments comprised a population of cells producing varying amounts of LMP. It is possible, therefore, that the small amount of neutrophil production seen on exposure of LMP virus-infected FDCP-mix to these differentiation-inducing conditions was due to differentiation of a subpopulation of cells that were not expressing LMP.

All these data, therefore, are consistent with the suggestion that LMP expression has resulted not only in a reduction in the ability of FDCP-mix cells to self-renew and produce cells with purely stem cell characteristics, but also in constitutive activation of a differentiation programme leading to production of cells of the monocyte/macrophage lineage in the absence of added differentiation-inducing factors. Moreover, the reduction in neutrophil production by LMP virus-infected cells in culture conditions that allow preferential expression of this developmental option in non-infected and control virus-infected cells suggests that LMP expression has resulted in a subversion of the developmental potential of the FDCP-mix cells. Other work has demonstrated that expression of LMP in a human epithelial cell line, SCC12F, results in a block in differentiation (Dawson *et al.*, 1990). The effects of LMP expression on the developmental potential of FDCP-mix might be similarly interpreted as representing a 'block' in differentiation along the neutrophil lineage as a result of LMP expression, while at the same time promoting monocyte/

macrophage differentiation. An alternative explanation can be suggested in light of the known toxicity of high levels of LMP expression to some cells (Hammerschmidt *et al.*, 1989), i.e. that the maturing neutrophils or their direct precursors are extremely sensitive to this 'toxicity'. At present, it is not possible to distinguish between these two possible mechanisms.

Whatever the mechanism by which LMP influences lineage choice and causes suppression of neutrophil production in these cells, however, a prediction from our results is that haemopoietic progenitor cells containing and expressing EBV LMP sequences will be unable to interact effectively in haemopoietic development, since they will be unable to produce mature neutrophils. The question of whether the finding of EBV sequences in monocyte cell lines derived from patients with myelopoietic defects is connected with the aetiology or pathology of these disorders is unresolved. The present situation is, however, similar to that which once existed for the link between EBV and lymphoid disease. The data presented here, although not definitively linking EBV with disorders in myelopoiesis, do show that the potential exists for a contribution by EBV sequences to the pathogenesis of some maturation defects of myelopoiesis. Furthermore, the system may provide a basis for examining the effects of LMP at the mechanistic level.

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