Epstein–Barr virus (EBV) nuclear antigen 6 induces expression of the EBV latent membrane protein and an activated phenotype in Raji cells

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Epstein–Barr virus (EBV) nuclear antigen (EBNA) 6 (also known as 3c) is a latent nuclear protein with an Mr of about 160K which is invariably expressed in EBV-immortalized B cells. It includes a putative basic leucine zipper domain; as such it is a good candidate for a regulator of viral gene expression. More than 75% of the EBNA 6 coding sequence is deleted from viral genomes carried in the Burkitt’s lymphoma (BL) tumour-derived cell line, Raji. Thus although Raji cells express normal levels of the remaining five EBNAs and low levels of latent membrane protein (LMP), EBNA 6 protein is completely absent. In this study we have established Raji clones stably expressing EBNA 6 after cotransfection of an EBNA 6 gene under the control of the simian virus 40 early promoter with a selectable marker. Analysis of these clones has revealed that EBNA 6 induces a significant increase in the expression of LMP. In addition the cells have undergone a number of morphological and phenotypic changes consistent with blast-activation of normal B lymphocytes. The Raji cells expressing EBNA 6 show ruffling of the cell membrane and the development of a polarity defined by multiple villous (‘spiky’) projections at one end of the cell. This morphological change is associated with a dramatic increase in the expression of the cytoskeletal protein, vimentin. The EBV-associated B cell activation marker CD23 (blast 2) is induced to high levels although other activation markers such as CD30 and CD39 are unaffected. All these changes appear to be independent of the precise levels of EBNA 6 protein expressed. EBNA 2 has been shown previously to trans-activate the LMP gene and in the control Raji cells, EBNA 6-positive Raji cells and in B lymphoblastoid cells similar levels of EBNA 2 are expressed. Our findings are therefore most consistent with a model in which EBNA 6 either augments or complements the action of EBNA 2 in the induction of LMP and the cascade of gene expression which leads to B cell activation and immortalization by EBV.

Introduction

Epstein–Barr virus (EBV) is the causative agent in the benign lymphoproliferation known as infectious mononucleosis; it is also associated with the aetiology of at least three human tumours, Burkitt’s lymphoma (BL), nasopharyngeal carcinoma and B cell lymphoma in immunocompromised individuals (Epstein & Achong, 1986; Miller 1990; Thomas et al., 1991). In vitro EBV can infect, activate and immortalize a subpopulation of normal resting human B lymphocytes to produce continuously proliferating lymphoblastoid cell lines (LCLs) (Pope et al., 1968). The phenotype of EBV-immortalized cells resembles that of antigen- or mitogen-activated B lymphoblasts (Nilsson & Klein, 1982). Viral gene expression in these cells is limited to a small number of genes which include those encoding six nuclear antigens (EBNAs 1 to 6) and three membrane proteins [latent membrane protein (LMP) and the terminal proteins 1 and 2] (reviewed by Kieff & Liebowitz, 1990). It is generally assumed that together these proteins are responsible for the activation of the resting B cell, induction of continuous proliferation and maintenance of the EBV genome in a latent form. The timing and sequence of viral and cellular gene expression immediately after infection suggests that an ordered cascade of gene expression is necessary for cellular immortalization to occur. EBV gene expression is initiated with the synthesis of EBNAs 2 and 5 which are soon followed by the remaining EBNAs (Alfieri et al., 1991; Allday et al., 1989; Moss et al., 1986; Rooney et al., 1989). There is then a delay during which the cell progresses towards DNA synthesis (S-phase of the cell cycle); LMP expression appears to coincide with this entry into the S phase (Alfieri et al., 1991; Allday et al., 1989; Walls et al., 1989). From this point on the cell is a fully activated, proliferating lymphoblast.

Both EBNA 2 and LMP have been shown, in gene
transfer experiments, to induce cellular changes consistent with B cell activation; in particular, both stimulate expression of the cell surface antigen CD23 (Wang et al., 1988; Wang et al., 1987, 1990a). In addition, EBNA 2 is a transcription factor which can activate the expression of LMP (Fahraeus et al., 1990; Wang et al., 1990b) and, by acting on the major rightward promoter (Cp or BCR 2) in the BamHI C region of EBV (Sung et al., 1991; Woisetschlaeger et al., 1991), may also initiate or enhance the expression of EBNAs 1, 3, 4 and 6 (Woisetschlaeger et al., 1991). It is not surprising, therefore, that EBNA 2 has been shown to be essential for immortalization (Hammerschmidt & Sugden, 1989). EBNA 1 binds to the latent origin of replication (oriP) and is necessary for the replication of EBV DNA. It is probably primarily concerned with maintenance of the latent EBV genomes in an episomal form (Yates et al., 1985). Little is known about the functions of the remaining nuclear antigens or the terminal proteins.

EBNA 6 (also known as EBNA 3c) is a latent nuclear protein with an Mr of about 160K which is expressed from a pair of exons located in the BamHI E, c, and e fragments of the EBV genome (Allday et al., 1988; Petti et al., 1988; Ricksten et al., 1988). An analysis of its amino acid sequence reveals a motif which resembles the basic region leucine zipper domain found in a number of well characterized transcription factors such as c-fos and c-jun (Vinson et al., 1989; Fig. 1). Constitutive expression of EBNA 6 after single gene transfer into the EBV-negative B cell line BJAB induced expression of the CD21 antigen showing that it can exert an effect on at least one cellular gene (Wang et al., 1990a). This, taken together with the demonstration that it is a DNA-binding protein (Kallin et al., 1986), suggests that it is a regulator of gene expression. More than 75% of the EBNA 6-coding sequence is deleted from the EBV genomes carried in the BL tumour cell line, Raji (Hatfull et al., 1988; Fig. 1). Thus, although Raji cells express the remainder of the proteins which constitute the EBNA complex, they are EBNA 6-negative (Allday et al., 1988; Petti et al., 1988). Owing to a second deletion from the Raji virus genome, which affects the expression of late genes (Hatfull et al., 1988), these cells cannot be induced to produce infectious virus; it has not therefore been possible to ask directly whether EBNA 6-negative virus activates and/or immortalizes normal B cells. However, in a series of experiments performed by Skare et al. (1985) the EBNA 2-negative, non-immortalizing P3HR1 strain of virus was used to superinfect Raji cells and, as a result of homologous recombination between resident Raji cell episomes and input P3HR1 genomes, immortalizing virus was produced. Analyses of these virus isolates showed that they had all rescued the BamHI Y and H, EBNA 2-encoding, region from Raji cell genomes and this was taken as evidence that the DNA fragment and corresponding protein(s) are vital for immortalization. As the BamHI E/c1/c2 sequences which encode EBNA 6 were similarly rescued from P3HR1 genomes, it was suggested that this region, and therefore EBNA 6, may also be necessary for the immortalization of normal B cells (Skare et al., 1985; Wang et al., 1990a). Here we have introduced a functional EBNA 6 gene, under control of a simian virus 40 (SV40) promoter, into Raji cells and by drug selection produced clones which stably, constitutively express EBNA 6. These cells have been analysed to determine whether EBNA 6 influences the expression of any viral gene(s) and/or, against a background of EBV latent gene products, exerts any effect on the phenotype of Raji cells. The data suggest that EBNA 6 plays a key role in the activation, and therefore immortalization, of B cells by EBV.
Methods

Cell culture. All cell lines, Raji (a BL cell line; Pulvertaft, 1965), a lymphoblastoid cell line, B-LCL (established by infection of adult peripheral B cells with B95-8 virus) and BJAB (an EBV-negative B lymphoma cell line; Menezes et al., 1975) were routinely grown at 37 °C in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS).

Plasmid constructions. The recombinant expression vector pSV2E3/4 which includes the EBNA 6-coding region cloned from B95-8 virus genomic DNA under control of the SV40 early region promoter/enhancer element has been described previously (Allday et al., 1988). The selectable vector pSV2Hyg, which has the hph gene encoding hygromycin B phosphotransferase under control of the SV40 early region promoter/enhancer element, has also been described (Gritz & Davis, 1983).

Transfections. EBNA 6-expressing and control hygromycin B-resistant Raji cells were generated by electroporation with a Gene Pulser apparatus (Bio-Rad) in disposable electroporation cuvettes with a 0.4 cm electrode gap. Briefly, 10⁷ cells were washed once in RPMI 1640 medium, resuspended in 0.4 ml Optimem 1 (Gibco-BRL) containing either 2 μg of pSV2Hyg DNA or 2 μg of pSV2Hyg mixed with 10 μg of pSV2E3/4 DNA and added to a cuvette. After 5 to 10 min on ice the cells were electroporated by pulsing at 260 V with a capacitance of 960 μF, placed on ice for a further 10 min and then transferred to 10 ml RPMI 1640 with 20% FCS. Two days after electroporation the transfected cells were selected in RPMI 1640 with 10% FCS containing 400 μg/ml of hygromycin B (Sigma). Cloning was performed by limiting dilution into 96-well plates. This was done in the presence of 20% FCS and 400 μg/ml hygromycin B on a fibroblast feeder layer. The clones and uncloned pSV2Hyg-positive control cells were screened by immunoblotting. The cells were subsequently maintained in 200 μg/ml hygromycin B.

Immunoblotting. Protein extracts were made by sonicating washed cell pellets in SDS sample buffer and after boiling these were separated on 7.5% SDS–polyacrylamide gels and Western-blotted as described previously (Allday et al., 1988, 1989). Human serum RT was used to detect EBNA1, 2, 3, 4 and 6 and the monoclonal antibody (MAb) cocktail CS1–4 (a gift from M. Rowe, University of Birmingham, U.K.); Rowe et al., 1987) to detect LMP. In both cases the final antibody was the appropriate immunoperoxidase–antibody conjugate diluted 1:10000. For LMP, a second layer of rabbit anti-mouse immunoglobulin (diluted 1:1000) was included to amplify the signal. Protein bands were visualized using an enhanced chemiluminescence system (Amersham) according to the manufacturer’s instructions.

Immunochemistry. Cytocentrifuge preparations of cell lines were air-dried (30 to 60 min; 22 °C) fixed in cold (4 °C) acetone for 5 min and examined immunochemically by the peroxidase–anti-peroxidase (PAP) method (Smith & Thomas, 1990). MAbS were used to define the following cellular antigens: vimentin (V9, Dako); CD23 (MHM6 from A. McMichael, University of Oxford, U.K.); CD21 (HBS, Becton Dickinson); CD30 (BerH2, Dako); CD39 (AC2, Serotec); CD11a [leukocyte function antigen (LFA-1), 1MHM24 from A. McMichael]; CD54 (intercellular adhesion molecule (ICAM)-1, RR1/1) and CD58 (LFA-3, TS2/9) (both from T. Springer, Harvard Medical School, U.S.A.); CD77 (BL antigen from J. Wiels, Institut Gustav Roussy, France); and CD10 (J5, Orthomune). Primary layer reagents, used at between saturating and 10-fold dilution were detected with rabbit anti-mouse (m) Ig (Dako) at a 1:25 final dilution and mPAP (Dako); dilution 1:100. Sections were developed with 3′-3′-diaminobenzidine tetrahydrochloride (1 mg/ml) with 30% (w/v) hydrogen peroxide (1 μl/ml: Sigma) and counterstained in Mayer’s haemalum.

Results

Expression of EBNA 6 in Raji cells

EBNA 6-positive and control, hygromycin B-resistant Raji cells were established as described in Methods. Preliminary screening, by immunoblotting, of clones derived from cells cotransfected with EBNA 6-encoding pSV2E3/4 DNA and pSV2Hyg DNA revealed that approximately half were EBNA 6-positive. Three positive clones, one negative clone and uncloned hygromycin B-resistant cells which had been transfected with pSV2Hyg DNA alone were selected at random for detailed analysis. Protein extracts were made from these cells and analysed by Western immunoblotting using a

Fig. 2. Immunoblot analysis showing EBNA expression in Raji transfectants. Cell extracts were separated by SDS–PAGE, transferred to nitrocellulose and probed with human serum RT which recognizes EBNA 1, 2, 3, 4 and 6. Three representative EBNA 6-positive Raji clones are shown: 11. 2. 1 (lane 5), 11. 5. 3 (lane 6) and 5. 8 (lane 7). pSV2Hyg vector controls are shown: uncloned cells, 13. 6 (lane 3) and an EBNA 6-negative clone, 1. 2. 5 (lane 4). A B-LCL and the EBV-negative B cell line BJAB are shown as controls (lanes 1 and 2 respectively). The position of the various EBNA and Mr markers are indicated. In addition to the full-size EBNA 6 a smaller EBNA 6-related species is also indicated in the Raji transfectants.
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(b)  1 2 3 4 5 6

---, EBNA 6

(a) 67K

LMP

EBNAS 1 and 2

98K

EBNA 6

67K

Fig. 3. (a) Immunoblot analysis of LMP expression in EBNA 6-expressing Raji cell clones. Cell extracts were separated by SDS-PAGE, transferred to nitrocellulose and probed with the cocktail of anti-LMP MAbs, CS1-4. EBNA 6-expressing clones: 11.5.8 (lane 2), 11.5.3 (lane 3) and 11.2.1 (lane 5). pSV2Hyg transfected controls: 13.6 (lane 1) and 11.2.5 (lane 4). A B-LCL is also shown (lane 6). The positions of LMP and $M_r$ markers are indicated. (b) The above Western blot was reprobed with human serum RT to demonstrate EBNA expression and shows that all the lanes are equivalently loaded with protein. EBNA species and positions of $M_r$ markers are indicated. (c) Photomicrographs showing anti-LMP immunoperoxidase staining of pSV2Hyg control Raji cells 13.6 (panel 1), representative EBNA 6-expressing Raji clone 11.5.3 (panel 2) and a B-LCL (panel 3). Bar marker represents 25 μm.

human serum (RT) which recognizes EBNA 1, 2, 3, 4 and 6. It can be seen that clones 11.2.1 (Fig. 2, lane 5) and 11.5.3 (lane 6) express lower levels of EBNA 6 than those found in the control B-LCL (lane 1). However, clone 11.5.8 (lane 7) expresses significantly higher levels than both the other Raji clones and B-LCL. In all three EBNA 6-positive clones a second protein can be seen migrating just below EBNA 3 (the bottom band of the 3, 6 and 4 triplet). This protein band is recognized by other EBNA 6-positive antibodies (data not shown) indicating that it is EBNA 6-related. It is probably the result of proteolytic cleavage. However, at present we cannot rule out the possibility of it resulting from aberrant splicing or translation of the SV2E3/4 derived message. The monoclonal nature of the EBNA 6-expressing cells was confirmed by recloning at one cell per well and Western immunoblotting extracts from 10 subclones. All three clones were judged to be monoclonal because only EBNA 6-expressing subclones were detected.

Expression of EBNA 6 induces an increase in LMP expression

Protein extracts were made from EBNA 6-positive Raji cell clones, hygromycin B-resistant control Raji cells and a B-LCL; these were analysed by Western immunoblotting for LMP using the cocktail of MAbs CS1–4. Fig. 3(a) shows that the EBNA 6-positive clones (lanes 2, 3 and 5) express significantly higher levels of LMP than the control Raji cells (lanes 1 and 4). Expression at this high level reveals LMP as a doublet of two closely migrating species. A comparison of lanes 2, 3 and 5 with lane 6 shows that EBNA 6 induces levels of LMP expression in Raji cells of a similar magnitude as that found in the B-LCL (lane 6). To establish that equal amounts of protein extract were loaded in each lane the nitrocellulose filter was rinsed in PBS and reprobed with human serum RT. An equal level of EBNA 1, 2, 3 and 4 can be seen in the various Raji clones (Fig. 3b, lanes
EBNA 6 expression in Raji cells

Fig. 4. (a) Morphological changes in EBNA 6-expressing Raji cells as revealed by low power scanning electron microscopy: pSV2Hyg control cells, 13.6 (panel 1), a representative EBNA 6-expressing clone 11.5.8 (panel 2) and a B-LCL (panel 3). Bar marker in panel 1 represents 10 μm, for all three panels. (b) Photomicrographs showing anti-vimentin immunoperoxidase staining of pSV2Hyg control cells, 13.6 (panel 1), a representative EBNA 6-expressing Raji clone 11.5.3 (panel 2) and a B-LCL (panel 3). Bar marker represents 25 μm.

1 to 5). It can also be seen by comparing this blot with the previous one (Fig. 3a) that the precise level of EBNA 6 does not affect the amount of LMP synthesized. Levels of LMP are no higher in the 11.5.8 Raji cell clone (lane 2), which expresses very high levels of EBNA 6, than in either of the other two. To determine whether the increase in LMP was restricted to a small number of cells expressing at a very high level or whether it is increased in all the cells, cytospin preparations were made from the various Raji clones and control cells and they were analysed by immunoperoxidase staining using CS1–4 MAbs. Clone 11.5.3 is shown as being representative of the EBNA 6-positive clones (Fig. 3c, panel 2). A comparison of this with the uncloned control cells 13.6 (panel 1) shows that, indeed, most cells express higher levels of LMP and that the levels appear similar to those in the B-LCL (panel 3). These results are entirely consistent with the results of the immunoblotting in Fig. 3(a).

Induction of an activated, lymphoblast-like phenotype in the EBNA 6-expressing Raji

Examination of Raji cells expressing EBNA 6 with an inverted light microscope revealed that many of the cells had developed 'spiky' membranous projections which were often associated with a pronounced polarity (projections at one end and a smooth rounded membrane at the other). A scanning electron micrograph of a representative clone (11.5.8) shows this morphology more clearly (Fig. 4, panel 2). In addition to the villous projections, membranous ruffles can be seen which together with the spikes often appear to be attached to other cells or the substratum. A comparison of these cells with the B-LCL in panel 3 shows that both have a similar growth pattern and morphology which is quite different to the pSV2Hyg-positive control Raji cells in panel 1. The EBNA 6-negative cells retain the largely smooth, spherical appearance of parental Raji cells.
As any change in morphology is likely to be accompanied by changes in the level or distribution of cytoskeletal components, cytospin preparations of EBNA 6-positive and negative Raji cells were stained for vimentin using the V9 MAb. In parallel with their morphological changes, the EBNA 6-positive Raji cells (Fig. 4b, panel 2) more closely resembled the B-LCL (panel 3) both in the level and distribution of vimentin. The polar distribution of the protein (‘patching’), which has been described in LCLs (Kieff & Liebowitz, 1990), can clearly be seen in a number of cells in panel 2; we assume the apparently nuclear distribution seen in some cells results from the cytoskeleton collapsing during cytospin preparation. The control Raji cells, on the other hand, were essentially vimentin-negative (panel 1).

Further analysis, using the anti-CD23 MAb MHM6, produced additional results consistent with the more blast-like phenotype of the EBNA 6-expressing clones described above. EBNA 6-positive Raji cells (Fig. 5a, panel 2) stained by the immunoperoxidase method showed a large number expressing high levels of the CD23 antigen. Similar staining of pSV2Hyg-positive control Raji cells (panel 1) shows that the vast majority of cells are either negative for CD23 or express only low levels of the antigen. EBNA 6 expression appears to induce in many Raji cells levels of CD23 which give a staining intensity similar to that found in the fully activated B-LCLs (panel 3). As shown for the vimentin immunocytochemistry we assume that the appearance of nuclear staining in some cells is an artefact created by the cytospin preparation. FACS analysis of similar cells stained in suspension with MHM6 and a fluorescent second-layer antibody produced results which are entirely consistent with the peroxidase staining: compare Fig. 5(b) panels 1 (EBNA 6-negative), 2 (EBNA 6-positive) and 3 (B-LCL) with Fig. 5(a) panels 1, 2 and 3.

In addition, all the Raji cell clones and controls were analysed by immunoperoxidase staining and/or FACS
EBNA 6 expression in Raji cells

Table 1. Summary* of the effects of EBNA 6 on the phenotype† of Raji cells

<table>
<thead>
<tr>
<th>Cell surface markers</th>
<th>pSV2Hyg-transfected Raji cells</th>
<th>EBNA 6-expressing Raji cells</th>
<th>B-LCL</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMP</td>
<td>+</td>
<td>+ + + (†)‡</td>
<td>+ + +</td>
</tr>
<tr>
<td>Membrane ruffling</td>
<td>+/−</td>
<td>+ + (†)</td>
<td>+ + +</td>
</tr>
<tr>
<td>Polarity/villous projections</td>
<td>+/−</td>
<td>+ + (†)</td>
<td>+ + +</td>
</tr>
<tr>
<td>Vimentin</td>
<td>−</td>
<td>+ + + (†)</td>
<td>+ + +</td>
</tr>
<tr>
<td>CD23</td>
<td>+</td>
<td>+ + + (†)</td>
<td>+ + +</td>
</tr>
<tr>
<td>CD21</td>
<td>+</td>
<td>+</td>
<td>ND§</td>
</tr>
<tr>
<td>CD30</td>
<td>−/−</td>
<td>−/−</td>
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<td>+ + +</td>
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<tr>
<td>ICAM-1</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+ + +</td>
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<tr>
<td>BLA (CD77)</td>
<td>+ + +</td>
<td>+ + +</td>
<td>−</td>
</tr>
<tr>
<td>CALLA (CD10)</td>
<td>+ + +</td>
<td>+ + +</td>
<td>+/−</td>
</tr>
</tbody>
</table>

* This summarizes data from analyses of all EBNA 6-positive Raji clones by Western blotting (LMP), scanning electron microscopy and/or light microscopy (morphology), immunoperoxidase staining (LMP, vimentin and all the cell surface markers) and/or flow cytometry (cell surface markers with the exception of BLA and CALLA).
† Individual characteristics are expressed on an arbitrary scale (− to + + +) relative to a representative B-LCL.
‡ † Indicates a significant increase relative to control Raji cells.
§ ND, Not determined.

Discussion

These experiments have demonstrated that when EBNA 6 is expressed in Raji cells the level of LMP is increased. It has also been shown that, compared with a representative B-LCL cell, the control Raji cells express very little LMP; this would appear to be due to the absence of EBNA 6 and may be functionally significant (see below). Levels of EBNA 6 below that found in B-LCLs are sufficient to induce this change and the response does not appear to increase further even when EBNA 6 expression is significantly higher than that in B-LCLs. The precise level of EBNA 6 is therefore not critical for this particular response. In this respect the effect of EBNA 6 is unlike that demonstrated for EBNA 2 where the magnitude of LMP expression was determined by the levels of EBNA 2 present (Abbott et al., 1990). When the LMP extracted from the EBNA 6-positive Raji is visualized by Western blotting (from a 7.5% SDS-polyacrylamide gel), it is revealed as a doublet of closely migrating species (Fig. 3a). The much lower levels seen in the control Raji cell extracts appear as a single band with the lower Mr. Thus in addition to the level increasing, it is possible that there may be a modification, such as phosphorylation, of the protein associated with, and revealed by, the increased expression. The nature of the difference between these two species is unclear and it will require a detailed biochemical analysis to determine its significance. Immunoperoxidase staining of cytospin preparations shows that EBNA 6 exerts its effect and induces LMP in the vast majority of cells and not just in a subpopulation which expresses at a very high level.

In addition to an increase in the expression of LMP, changes in the phenotype of the Raji cells have been induced by EBNA 6 expression. These include an altered morphology, a dramatic increase in the expression of the cytoskeletal protein vimentin and an increase in the expression of the activation antigen, CD23. All these changes are consistent with expression of LMP at the levels found in B-LCLs. It was previously shown, by single gene transfer experiments, that LMP expression at these LCL-like levels in EBV-negative BL cells produces changes in the cellular phenotype very similar to those described in the EBNA 6-positive Raji cells (Wang et al., 1988; Wang et al., 1990a; Birkenbach et al., 1989). It is possible therefore that the shift seen in the phenotype of EBNA 6-positive Raji cells is the direct result of increasing LMP above a critical threshold. This would mean that the amount of LMP normally found in Raji cells is suboptimal for any significant effect on cellular
phenotype. However, it is also possible that a modification of LMP is required for activity and that this occurs only in the EBNA 6-positive cells or cells activated by some other means. Furthermore, in relatively non-activated cells such as control Raji cells, EBNA 2 alone is apparently unable to activate the LMP gene sufficiently to induce an alteration in cellular phenotype. Because similar quantities of EBNA 2 are found in Raji cells, EBNA 6-positive Raji cells and B-LCLs, we can reasonably exclude the possibility that EBNA 6 is acting indirectly on the LMP gene by increasing the levels of EBNA 2. An alternative, and perhaps more likely, explanation of these data is that EBNA 6 in some way augments the action of, or cooperates with, EBNA 2 in increasing the expression of LMP. EBNA 2 activates the LMP gene by inducing transcription through an EBNA 2-responsive element located approximately 50 to 100 bp upstream of the transcriptional initiation site (Fahraeus et al., 1990; Wang et al., 1990b). EBNA 2 protein does not appear to bind directly to the DNA at this site and may act by counteracting the effects of a cellular repressor protein (Fahraeus et al., 1990). At this stage we cannot exclude the possibility that EBNA 6 acts indirectly through some undefined cellular pathway or even acts post-translationally. However, because of its similarity to transcription factors such as c-fos and c-jun, it is quite feasible that it directly cooperates with EBNA 2 in the stimulation of LMP transcription. It will now be possible to test this hypothesis by cotransfecting EBNA 6 expression vectors with the LMP 5′ regulatory region linked to a reporter gene. Whereas EBNA 2 derepresses the LMP gene, EBNA 6 may trans-activate it. Whatever the mechanism of this association, to our knowledge this is the first evidence that EBV nuclear proteins may cooperate in the cascade of events which lead to B cell activation. Given that EBNA 2 and EBNA 6 co-localize to the nucleus and produce a similar punctate staining pattern (Allday et al., 1988; Kieff & Liebowitz, 1990) it will be interesting to determine whether they form part of the same protein complex or even make direct contact with each other.

In the preceding paragraph it was implied that the changes seen in the phenotype of EBNA 6-positive Raji cells could be accounted for solely by the increased levels of LMP. However, the action of EBNA 6 may be more extensive and it could also complement EBNA 2 in the activation of cellular genes. It was demonstrated previously that EBNA 2 directly induces CD23 expression (Wang et al., 1987) and the 5′ control region of the CD23 gene has been shown to contain an EBNA 2-responsive element similar to that found in the LMP promoter (Wang et al., 1991). Therefore, if EBNA 6 can complement the action of EBNA 2 in the induction of LMP gene expression, it is a possibility that it may also do the same in the stimulation of CD23 or other cellular genes and so make a more direct contribution to B cell activation. A final point regarding LMP expression in these experiments should be noted: LMP has been shown to be toxic in a variety of cells (Hammerschmidt et al., 1989) and it has been suggested that vimentin could possibly protect against these toxic effects (Birkenbach et al., 1989). Thus, as the apparent activation of vimentin synthesis appears as one of the most striking and clear-cut changes seen in the EBNA 6-positive Raji cells, we should not rule out the possibility that this is a primary result of EBNA 6 expression. The high(er) levels of LMP observed could be a consequence of selective outgrowth of these more robust cells.

EBNA 6 expression in Raji cells does not induce a change in the expression of CD21 although in an earlier study it was shown to induce CD21 in the EBV-negative B cell line BJAB (Wang et al., 1990a). The precise reason for this difference in response is not apparent but probably relates to the cell type used. BJAB was CD21-negative prior to stable introduction of EBNA 6 (Wang et al., 1990a). Raji cells, on the other hand, already express high levels of the antigen (Table 1); we assume that this accounts for the lack of any detectable responsiveness. Both LMP and EBNA 2 have been reported to stimulate the expression of CD39 and reduce the expression of CALLA and CD10 (Wang et al., 1990a). Raji cells are also atypical in this respect as both the EBNA 6-negative and EBNA 6-positive cells are CD39-negative and CALLA-positive.

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