Epstein-Barr Virus Latent Gene Expression during the Initiation of B Cell Immortalization

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SUMMARY

Epstein-Barr virus (EBV) has the capacity to immortalize a subpopulation of resting B lymphocytes. Lymphoblastoid cell lines (LCL) established in this way carry the latent EBV genome as multiple copies of an extrachromosomal episome. Viral gene expression in LCLs is highly restricted; products identified correspond to a membrane protein (latent membrane protein; LMP), a nuclear antigen complex (Epstein–Barr nuclear antigens; EBNAs 1 to 6), two small RNA species (EBERs 1 and 2) and RNA species thought to encode a second membrane-associated polypeptide designated terminal protein (TP). Here we have investigated the temporal sequence of expression of the characterized 'latent' proteins during the initiation of immortalization when resting B cells are stimulated to enter and traverse the cell cycle. The analysis has been carried out on prolymphocytic leukaemia cells infected in vitro with either the immortalizing B95-8 strain of virus or the non-immortalizing P3HR1 strain. The results reveal that following B95-8 infection, a sequence of EBV expression is initiated within approximately 8 h with the synthesis of detectable levels of EBNA 2 shortly followed by EBNAs 1, 3, 4, 5 and 6. There is then a delay of approximately 40 h until the expression of LMP completes the latent pattern of proteins found in LCLs. P3HR1 infection, however, produces only transient expression of some EBNA species in a small percentage of cells after approximately 48 h. These observations suggest the failure of P3HR1 virus to immortalize may not be due solely to the absence of EBNA 2 expression and that cellular and/or virus-mediated events occur after EBNA synthesis which then facilitate efficient LMP expression and immortalization.

INTRODUCTION

In vitro, Epstein–Barr virus (EBV) can infect B lymphocytes via the C3d receptor molecule CR2 (CD21) (Nemerow et al., 1986) and a subpopulation of these cells are transformed into continuously proliferating lymphoblastoid cell lines (LCLs) (Pattengale et al., 1973). Estimates, based on limiting dilution analysis, suggest that the number which are immortalized is between 1 and 10% of infected cells (Tosato & Blaese, 1985). EBV DNA is carried in LCLs primarily as multiple copies of an autonomously replicating extrachromosomal episome (Lindahl et al., 1976). The cells are described as being latently infected with, and growth-transformed or immortalized by, EBV. Viral expression is restricted to a small number of latent genes. The protein products of these genes include a membrane protein (latent membrane protein; LMP) and a nuclear antigen complex recently shown to consist of six proteins designated EBNAs 1, 2 (of which two variants, A and B, exist), 3, 4, 5 (also known as leader protein; LP) and 6 (Dillner & Kallin, 1988; Allday et al., 1988). Recently, RNA species that are transcribed across the linked termini of the EBV episome have been described. These are thought to encode a further membrane-associated protein (Laux et al., 1988).

EBNA 1 is involved in the extrachromosomal replication of the EBV episomes and appears to be an absolute requirement for viral latency (Yates et al., 1985). It is assumed that both EBNA 2 and LMP play important roles in the process of B cell immortalization, as both influence cellular
growth properties. Virus derived from the P3HR1 Burkitt’s lymphoma (BL) cell line which is non-immortalizing has the gene encoding EBNA 2 deleted (Miller et al., 1974; Rabson et al., 1982). Furthermore, DNA-mediated gene transfer has shown that EBNA 2 enhances the ability of rodent cells to grow in culture by reducing their dependence on serum factors (Dambaugh et al., 1986). It also stimulates cellular DNA synthesis in human lymphocytes (Dambaugh et al., 1986). The entry of cells into the S phase of the cell cycle involves increased expression of a number of cellular genes, the best characterized of which is CD23, Blast 2 (Thorley-Lawson et al., 1985). This is a cell surface B cell activation marker, thought to be involved in the autocrine-regulated growth of both normal and EBV-immortalized B cells (Swendeman & Thorley-Lawson, 1987). Infection and transfection of EBV-negative B cell lines has clearly demonstrated that EBNA 2 plays a direct role in the superinduction of CD23 (Wang et al., 1987). Expression of LMP (p63) in the Rat-1 cell line transforms these immortal, but non-tumorigenic, cells into cells that are fully tumorigenic in a nude mouse assay. They also show a loss of contact inhibition, an increased anchorage independence and an ability to grow in soft agar (Wang et al., 1985). The precise role of the other latent proteins in either immortalization or EBV latency is unclear. The two small latent RNA species, the EBERs, which are transcribed by RNA polymerase III, are not polyadenylated, not translated and form a complex with the cellular protein called La (Lerner et al., 1981). As with most of the latent proteins, the function of the EBERs has yet to be established.

In order to gain some insight into the functions of the EBV latent gene products we have monitored the time course of expression of the latent proteins described above from the onset of infection until the full latent pattern is established and the infected cells are proliferating. The B cells used in the study are of leukaemic origin, prolymphocytic leukaemia (PLL) cells (Melo et al., 1986). These are ‘frozen’ at an early lymphoblastic stage of differentiation. They were chosen because they form a monoclonal, relatively homogeneous population of non-proliferating B cells which are efficiently activated and immortalized by EBV (up to 60% of the cells can be immortalized). In addition they appear to behave in a very similar, if not identical manner to normal resting B cells with respect to EBV and other B cell activators (Walls et al., 1989). Because these cells are often available in large numbers from a single individual it has been possible to carry out many experiments on an identical, monoclonal, population. We have analysed latent viral expression during the first 96 h after infection in vitro with immortalizing (B95-8) and non-immortalizing (P3HR1) strains of EBV.

METHODS

Leukaemic cells and cell lines. Blood samples were obtained from a patient (not undergoing treatment) with chronic B cell leukaemia which was diagnosed as PLL by clinical, morphological and phenotypic criteria (Melo et al., 1986). Mononuclear cells were separated by centrifugation over Ficoll–Hypaque, washed in RPMI 1640 medium containing antibiotics and 2% (v/v) foetal calf serum (FCS). Since the leukaemic cells constituted at least 90% of the total peripheral blood mononuclear cells, enrichment for B lymphocytes was not carried out.

Cell lines B95-8 (Miller et al., 1974), IB4 (King et al., 1980), P3HR1 (Hinuma et al., 1967) and Ramos (Klein et al., 1975) were grown in RPMI 1640 containing antibiotics and 10% (v/v) FCS.

Virus and infections. Supernatants from a culture of B95-8 marmoset LCL were used as a source of infectious B95-8 virus (Miller et al., 1972). Virus was concentrated by ultracentrifugation and titrated by immortalization of umbilical cord blood mononuclear cells (transforming titre of approx. 10–4). Virus from P3HR1 supernatants was concentrated by ultracentrifugation and titered by scoring EBNA-positive cells after infecting the EBV-negative BL line BJAB (80% EBNA-positive after 24 h). Cells were suspended at 107 to 2 x 107/ml in the virus preparation, incubated for 1 h at 37 °C and then diluted 10-fold in culture medium (RPMI with 10% FCS). They were then incubated at 37 °C in 24-well flat-bottom plates. The point at which the cells were diluted after infection was taken as time zero in all experiments.

Sera and monoclonal antibodies. EBV-positive serum RT was from a normal healthy donor and contains antibodies to EBNA 1, 2, 3, 4 and 6. EBV-positive serum RM which contains high titres of antibodies to EBNA 5 (and also antibodies to 1, 2, 3 and 6) was obtained from C. Rooney (Ludwig Institute, London, U.K.) and G. Miller (Yale Medical School, New Haven, Conn., U.S.A.). Serum ST which contains high titres of antibodies to EBNA 1 and 5 was obtained from P. Venables (Kennedy Institute, London, U.K.). Anti-LMP monoclonal antibodies CS1 to 4 (Rowe et al., 1987) were from M. Rowe (University of Birmingham, U.K.).
EBV latent gene expression

SDS–PAGE and immunoblotting. Total protein from $10^6$ cells (for EBNA) and $2 \times 10^6$ cells (for LMP) was solubilized in SDS sample buffer and separated as previously described (Allday & MacGillivray, 1985) by SDS–PAGE on 7.5% gels. Western immunoblotting for EBNA species was performed as described (Allday et al., 1988) using an immunoperoxidase detection system. LMP immunoblots were probed with $^{125}$I-labelled Protein A (Amersham) as described by Rowe et al. (1987).

Immunofluorescence (IF) and anti-complement immunofluorescence ACIF. Staining for LMP was carried out by routine indirect IF tests on methanol-fixed cytospin preparations using monoclonal antibodies CS1 to 4. Staining for the EBNA complex was carried out on methanol-acetone-fixed cytospin preparations using positive and negative sera, by ACIF tests (Reedman & Klein, 1973) with the added complement reinforcement step described by Klein et al. (1976).

RESULTS

EBNA expression

Eight h after infection of PLL cells with B95-8 EBV, ACIF using a human serum (RT) that recognizes EBNA 1, 2, 3, 4 and 6 produced a faint but characteristic EBNA fluorescence pattern in approximately 50% of cells (Fig. 1 a). Prior to this time no staining was detectable (see the uninfected cells stained with RT in f). From 8 to 48 h the intensity of staining increased and the pattern altered slightly with some cells exhibiting pronounced nucleolar staining (Fig. 1 d, arrowed). The number of clearly positive cells rose slightly as staining became more intense.

To establish which EBNA species contribute to this fluorescence pattern, samples of cells were taken at various time points after infection and protein extracts were immunoblated with serum RT. At 10 h post-infection EBNA 2 was just detectable (Fig. 2a, lane 6). The level of this protein had increased by 12 h and by 18 h (Fig. 2b, lane 2) EBNA 1, 3, 4 and 6 could also be clearly seen. Between 18 and 24 h the levels of EBNA 1 and 2 reached that found in established LCLs (compare Fig. 2a, lane 8 with lanes 10 and 11). Although during this period EBNA 3, 4 and 6 were detectable it was not until the 40 h time point that they were present at levels equivalent to those in established cell lines (compare Fig. 2b, lane 3 with lane 6). Immunoblotting with a serum (RM) that contains high titres of antibody to EBNA 5 shows a characteristic ladder of EBNA 5 species (see Finke et al., 1987) appearing between 12 and 24 h (Fig. 3, lanes 2 and 3), at about the same time as EBNA 1, 3, 4 and 6. The apparently low levels of EBNA 2 in this blot merely reflect the low titre of anti-EBNA 2 antibodies in serum RM with respect to RT; compare Fig. 2a lanes 8 and 10 with Fig. 3, lanes 1 and 3.

LMP expression

Indirect IF staining with a cocktail of four anti-LMP monoclonal antibodies produced the cytoplasmic staining pattern (in 5 to 10% of PLL cells) typical of LMP (Rowe et al., 1987) 48 h after infection with B95-8 virus (Fig. 4b). Before this, cells were completely negative (see Fig. 4a). The number of positive cells then increased over the next 48 h to approximately 30% (c and d). To confirm these data, cell extracts were immunoblated with the same cocktail of monoclonal antibodies. The results (Fig. 5a) showed that LMP was detectable only after 72 h (lane 2). The failure to detect LMP at 48 h by immunoblotting is consistent with the relatively small percentage of cells observed to be positive by IF.

Infection with P3HR1 strain of EBV

We analysed expression of the latent genes after P3HR1 infection of PLL cells in studies similar to those carried out with B95-8. EBNA 1 and 3 (Fig. 2c, lane 13) and EBNA 5 (see Finke et al., 1987) were expressed in the parent P3HR1 cell line. However, in the PLL cells during the first 24 h post-infection, when B95-8-infected cells have synthesized EBNA 1 to 6, there appeared to be no expression of the P3HR1 EBNA genes as determined by fluorescence (not shown) or immunoblotting with either serum RT (Fig. 2c, lanes 1 to 8) or RM (Fig. 3, lanes 4 and 5). At 48 h after infection a small number of cells (approx. 10%) showed a punctate nuclear staining characteristic of EBNA 1 with various sera, including RT and RM (compare Fig. 1e, for example, with the uninfected cells in f). There was no evidence to suggest that these cells proliferated and by 96 h fluorescent staining was no longer detectable (not shown). Attempts to
Fig. 1. Photomicrographs showing anti-EBNA ACIF staining of B95-8 virus-infected PLL cells at (a) 8 h, (b) 12 h, (c) 24 h and (d) 48 h post-infection with serum RT. Panel (e) shows PLL cells 48 h after infection with P3HR1 virus and (f) uninfected cells, both similarly stained with serum RT. Bar marker represents 20 μm.
**EBV latent gene expression**

Fig. 2. Immunoblots of EBNAs 1, 2, 3, 4 and 6. (a) SDS-solubilized proteins from 10^6 PLL cells were taken at 0, 2, 4, 6, 8, 10, 12 or 24 h (lanes 1 to 8) after infection with B95-8 virus, and after separation on a 7.5% SDS polyacrylamide gel and transfer to nitrocellulose were probed with serum RT. Control (lane 9) represents uninfected PLL cells at 24 h. B95-8 (lane 10) and IB4 (lane 11) are EBV-positive LCLs and Ramos (lane 12) an EBV-negative BL cell line. (b) Similar immunoblot carried out on proteins from B95-8-infected PLL cells 18, 40, 65 or 90 h post-infection (lanes 2 to 5). Lane 1 represents uninfected PLL cells at 18 h, lane 6 IB4. (c) Similar immunoblot of proteins from PLL cells 0, 2, 4, 6, 8, 10, 12 or 24 h (lanes 1 to 8) after infection with P3HR1 virus. Proteins from the parent P3HR1 cell line are shown (lane 13). Lanes 9 to 12 are as in (a). In each blot the positions of EBNAs 1, 2, 3, 4 and 6 are indicated (↑) and the approximate positions of Mr standards are shown.

Analyse this transient expression were hampered by the small number of EBNA-expressing cells and the low levels of antigen. A representative immunoblot of extracts made at 48 h post-infection (Fig. 6) (probed with serum ST) shows a low level of EBNA 1 and a single protein band which may be an EBNA 5 species (compare lanes 1, 2 and 4). Both fluorescence (not shown) and immunoblotting (Fig. 5b, lanes 1 to 3) failed to detect LMP expression in the PLL cells up to 4 days post-infection although the parent P3HR1 cell line synthesized readily detectable levels of LMP (Fig. 5b, lane 4).

**Immortalization**

It was established on several occasions that PLL cells could be immortalized with B95-8 virus; cells from various experiments were allowed to proliferate and were passaged for several
Fig. 3. Immunoblot probed with serum RM showing EBNA 5 expression in B95-8 virus-infected PLL cells (lanes 2 and 3; 12 and 24 h post-infection) and P3HR1 virus-infected PLL cells (lanes 4 and 5; 12 and 24 h post-infection). B95-8 cells (lane 1) and P3HR1 cells (lane 6) were similarly blotted and probed. EBNA species are indicated (△) and the approximate positions of M, standards are indicated.

Fig. 4. Photomicrographs showing anti-LMP indirect IF of B95-8 virus-infected PLL cells at (a) 24 h, (b) 48 h, (c) 72 h and (d) 96 h post-infection. Bar marker represents 20 μm.
DISCUSSION

In this study we have utilized well characterized human sera and murine monoclonal antibodies to investigate expression of the EBV latent proteins at the very onset of immortalization, when resting B cells are stimulated by viral infection to enter and traverse the cell cycle. PLL cells have been employed because they are a relatively homogeneous (monoclonal) population of resting B cells which can be very efficiently immortalized by EBV. As 50% or more are infected and immortalized (Walls et al., 1989) they provide a very sensitive target population for analysing early viral gene expression before cellular proliferation begins between 48 and 72 h post-infection.

EBNA expression can be demonstrated by ACIF 8 h after infection of PLL cells with B95-8 virus and soon after this it can be analysed by Western immunoblotting. EBNA 2 is the first antigen to be detected (8 to 10 h) and the remainder of the EBNA5s appear more or less synchronously between 12 and 18 h post-infection. By 24 h the latent pattern of EBNA expression found in long term LCLs is established. The appearance of EBNA 2 first is reproducible and consistent with previously published data on the infection of tonsil and cord blood B cells (Kallin et al., 1986; Moss et al., 1986) and our own experiments with peripheral B lymphocytes (unpublished). Whether EBNA 2 is actually transcribed and translated before the other EBNA species or whether it is just more readily detected by the immunological techniques used is unclear. Nevertheless, it seems that all the EBNA5s are synthesized at, or about, the same time early after infection and long before cellular or viral DNA synthesis. Apart from the early appearance of EBNA 2, no differential expression of the individual EBNA species was apparent in any experiment performed with B95-8 virus.
Although the pattern of EBNA expression is identical to that in established LCLs approximately 24 h after infection of PLLs with B95-8 virus, LMP cannot be detected by either IF or immunoblotting at this time. It is only after 48 h that LMP is observed. This delay in expression of LMP correlates with an observation that the cell surface adhesion molecule LFA1 (on PLL cells) is induced 48 to 72 h after infection (our unpublished data). LFA1 is known to be induced by LMP (Murray et al., 1988a). The findings suggest that cellular and/or virus-mediated events occur after EBNA synthesis which can then facilitate efficient LMP expression; one possibility is that a threshold level of EBNA 2 (or any other EBNA species) acting in trans either directly or indirectly by stimulating cellular activation, is necessary. Evidence for this hypothesis comes from reports that P3HR1 (EBNA 2-negative)-converted EBV-negative BL lines do not synthesize LMP whereas B95-8 conversion of the same lines allows LMP expression (Murray et al., 1988b). However, it should be noted that the parent P3HR1 cell line readily synthesizes LMP in the absence of EBNA 2 expression (see Fig. 5b, lane 4). Another contributory factor may be the circularization of the EBV genome. This is thought to occur between 16 and 24 h after infection (Hurley & Thorley-Lawson, 1988; Thorley-Lawson, 1988), that is, before LMP expression. Whereas EBNA expression clearly does not require genome circularization, the LMP gene, located near the end of the linear EBV genome, may be efficiently expressed only after circularization has occurred and cis-acting sequences are juxtaposed to the region controlling LMP transcription. The failure of P3HR1-converted BL lines such as AW Ramos to express LMP (M. J. Allday, unpublished and Murray et al., 1988b) would then be explained by the fact that the EBV genome is present only as linear molecules integrated into host chromosomes (see Teo & Griffin, 1987) where the cis-acting elements need not be correctly sited.

When EBV infects resting B cells the binding of virus to CD21 (Nemerow et al., 1986) and possibly the insertion of virion-associated LMP into the membrane by envelope fusion (Mann et al., 1985) stimulate the cells to enter the cell cycle (G0–G1). EBNA 2 has been implicated in the progression towards S phase (Gordon et al., 1986) and superinduction of the B cell activation marker CD23, Blast 2 (Wang et al., 1987; Thorley-Lawson et al., 1985). EBNA 1 becomes necessary for replication and correct partitioning of the viral episome after it has circularized and cells start to proliferate. LMP appears to be synthesized at or around the time cells start to synthesize DNA (S) and its expression appears to be essential for immortalization (Walls et al., 1989; and with T. Azim, unpublished observations). The roles of EBNA 3, 4, 5 and 6 have not yet been determined but their temporal expression in cells becoming immortalized suggests that functions associated with progression through the cell cycle including the
transition from G2 to M (Melchers & Lernhardt, 1985; Gordon & Guy, 1987) should be considered (see Fig. 7).

At least one of the latent proteins, EBNA 4, cannot be absolutely necessary for the maintenance of cell proliferation although it may be necessary during initiation. For example, the LCL IB4 (see Fig. 2a, lane 11) established with the B95-8 virus (King et al., 1980) has lost the ability to express EBNA 4. We have observed two other LCLs which were established with B95-8 and do not express EBNA 4 (unpublished). This suggests EBNA 4 is not only unnecessary but that failure to synthesize it may actually give a growth advantage in vitro.

Previous reports (see Thorley-Lawson, 1988) suggest that P3HR1 virus infection of resting B cells does not result in EBNA expression. The delayed and transient nature of EBNA expression seen in our experiments may account for this observation. A small number of P3HR1-infected cells transiently synthesize EBNA 1 and possibly EBNA 5. These cells do not express EBNA 2, 4 and 6 nor, as far as we can determine from a number of experiments, LMP. Neither have we been able consistently to show EBNA 3 expression. It is not surprising that 5 to 6 days after infection the cells die. However, it is unclear whether this failure to synthesize some of the latent proteins and start continuously cycling (immortalization) results solely from the deletion in the BamHI YH region that removes the EBNA 2 gene (Rabson et al., 1982) or whether other genomic lesions are involved. In this respect it should be noted that the successful conversion of EBV-negative BL cells with P3HR1 shows that even a defective, non-immortalizing virus can infect, express EBNA 1, 3 and 5 and presumably give a growth advantage to cells which are already proliferating (see Murray et al., 1988a). This raises the possibility that EBNA 2 expression and concomitant cellular activation may be necessary for efficient expression of the other latent proteins only in resting B cells. Infection of the EBV-negative B cell line BJAB with the P3HR1 virus used in this study results in more than 80% of cells becoming EBNA-positive after 24 h. It is therefore tempting to speculate that the PLL cells which are permissive for EBNA expression after P3HR1 infection are those which have been spontaneously activated into the cell cycle. These may then bypass a requirement for EBNA 2 in the efficient expression of other EBV species.

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