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The Epstein-Barr virus encoded cytokine viral interleukin-10 enhances transformation of human B lymphocytes

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In vitro infection of human B lymphocytes with Epstein-Barr virus (EBV) results in their growth transformation and establishment of immortalised lymphoblastoid cell lines. The virus was recently found to encode a homologue of the pleiotropic cytokine interleukin-10 (IL-10), which has wide ranging effects on the immune system. We have investigated the effect of this virally encoded growth factor on the ability of EBV to immortalize B lymphocytes from tonsils and from adult and neonatal blood. Recombinant viral interleukin-10 (vIL-10) was found to increase dramatically the growth transformation of B cells from all three populations infected with either the highly transforming type 1 strain B95-8 or the less efficient type 2 strain BL16. This striking enhancement of transforming ability in the presence of viral IL-10 may be in part due to increased viability of the B cells during infection and decreased levels of interferon- γ , a cytokine known to inhibit EBV transformation. Thus viral IL-10 influences a number of cell types of the immune system to allow the enhanced outgrowth of EBV transformed cells.

Keywords: Epstein-Barr virus; transformation; viral interleukin-10 viability; interferon- γ

Introduction

Epstein-Barr virus is a ubiquitous human γ -herpesvirus. Initial infection with the virus at an early age is usually asymptomatic and it is only in more developed countries where primary infection is delayed that disease manifests as infectious mononucleosis (Henle and Henle, 1979). Infection with EBV has also been linked to a number of cancers including Burkitt's lymphoma (Geser *et al.*, 1983), nasopharyngeal carcinoma (de The, 1980), Hodgkin's lymphoma (Mueller *et al.*, 1989) as well as B lymphoproliferative disease arising in immunocompromised individuals (Purtilo *et al.*, 1981).

These neoplasias reflect the tissue tropism of the virus with the potential to infect both epithelial cells (Sixbey *et al.*, 1984) and B lymphocytes (Pope *et al.*, 1968). Infection of B lymphocytes with EBV results in the establishment of long term latency. During latency

in B cells the virus expresses a limited number of proteins and two small RNA molecules (EBERs—Arrand *et al.*, 1989). In type I latency, seen in Burkitt's lymphoma and recently in the peripheral blood of EBV seropositive individuals (Tierney *et al.*, 1994), only the EBNA1 protein and EBERs are detected; EBNA1 is required for maintaining the virus genome through subsequent cell divisions and the infected cells display a resting phenotype. Ten viral proteins are expressed during type III latency, these include six nuclear antigens: EBNA1, EBNA2, EBNA3a, EBNA3b, EBNA3c and EBNA-LP, BARF0 and three membrane proteins LMP1, LMP2A and LMP2B. B lymphocytes expressing the full range of EBV latent proteins show an activated phenotype with high surface levels of class II HLA, CD23 and CD54 (ICAM-1) (Wang *et al.*, 1987; Alfieri *et al.*, 1991; Kieff and Liebowitz, 1990).

In vitro infection of B lymphocytes with EBV results in growth transformation leading to immortalized lymphoblastoid cell lines (LCL) expressing all ten latent proteins and displaying an activated phenotype.

Two subtypes of EBV exist in the population as determined by significant variations in EBNA2, EBNA3 and EBERs (Addinger *et al.*, 1985; Arrand *et al.*, 1989; Rowe *et al.*, 1989). Type 1 virus is highly transforming (the prototypic type 1 strain is B95-8, the commonly used laboratory strain), whereas type 2 virus is much less efficient at transformation. These differences in transforming ability have been attributed to the variations in the coding sequence of EBNA2 (Rickinson *et al.*, 1987), although sequence variations in EBNA3a and EBNA3c may also contribute (Rowe *et al.*, 1989).

The EBV open-reading frame BCRF1 shares extensive sequence homology with the cytokine interleukin-10 (IL-10; Moore *et al.*, 1990). In addition, the product of BCRF1 and IL-10 are functionally analogous and therefore the BCRF1 product has been named viral IL-10 (vIL-10). Both IL-10 and vIL-10 have been shown to regulate the functions of many cell types of the immune system, including both lymphoid and myeloid cells. These functions include deactivation of macrophages, inhibition of cytokine synthesis by CD4⁺ helper T cells and proliferation and differentiation of activated B cells (for review see Moore *et al.*, 1993). The function of vIL-10 in the EBV life cycle is not known, however, using a recombinant EBV lacking the vIL-10 open-reading frame, it has been shown that vIL-10 is non-essential for the transformation of B cells *in vitro* (Swaminathan *et al.*, 1993). vIL-10 is expressed at late times during the productive phase of the EBV life cycle when the virus particles are being produced

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(Stewart and Rooney, 1992; Stewart *et al.*, 1994). Therefore vIL-10 is likely to act as a pathogenesis factor *in vivo* where productive replication is occurring and vIL-10 would be present during the initial infection of B cells. In our experiments we have attempted to mimic this situation *in vitro* by the addition of exogenous recombinant vIL-10 during the transformation of B cells. Purified tonsillar B lymphocytes, adult peripheral mononuclear cells and neonatal cord blood mononuclear cells were used in transformation assays with both type 1 and type 2 EBV. These assays were then used to assess the effect of exogenous vIL-10 on the ability of EBV to transform B lymphocytes from these different cell populations and further experiments were carried out to study possible mechanisms of action for vIL-10 in these assays. Our results clearly demonstrated that vIL-10 enhanced the transformation of B cells from all three populations and that the possible mechanisms of action included increased cell viability and inhibition of interferon- γ secretion.

Results

Effect of recombinant viral IL-10 on EBV transformation

The effects of vIL-10 on the transformation of B cells by EBV were studied using fetal cord blood mononuclear cells (CBMC), adult peripheral blood mononuclear cells (PBMC) derived from EBV seronegative donors and purified tonsillar B cells. These cell populations were used because they should not contain immune cells which could mediate regression of newly transformed B cells. Cells were infected with either the highly transforming prototypic type 1 EBV strain B95-8 or a type 2 strain BL16 which transforms B cells less well. To mimic the *in vivo* situation, infected cells were fed with medium containing vIL-10 for the first week only and thereafter with medium alone. This method would have the result of serially diluting vIL-10 over the period of the assay, however it appears that vIL-10 is active for only 5 days in culture (our unpublished results) and would therefore effectively be present for the first week only. The assays were maintained over a period of 6 weeks and scored weekly for the appearance of multiple transformed foci as described by Moss and Pope (1972). The presence of EBV-specific transformation as opposed to merely non-specific activation was confirmed by immunostaining the foci with a monoclonal antibody to EBNA2 and subsequently the transformed foci were used to establish long term lymphoblastoid cell lines which retained positive staining with the anti-EBNA2 monoclonal antibody 6 months post infection.

The initial set of experiments used CBMC. At the highest virus concentrations, evidence of B95-8-mediated transformation was apparent after 1 week irrespective of the presence of vIL-10, although the foci appeared to be fewer and smaller in the absence of the cytokine. This difference in size and frequency remained throughout the assay. Figure 1C shows an example of B95-8-transformed foci in the absence of vIL-10 at 5 weeks post-infection. In the presence of

vIL-10, as can be seen in Figure 1D, there were more transformed foci which appeared to be larger. The BL16 strain of EBV was, as expected, much less efficient at transforming CBMC. In the absence of vIL-10, foci did not appear until 2 weeks post-infection and these were few and small in size. However, in the presence of vIL-10, foci were, in all cases, apparent after only 1 week in culture. Figure 1A and B show examples of CBMC transformed with BL16 EBV at 5 weeks post-infection. It can be seen that foci in the absence of vIL-10 (Figure 1A) were very small and fewer in number than the foci in wells fed with vIL-10 (Figure 1B).

The transformed foci shown in Figure 1 were from the highest virus concentrations only. Figure 2 shows the results derived using a range of virus concentrations. In the absence of vIL-10, both B95-8 and BL16 virus could be titrated to an end point where transformation could no longer be seen. In the presence of vIL-10, however, transformation was observed in all virus concentrations tested, although at the lower concentrations the foci tended to be fewer and smaller.

PBMC and purified tonsillar B cells were used in subsequent experiments to assay transformation. The effects observed were generally the same as those found with CBMC, except that the addition of vIL-10 in this case had a more pronounced effect. In this case transformation by B95-8 EBV in the absence of vIL-10 did not appear until 2 weeks post-infection, whereas in the presence of the cytokine foci were evident, at all virus concentrations, 1 week earlier. Figure 1E and G show examples of transformed PBMC and tonsillar B cells respectively in the absence of vIL-10 at 4 weeks post-infection. It can clearly be seen that the size and frequency of these foci are much less than those observed in the presence of vIL-10 (Figure 1F and H).

As for CBMC, vIL-10 enhanced the transformation of PBMC at a range of virus concentrations. This can be seen in Figure 3A and B, where transformation was seen at the highest virus concentrations only in the absence of vIL-10. However, in the presence of vIL-10, transformed foci were apparent at all virus concentrations tested, although in contrast to CBMC this activity could be titrated to an end point. This biological activity of vIL-10 could be completely neutralised by the addition of anti-sera raised against a glutathione-S-transferase fusion protein of vIL-10 (data not shown).

The above results showed that vIL-10 had the effect of enhancing the transformation of human B cells by EBV.

Effect of viral IL-10 on the cell viability during EBV infection

We next examined potential mechanisms by which vIL-10 enhanced the transformation process. Interleukin-10 has been previously shown to increase the viability of resting murine B cells in a dose dependent manner (Go *et al.*, 1990). To study the effect of vIL-10 on the viability of cells infected with type 1 and type 2 EBV, tonsillar B cells, CBMC and PBMC were infected with B95-8 and BL16 and viability was assessed using trypan blue. Viable cells appeared

unstained with the dye and had a rounded morphology. As can be seen in Figure 4 the viability of the uninfected controls in all three cell populations was reduced to between 5–10% after 3 days. Infection with

EBV could partially rescue some of these cells, by increasing the cell survival by 20–40% and the addition of vIL-10 to these infected cells resulted in a further increase in survival with 80–90% of the cells

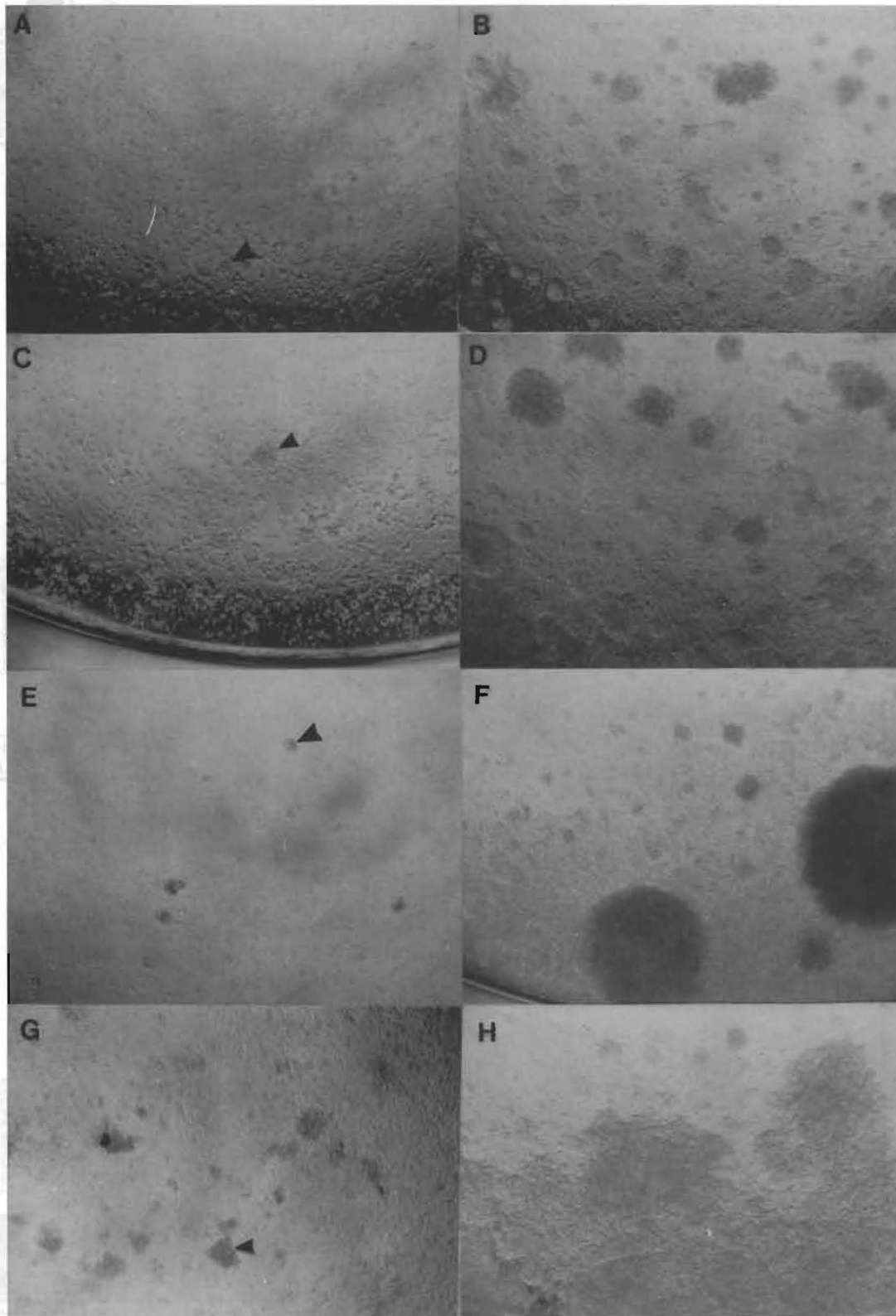


Figure 1 Transformed foci from assays (pictures were taken from the lowest virus dilutions only: 10^{-3} for B95-8 and 2×10^{-1} for BL16 at 5 weeks post infection. A–D cord blood mononuclear cells (CBMC), A and B infected with BL16; C and D infected with B95-8. E and F peripheral blood mononuclear cells infected with B95-8. G and H purified tonsillar B cells infected with B95-8. A, C, E and G transformation assays were set up in the absence of viral IL-10; B, D, F, and H in the presence of 100 ng ml^{-1} viral IL-10. Arrows show transformed foci

remaining viable after 3 days post-infection. These results would suggest that one possible mechanism of vIL-10 is to increase cell viability during EBV infection.

Effect of vIL-10 on the secretion of the interferon- γ

It has been demonstrated previously that EBV transformation is highly susceptible to the anti-viral

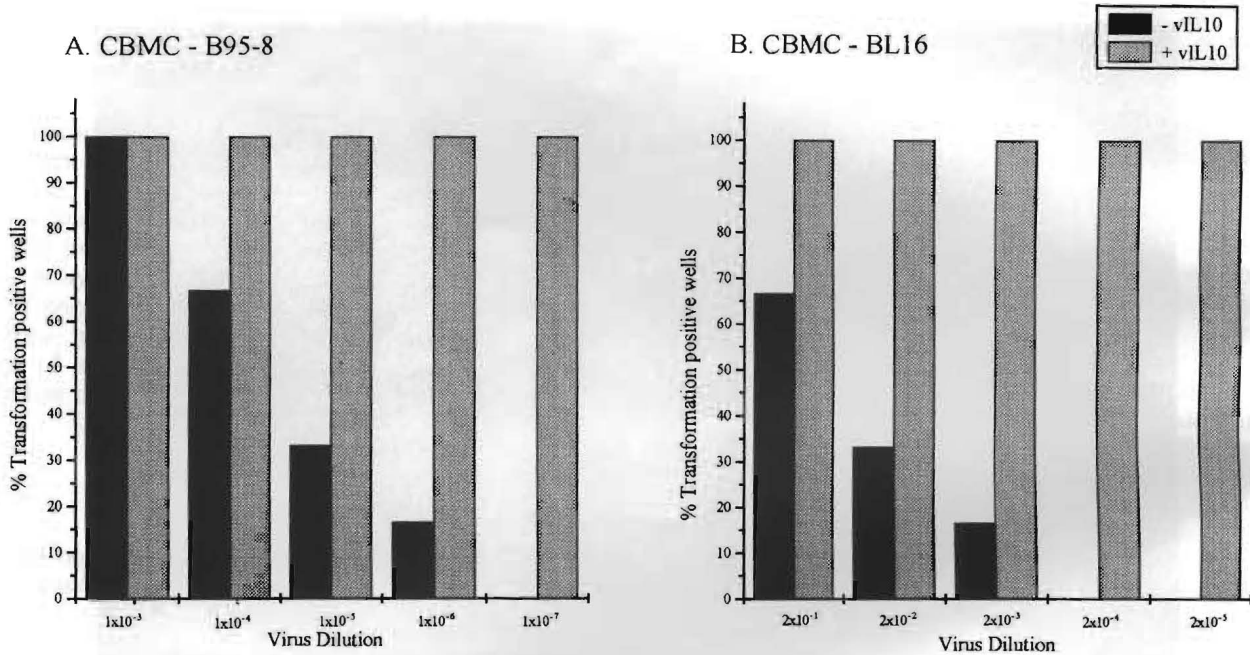


Figure 2 Transformation assays for CBMC infected with type 1 and type 2 EBV. 2×10^5 CBMC were infected with 10-fold serial dilutions of either type 1 (B95-8) or type 2 (BL16) EBV in the absence or presence of 100 ng ml^{-1} viral IL-10 and fed at weekly intervals for the duration of the assay. Transformation positive wells were scored after 6 weeks in culture

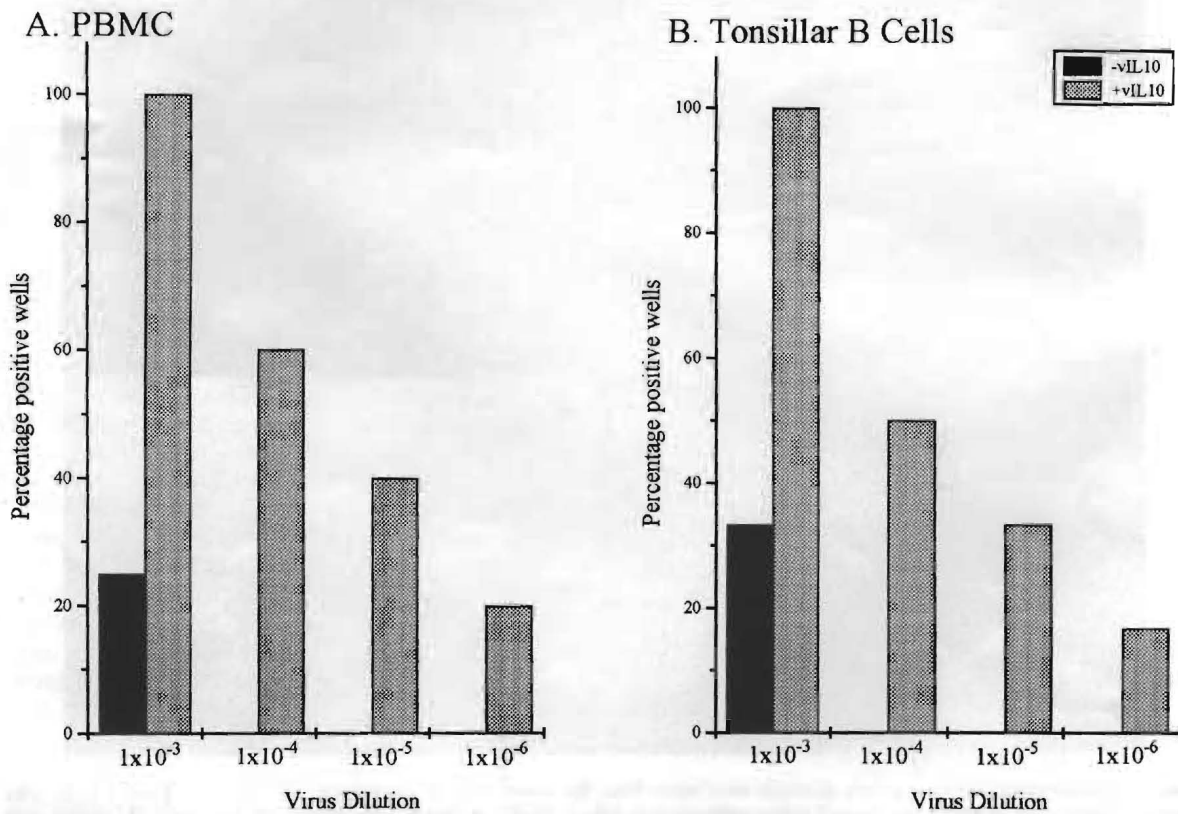


Figure 3 Transformation assays for PBMC and purified tonsillar B cells infected with B95-8. 2×10^5 cells were infected with 10-fold serial dilutions of a concentrated B95-8 preparation in the absence or presence of 100 ng ml^{-1} viral IL-10. The transformation assays were fed once per week and transformation positive wells were scored after 6 weeks

Figure
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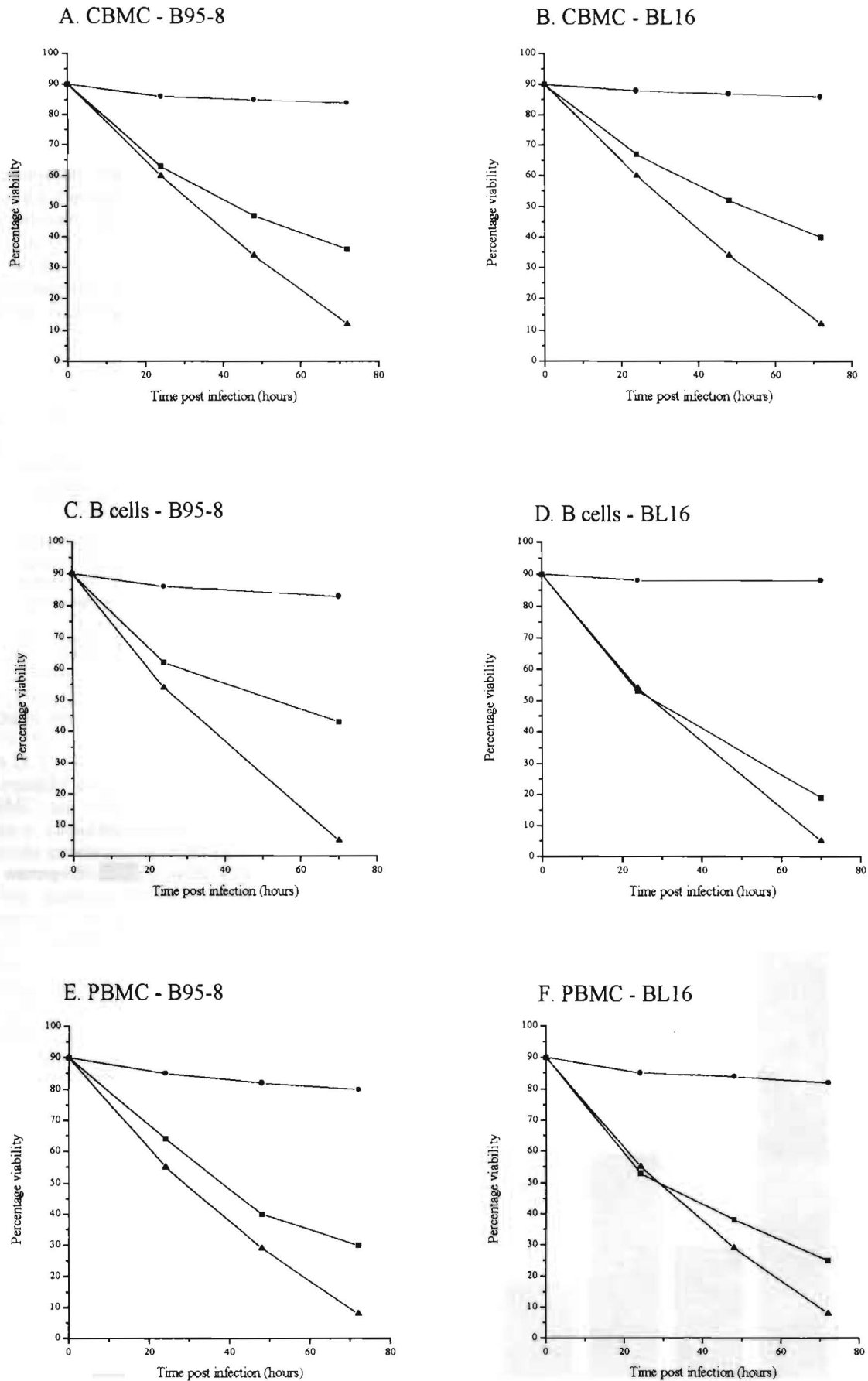


Figure 4 Cell viability after EBV infection 1×10^6 cells per ml were infected with B95-8 (10^{-3} dilution) or BL16 (2×10^{-1} dilution), cell viability was measured using trypan blue exclusion where viable cells showed a rounded morphology and did not take up the dye. Samples were tested daily up to 3 days post-infection - (▲) uninfected control; (■) virus infected no vIL-10 added; (●) virus infected 100 ng ml^{-1} vIL-10 added

effects of interferon- γ (IFN- γ) (Lotz *et al.*, 1985) and that this cytokine is produced at high levels during *in vitro* infection of PBMC with EBV (Whittingham *et al.*, 1993). Since vIL-10 can suppress production of IFN- γ from activated helper T cells and natural killer (NK) cells (Hsu *et al.*, 1990), we measured the production of IFN- γ during EBV infection of CBMC and PBMC using a specific capture ELISA. Supernatants were harvested 1 week post infection when the levels of IFN- γ released peak (Whittingham *et al.*, 1993) and the effect of vIL-10 on the endogenous IFN- γ was studied.

As is shown in Figure 5A, PBMC infected with EBV responded by producing interferon- γ , this cytokine production was reduced when vIL-10 was added to the cultures. vIL-10 inhibited IFN- γ synthesis by 70% and 60% for B95-8 and BL16 respectively. Infection of CBMC with both type 1 and type 2 EBV resulted in only minimal amounts of IFN- γ .

Sequence variation of vIL-10 open-reading frame

To exclude the possibility that functionally different open-reading frames (ORF) of vIL-10 exist we sequenced the vIL-10 ORF from a number of strains of EBV, by using polymerase chain amplification (PCR) to amplify the region of virus DNA containing the open reading frame and then directly sequencing the PCR product using an ABI automated sequencer (as described by Wright *et al.*, in press). The results shown in Table 1 indicate that there were no variations in BCRF1 sequences between any of the type 1 viruses (comparing B95-8 to virus DNA derived from spontaneous cell lines). Although there were differ-

ences at the nucleotide level for the type 2 virus sequenced, these produced no change in the amino acid sequence of the protein, thus both type 1 and type 2 EBV encode a functional viral IL-10.

Discussion

The results from our work clearly demonstrate the ability of viral interleukin-10 to enhance Epstein-Barr virus transformation of human B lymphocytes. A number of cell populations were used in the transformation assays: neonatal cord blood mononuclear cells, adult peripheral blood mononuclear cells from seronegative donors and purified tonsillar B

Table 1 Sequence analysis of BCRF1 from different strains of Epstein-Barr virus

Virus	Nucleotide Changes in BCRF1
Type 1	
B95-8	No change
EG	No change
SB	No change
JF-1	No change
Type 2	
AG876	A→G (114) ¹ T→A (171)
C2-BL16	A→G (114) C→T (306)

¹Figures in parentheses show the nucleotide position in BCRF1

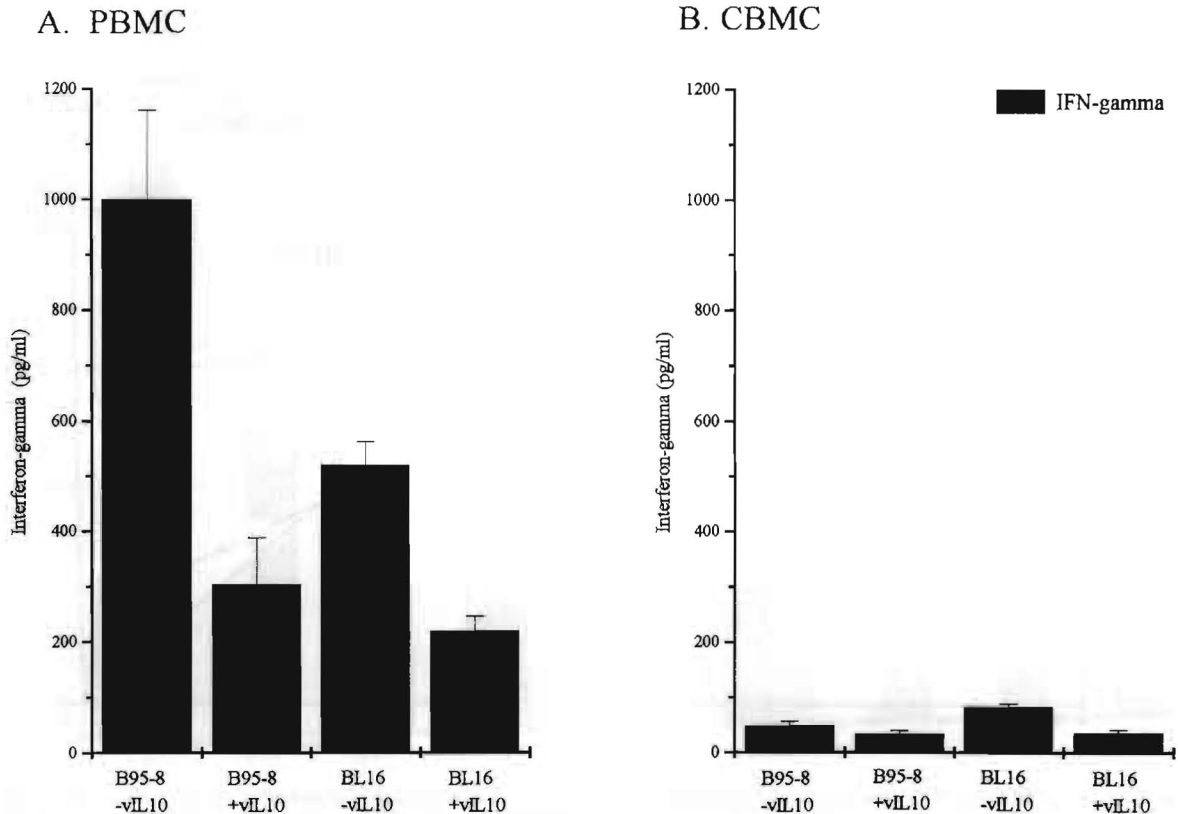


Figure 5 Interferon- γ synthesis in response to EBV infection. Supernatants were harvested from wells from the lowest virus dilutions in the transformation assays after 7 days post infection and measured for levels of interferon- γ by a specific capture ELISA

lymphocytes. In each case the addition of viral IL-10 dramatically increased the transforming ability of the virus.

There did however appear to be differences in the extent to which vIL-10 enhanced transformation. Both type 1 and type 2 EBV transformed fetal cord blood mononuclear cells across a range of virus dilutions. B95-8 (type 1) was more efficient than BL16 (type 2) at higher virus dilutions due to functional variations in EBNA 2 (Rickinson *et al.*, 1987) and possibly EBNA 3a and b (Rowe *et al.*, 1989). The addition of viral IL-10 to the assays enhanced the transforming ability of both viruses such that 100% transformation could be seen across all virus dilutions. In contrast, transformation of PBMC and purified B cells was apparent only at the lowest virus dilutions in the absence of vIL-10; the presence of vIL-10 in the assays markedly increased the size and frequency of foci across the range of dilutions. The transformation assays involving purified tonsillar B cells produced similar results to those seen with PBMC. This is most likely due to residual T cell contamination of the B cell preparation providing both cytokine and cell mediated anti-viral effects.

The differences seen in the transformation assays can be partly explained by variations in the immune response generated by the different cell populations in response to infection with EBV. A number of cytokines such as interleukin-2, interferon- γ , interleukin-6 and granulocyte-macrophage colony stimulating factor are elevated in response to EBV infection of PBMC (Whittingham *et al.*, 1993). Cord blood mononuclear cells however appear to be deficient in the production of a number of cytokines including IFN- γ , IL-4, IL-5 and IL-10 (Kruse *et al.*, 1993); they also lack significant numbers of CD8⁺ cytotoxic and CD45RO⁺ memory T cells (Roncarlo *et al.*, 1994). It has also been shown that CBMC are refractory to the anti-viral effects of interferon- γ (Thorley-Lawson, 1981). In our assays IFN- γ levels produced as a result of EBV infection of PBMC were comparable with those previously published. The addition of viral IL-10 however reduced this expression by between 60–70%. Cord blood lymphocytes expressed significantly less IFN- γ as has been found for other stimuli such as PHA and an anti-CD3 monoclonal antibody (Roncarlo *et al.*, 1994).

The difference in the size and frequency of transformed foci between PBMC and CBMC points to an important role for IFN- γ in the control of EBV infection. This has been inferred from work that has shown that EBV infected B cells are extremely sensitive to the anti-viral effects of IFN- γ (Lotz *et al.*, 1985) and also suggests one of the main functions of viral IL-10 is to inhibit the production of this cytokine during transformation. Other EBV encoded genes have also been found to modulate cytokine expression. The protein product of BZLF1 (the Z transactivator) was recently shown to upregulate expression of TGF- β 1 (Cayrol and Flemington, 1995), a potent immunosuppressor. Tumour necrosis factor- α , a cytokine that synergizes with interferon- γ to produce an anti-viral state, is inhibited at the transcriptional level upon EBV infection of human monocytes (Gosselin *et al.*, 1991) – this inhibition requires the expression of the latent proteins. It was also recently reported that human IL-10 production was induced in response to *in vitro* EBV infection of B lymphocytes (Burdin *et al.*, 1993) and

that LMP-1 was responsible for this induction (Nakagomi *et al.*, 1994). The EBV infected B cells could be induced to secrete large amounts of human IL-10 (4–9 ng ml⁻¹) into the media, with protein first appearing at 3–4 days post infection and peaking at 14 days post infection. This time course would be consistent with the requirement for viral IL-10 during the initial stages of infection only, after which time the transformed cells produced their own cytokines to support their growth. The levels of vIL-10 used in our assays were 10-fold higher than this, but in the microenvironment surrounding an infected cell it is possible that local concentrations of vIL-10 could exceed this value through interaction with the cell surface glycosaminoglycans (GAG) such as heparin or heparan sulphate (our unpublished results demonstrate the ability of vIL-10 to interact with both of these GAG).

The increase in cell survival during EBV infection in the presence of vIL-10 is most likely due to inhibition of apoptosis, as the resting B cells that EBV typically infects are highly susceptible to this mechanism of programmed cell death. This activity has been previously described for IL-10 with activated human T cells (Taga *et al.*, 1993).

The exact role of vIL-10 *in vitro* is unclear, previous studies using *in vitro* models have proposed two, conflicting, theories. Swaminathan *et al.* (1993) described the use of a recombinant EBV lacking the open reading frame coding for vIL-10. The deletion of the vIL-10 ORF had no effect on the transforming ability of the virus compared with wild type, but reactivation of lymphoblastoid cell lines transformed with the mutant virus resulted in the induction of greater levels of interferon- γ . The absence of any differences in transformation by wild type EBV compared with the vIL-10 deletion mutant may have resulted from the fact that these assays were already optimised for transformation, whereas our assays used a range of suboptimal virus concentrations to investigate the activity of vIL-10 during transformation. Our data concerning inhibition of IFN- γ synthesis by addition of vIL-10 to transformation assays, does further support the conclusion that vIL-10 may act to suppress any immune response to EBV infection *in vivo*.

Miyzaki *et al.* (1993) described a critical role for vIL-10 during the transformation process by using antisense oligonucleotides to inhibit production of the protein and suggested that the protein was expressed at early times after infection and thus constituted a true latency gene. The expression of vIL-10 had previously been demonstrated to be at late times during the productive phase of the virus replication cycle (Hudson *et al.*, 1985; Stewart and Rooney 1992; Stewart *et al.*, 1994) with no detectable protein produced by latently infected cells. Our results do support a role for vIL-10 during the transformation process itself, however the data from Swaminathan *et al.* (1993) suggested that the protein was in fact non-essential for transformation and it may therefore act as a pathogenesis factor by enhancing transformation during B lymphocyte infection with sub-optimal amounts of virus.

Our results, along with previously published data, have led us to propose the model shown in Figure 6 to describe the possible role of vIL-10 during the EBV life

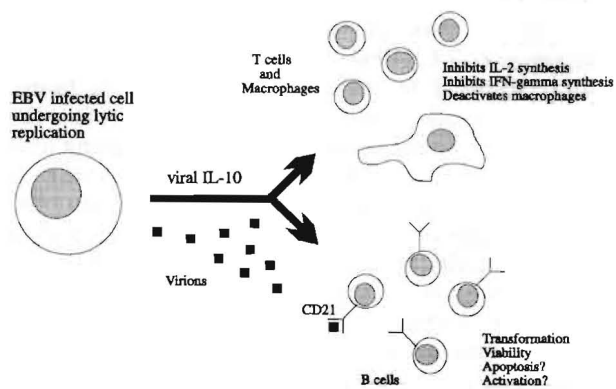


Figure 6 Schematic representation of the role of viral IL-10 in the Epstein-Barr virus life cycle. The diagram is primarily based upon results from this report, although it also includes activities previously described for IL-10 (for review see Moore *et al.*, 1993)

cycle. vIL-10 could act in two ways: to dampen any initial immune response to the infection and also enhance the transformation process itself.

The activities we have demonstrated for vIL-10 could be useful in a number of practical applications. It may allow recovery of low titres of type 1 and, more importantly, type 2 EBV from blood or clinical samples, type 2 EBV is very difficult to rescue from samples due to its low transforming ability. Viral IL-10 may also be useful for establishing spontaneous cell lines from EBV seropositive individuals, where an initial replication of endogenous virus is required and not simply outgrowth of existing transformed cells (Rickinson and Epstein 1975).

Viral interleukin-10 therefore appears to play a pivotal role in the establishment and maintenance of the EBV carrier state found in seropositive individuals through its ability enhance transformation of B cells during an initial infection and to suppress any immune response arising from this infection or any reactivation of virus.

Materials and methods

Expression of viral interleukin-10

BCRF1 was cloned, using conventional techniques (Sambrook *et al.*, 1989), as a RsaI fragment to remove the N-terminal signal sequence of the protein into the pMAL-c2 (New England Biolabs) prokaryotic expression vector. This created a fusion protein with maltose binding protein (MBP) that can be purified using affinity chromatography with amylose resin, as described by the manufacturer. After cleavage from MBP with the restriction protease factor Xa, the viral interleukin-10 was further purified using a heparin agarose column. The cleaved vIL-10 was eluted from the heparin column with 300 mM NaCl and resulted in a protein solution of approximately 90% purity.

Cells and virus

Umbilical cord blood samples were obtained from full term neonates and provided by Dr Fiona Clarke (Department of Immunology, Paterson Institute, Christie Hospital, Man-

chester). Cord blood erythrocytes were sedimented using 6% (w/v) Dextran (250 000 mol.wt., ICN) (Walls and Crawford, 1987). The erythrocyte depleted mononuclear cells were then isolated by density centrifugation with Lymphocyte Separation Medium (ICN). Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples from healthy EBV seronegative donors by centrifugation through Lymphocyte Separation Medium. Tonsillar B cells were kindly provided by Diane Johnson (Department of Gene Regulation, Paterson Institute, Manchester) and prepared using methods previously described (Weiner *et al.*, 1973).

Concentrated stocks of B95-8 (Miller *et al.*, 1972) and BL16 (Lenoir *et al.*, 1985) virus were prepared by inducing the cell lines to lytic cycle replication. The culture supernatants were centrifuged and filtered to remove cell debris before concentrating 100-fold using a Hollow Fibre & TurboTube Ultrafiltration Cartridge with a molecular weight exclusion of 500 000 Da (A/G Technology Corporation).

Transformation Assays

Transformation assays were performed as described previously (Walls and Crawford, 1987), briefly mononuclear cells or purified tonsillar B cells were plated at density of 1×10^6 cells per ml in 96 well tissue culture plate (Falcon) and infected with 10-fold serial dilutions of either B95-8 or BL16 (B95-8: 10^{-3} – 10^{-7} and BL16: 2×10^{-1} – 2×10^{-5}). Six replicates of each virus dilution were set up in the absence or presence of 10 ng ml^{-1} recombinant viral IL-10 in RPMI 1640 (Gibco BRL) containing 10% fetal calf serum (Globepharm). The transformation assays were incubated at $37^\circ\text{C}/5\% \text{ CO}_2$ and fed once per week by removing half the medium and replacing with fresh RPMI 1640 containing 10% FCS. Transformation positive wells were scored after 6 weeks in culture.

Immunofluorescence to determine cellular phenotype

Transformed cells from the above assays were removed and washed twice with PBS and then fixed with acetone:methanol (1:1). The slides were preincubated with PBS containing 0.2% Tween-20 and 5% normal rabbit serum (PBST/NRS) for 30 min at 37°C before the addition of antibody diluted in PBS/NRS. Excess antibody was removed by washing with PBST and then, if required, a secondary antibody conjugated to phycoerythrin (PE) was added. The slides were washed once more, mounted in PBS:glycerol (1:1) containing 2.5% Dabco (Sigma) and visualised using a uv microscope.

Anti-EBNA2: PE2 (Dako); anti-CD23 (Serotec); anti-CD19 FITC conjugate (Dako) and anti-mouse PE conjugate (Dako).

Cell viability

Peripheral blood mononuclear cells were plated at a density of 2×10^6 cells per ml in 96 well plate and infected with either 10^{-3} dilution of B95-8 or 2×10^{-1} dilution of BL16. Purified tonsillar B cells were infected with the same virus dilutions, but at a density of 1×10^6 cells per ml. Cell viability was measured using Trypan Blue (Sigma) exclusion.

Interferon- γ production during EBV infection

Supernatants were collected from the transformation assays at week 1 and 2 post infection and the levels of interferon- γ present in each sample were measured using an interferon- γ ELISA (Serotec).

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