Epstein–Barr virus nuclear antigen-1 renders lymphocytes responsive to IL-2 but not IL-15 for survival

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Epstein–Barr virus nuclear antigen-1 (EBNA-1) is the only latent protein expressed in all virus-associated tumours. It plays a critical role in viral propagation and in the replication, episomal maintenance and partitioning of the viral genome. However, its tumorigenic potential is debated. We have previously shown that lymphocytes from a tumour-prone, EBNA-1-expressing, transgenic mouse line show increased responsiveness to interleukin-2 (IL-2). It was important to determine whether this property was unique to the transgenic line or whether it is a general consequence of EBNA-1 expression in B cells. In order to distinguish between these possibilities, explanted lymphocytes from two independent transgenic mouse lines were examined. The lymphocytes from both lines showed enhanced proliferation rates compared with controls. The transgenic lymphocytes survived for extended periods in culture, dependent on the dose of IL-2, while IL-15 (the receptor of which shares the β and γ chain components of the IL-2 receptor) induced little effect. In accordance with this, transgenic B cells showed enhanced induction of expression of the IL-2 receptor α chain (CD25), which modulates affinity for the ligand. As this phenotype is evident in lymphocytes from mice of both lines, it is necessarily independent of any transgene insertion site effects and may be attributed to EBNA-1 expression. Furthermore, 10/12 tumour-bearing transgenic mice had elevated IL-2 levels in serum and 4/6 tumours were CD25 positive. IL-2 is normally produced by activated T cells in vivo; thus, chronic immune activation or modulation could elicit this unique mode of virus-infected cell survival.

INTRODUCTION

Epstein–Barr virus (EBV) is a human herpesvirus associated with a number of malignancies, including endemic Burkitt’s lymphoma (eBL), nasopharyngeal carcinoma (NPC), Hodgkin’s disease (HD) and lymphomas in immunosuppressed individuals (Kawa, 2000). The ability of EBV alone to induce B-cell proliferation is supported by the efficient transformation of primary B cells into permanent lymphoblastoid cell lines (LCLs) upon in vitro infection by the virus. In LCLs and tumour cells, EBV establishes a latent infection in which a subset of viral genes is expressed. Different tumour types express a different subset of these latent genes but in all cases, EBV nuclear antigen-1 (EBNA-1) is expressed; indeed, EBNA-1 is the only viral antigen consistently detected in eBL.

EBNA-1 is required for stable and efficient latent EBV infection (Humme et al., 2003; Lee et al., 1999). It activates replication of the viral episomal genome and plays a critical role in viral genome segregation at cell division (Ceccarelli & Frappier, 2000; Chittenden et al., 1989; Kapoor & Frappier, 2003; Lee et al., 1999). EBNA-1 also transactivates the expression of several viral latency genes from the viral Cp promoter (Gahn & Sugden, 1995; Zetterberg et al., 2004).

While EBNA-1 plays a crucial role in viral maintenance, it may also contribute to the development of malignancy. However, it is clearly not essential to the in vitro immortalizing property of the virus since a viral strain with a deletion in the EBNA-1 gene is still able to generate LCLs in culture, albeit at much-reduced efficiency (Humme et al., 2003). Nevertheless, Kennedy et al. (2003) showed that EBNA-1 contributes to the ongoing survival of EBV-positive BL cell lines, a property which was demonstrated to be independent of the viral genome maintenance function. Furthermore, EBNA-1 interacts with a ubiquitin-specific protease USP7 (Holowaty et al., 2003; Saridakis et al., 2005), which plays a role in regulating the p53 and Mdm2 pathway (Li et al., 2004). Thus, it is hypothesized that EBNA-1 could interfere with the stabilization of p53 and thereby affect cell survival and proliferation (Saridakis et al., 2005).
A potential oncogenic role of EBNA-1 in vivo is indicated by the predisposition of transgenic mice that express EBNA-1 in B cells to B-cell lymphoma (Wilson et al., 1996; Wilson & Levine, 1992). However, the causal association remains controversial on two counts. First, the transgenic mouse line expressing relatively low levels of EBNA-1 in lymphocytes (line E EBNA-1.26) succumbs to B-cell lymphoma with comparatively rapid onset, while an independent mouse line expressing EBNA-1 at higher levels (line E EBNA-1.59) exhibits a lymphoma incidence with low penetrance and long latency (Wilson et al., 1996). The insertion site of the transgene of the former line (currently under investigation) is complex and at present it is not clear whether this has made any contribution to the lymphoma phenotype, as previously noted (Wilson et al., 1996). Second, Kang et al. (2005), using a different series of transgenic mice expressing high levels of EBNA-1 in lymphocytes, did not detect the lymphoma phenotype. The level or pattern of transgene expression could explain the phenotypic difference between the mice strains. However, there are other possibilities, most notably the pathogen status of the animal housing could influence immune signalling in the mice. Thus, the precise predisposing event underlying the lymphoma phenotype of the mice developed in our laboratory is not clear.

In order to explore the consequences of EBNA-1 expression in vivo, independent of any potential transgene insertion site effects and tumour phenotype, the studies described here were conducted in mice from two independent transgenic lines prior to tumour development. As such, properties found in both lines that are distinct from controls can better be attributed to the expression and action of EBNA-1.

We previously reported that lymphoid cells explanted from line E EBNA-1.26 mice showed prolonged survival in culture compared with controls, but only when supplemented with interleukin-2 (IL-2) (Tsimbouri et al., 2002). In order to extend this observation and to exclude the possibility that this is due to insertion site effects in line 26, the response to IL-2 in the two independently derived EBNA-1 expressing transgenic mouse lines was examined. We demonstrate that the lymphocytes show enhanced proliferation compared with controls and extended survival when cultured in the presence of IL-2. As this property is evident in cells from both transgenic lines, it is associated with EBNA-1 expression. Moreover, since it is manifested upon addition of IL-2, normally produced by T cells, the full consequences may only become apparent in vivo in the context of an active immune system.

**METHODS**

**Transgenic mice and screening.** The two lines, E EBNA-1.26 (line 26) and E EBNA-1.59 (line 39), of transgenic mice used in this study have been described previously (Drotar et al., 2003; Wilson et al., 1996). Line 26 mice succumb to lymphoma between 4 and 12 months of age and line 59 mice usually from 18 months onwards. Mice were maintained in the C57BL/6 strain under conventional housing conditions. All procedures were conducted under Home Office licence and the research has complied with Home Office and institutional guidelines and policies. Tail genomic DNA was prepared and tested by Southern blot for E EBNA-1 transgenic status as described previously (Wilson & Drotar, 2001).

**Western blotting.** Cells were washed in PBS, recovered by centrifugation at 1500 g for 5 min, snap frozen in liquid N2 and stored at –70 °C. Cells were resuspended in 200 μl NE lysis buffer [20 mM HEPES/NaOH, pH 7.9, 0.4 M NaCl, 1 mM EDTA, pH 8, 1 mM EGTA, pH 8, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail (Sigma; 100 mM benzamidine, 200 μg leupeptin ml−1, 200 μg pepstatin A ml−1)] on ice for 15 min; insoluble matter was discarded after centrifugation at 16,000 g for 10 min, 4 °C. Proteins were separated by reducing SDS-PAGE (7.5%) and electroblotted as previously described (Wilson et al., 1996). Transferred immobilized EBNA-1 antigens were detected with the monoclonal antibody (mAb) 1H4 (Grasser et al., 1994) diluted 1:20 in blocking buffer [20 mM Tris/HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20 (TBST) with 5% non-fat milk], followed by goat-anti-rat horseradish peroxidase (HRP) secondary antibody (diluted 1:4000). Antigens were visualized using the luminol system (ECL; Amersham).

**Tissue culture.** Splenocytes and bone marrow cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U penicillin ml−1 and 100 μg streptomycin ml−1 (Gibco), with or without IL-2 or IL-15. Except where noted, cells were explanted prior to tumorigenesis (hence taken from 2-month-old line 26 mice and from line 59 mice aged up to 12 months). Viable cells (20 μl aliquots) were counted by trypan blue exclusion staining of dead cells (Tsimbouri et al., 2001) from cultures at 24 to 48 h intervals. Cultures below 10⁵ cells ml−1 were regarded as non-viable. For cells stimulated with goat F(ab')2 anti-mouse IgM (anti-μ) antibody (Caltag Laboratories), 10 μg antibody ml−1 was used for the first 2–4 days of culture as indicated, with or without IL-2.

**[^3H]Thymidine assay.** To measure proliferation, cells were cultured in 96-well plates at 2×10⁵ cells per well (in triplicate), with or without 60 ng IL-2 ml−1. Cultures were pulsed with 1 μCi (37 kBq)[^3H]Thymidine (ICN) for 16 h and then frozen at –20 °C. Subsequently, the cells were harvested onto 96-well filter-plates using a Filtermate cell harvester. Thymidine uptake was measured by adding 50 μl scintillation fluid per well and counting in a TopCount Microplate Scintillation Counter.

**Flow cytometry.** Splenocytes and bone marrow cells were isolated and red blood cells were then lysed in NH₄Cl/Tris buffer, pH 7.2, for 10 min (Tsimbouri et al., 2001), before pre-incubation for 20 min with 20 μl of either goat or rabbit whole serum. Cells (5×10⁵ in 0.5 ml volume) were stained by incubating for 45 min at 4 °C with different cell markers: 1 μg FITC- or PE-conjugated antibodies directed to B220, CD3, CD21, CD23, CD25, CD38, ThB, CD122, CD132 (Caltag), CD43 (BD Pharmingen), CD90 or Cy5-conjugated CD19 (eBioscience). Samples were washed in PBS and analysed using a Becton Dickinson flow cytometer using appropriate isotype controls (Serotec).

**B-cell and T-cell selection.** Cells were used for two purposes. Firstly, for Western blot analysis, explanted cells were washed in PBS, red blood cells were lysed, washed again and counted. Dynabeads mouse panB (B220) and panT (Thy1.2) were used for B-cell and T-cell selection, respectively (Tsimbouri et al., 2001). A ratio of 4:10 beads per target cell was used with 10² cells ml⁻¹, and incubated for 20 min at 4 °C with gentle agitation. Immuno-selected B cells were separated using the Dynal MPC magnetic device for 2 min at 4 °C. The supernatant containing unsellected cells was removed and kept for...
subsequent T cell selection. The selected cells were washed three times in 1 ml PBS/1% FCS, then pelleted and stored at –70 °C for analysis. Secondly, for culturing, in a preliminary experiment, mature B cells were negatively selected from splenocytes using MACS-CD43 and immature B cells were selected from bone marrow using Dynabead-B220; T-cells were selected using MACS-Thy1.2. The yield of relatively pure selected cells was low and cultures set up from individual mice were poorly viable. In a subsequent experiment, cells from 6-month-old mice (excluding line 26 mice showing signs of neoplasia) were pooled and cultures were set up from selected T cells using MACS-Thy1.2 (CD90.2) separate from the remaining population depleted of T cells (this was referred to as the B^B° population).

ELISA. Serum samples at 1:40 dilution were assayed for murine IL-2 by ELISA (eBioscience #88-7024) according to the manufacturer’s protocol. Briefly, 96-well plates were coated with capture antibody overnight at 4 °C and then blocked with assay diluent. Duplicate or triplicate samples and standards were incubated in the wells overnight at 4 °C and IL-2 was then detected with a biotin-conjugated detection antibody, followed by avidin–HRP and then HRP substrate. Multiple well washes were conducted between each step. Absorbance was read at 450 nm.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared by Polytron disruption of snap-frozen samples using NE lysis buffer (above); samples were incubated on ice for 15 min and insoluble matter was removed by centrifugation at 16 000 g for 15 min at 4 °C. Binding reactions were performed for 30 min on ice using 20 μg nuclear extract and 0.2–1 ng probe in binding buffer (10 mM HEPES/NaOH, pH 7.9, 0.2 mM EDTA, pH 8, 0.1 M NaCl, 4 mM DTT, 0.1 mg BSA ml⁻¹, 1 %, v/v, glycerol with 2 μg polyIdC). The double-stranded oligonucleotide probe incorporating the EBNA-1 binding site (5'-TGGATAGCTATGCTATCCA-3') was end labelled using T4 polynucleotide kinase and [³²P]dATP and column purified (NICK; Amersham). Excess unlabelled oligonucleotide competitor (100×) was added to one aliquot of each sample for 10 min prior to incubation with the probe. The reaction products were electrophoresed through a 6 % non-denaturing acrylamide gel, which was dried and exposed to film after completion.

RESULTS

EBNA-1 transgenic lymphocyte cultures show enhanced survival in response to IL-2

In order to investigate whether the enhanced response of transgenic lymphocytes to IL-2 in culture was a unique property of EμEBNA-1.26 (line 26) mice, we investigated whether this property was shared by another independent EBNA-1-expressing mouse line, EμEBNA-1.59 (line 59). In this study, all tissues were explanted prior to the development of any neoplastic changes in the respective lines, as assessed by gross inspection, histopathology and flow cytometry. Spleen and bone marrow cells from line 26, line 59 and their non-transgenic sibling controls (NSC) were cultured in medium with or without IL-2. Viable cell counts were taken at regular intervals. The experiment was repeated four times using three to eight mice of each genotype per group. The findings were consistent between the experiments and representative data for splenic (Fig. 1) and bone marrow (Fig. 2a) cell cultures are shown. In order to determine whether the age of the mouse affected the lymphocyte survival, cells from wild-type control mice at 3 and 12 months old were cultured in the presence or absence of IL-2. No difference was observed between the two age groups (data not shown).

**Fig. 1.** Effect of IL-2 on the survival of lymphocytes from EμEBNA-1 lines 26 and 59. Splenocytes from 12-month-old mice of line 59 and NSC and 2-month-old mice of line 26 and NSC were cultured in RPMI with 10 % FCS, with or without IL-2, at an initial density of 5×10⁶ cells ml⁻¹. (a) Cells were pooled from four EμEBNA-1.59 transgene-positive (line 59) and four NSC mice, and four EμEBNA-1.26 transgene-positive (line 26) and four NSC mice and cultured in the absence of IL-2. (b–d) Cells collected from eight individual mice were pooled in pairs and cultured as four separate cultures from each of the transgene positive EμEBNA-1.59 (line 59) and EμEBNA-1.26 (line 26) mice and from NSC mice (control), supplemented with 20 ng IL-2 ml⁻¹ (b), 40 ng IL-2 ml⁻¹ (c) or 60 ng IL-2 ml⁻¹ (d). Means ± SEM are shown. Note that, as individual cultures die out, the SEM increases to the point, in some instances, when only one of the four paired cultures remains alive. The cultures were continued until no viable cells could be detected (up to a maximum of day 43).
To examine the effect of different IL-2 concentrations upon the survival of transgenic lymphocytes compared to controls, parallel splenocyte cultures (from eight mice in each group) were supplemented with 20, 40 and 60 ng IL-2 ml$^{-1}$ (Fig. 1b–d) and compared with cultures with no IL-2 (Fig. 1a). In the absence of IL-2, line 59 splenocytes showed slight extended survival (up to 24 days) compared with the NSC (7–12 days), while there was no pronounced difference in survival between line 26 and NSC splenocyte cultures (Fig. 1a). As noted above, there was no difference in survival between the two control groups (NSC for both line 26 and 59 mice) derived from mice of 2 or 12 months old (controls 2 and 1, respectively; Fig. 1a). In the presence of 20 ng IL-2 ml$^{-1}$, the control cultures survived up to day 20, the line 26 cultures to day 30 and the line 59 cultures to day 35. At 40 ng ml$^{-1}$, only one of the four control cultures remained alive up to day 30, while line 26 and 59 cultures lasted to day 40 and 43, respectively. At 60 ng IL-2 ml$^{-1}$, the control cultures survived up to day 35, while the line 26 and 59 cultures remained viable to the end of the study period at day 43. Thus, at each IL-2 concentration, the transgenic splenocyte cultures showed extended survival compared with the NSC cultures. The survival extension was dose-dependent; survival of the transgenic samples in 20 ng IL-2 ml$^{-1}$ was roughly equivalent to the control samples at a 3-fold higher IL-2 concentration (60 ng IL-2 ml$^{-1}$).

In order to explore this effect on an immature lymphocyte population, bone marrow-derived cultures were similarly examined. Line 59 bone marrow cells showed no survival advantage over the controls in the absence of IL-2 (Fig. 2a), as previously shown for line 26 bone marrow cultures (Tsimbouri et al., 2002); all were dead within 2 weeks of explant. However, in the presence of IL-2, bone marrow cultures from lines 59 and 26 showed a significant prolongation of survival compared with NSC cultures (Fig. 2a). In the representative experiment that is shown, seven of eight NSC cultures (four in each of control groups 1 and 2) died between days 9 and 12, with only one of the NSC cultures in control group 2 (control 2 + IL-2) lasting to day 19. In contrast, line 26 and 59 bone marrow cultures survived up to day 34 and 72, respectively.

**EBNA-1 lymphocyte survival is dependent on IL-2 but not IL-15**

The IL-2 high-affinity receptor is trimeric, composed of IL-2R$\alpha$ (CD25), IL-2R$\beta$ (CD122) and IL-2R$\gamma_c$ (CD132) chains. IL-2R$\alpha$ contributes to IL-2 binding affinity but does not contribute to signalling directly, due to its short cytoplasmic tail. The intermediate affinity dimeric receptor, composed of IL-2R$\beta$ and IL-2R$\gamma_c$ chains, is necessary and sufficient for IL-2 signalling but requires 10–50-fold higher concentrations of IL-2 than typically found in vivo (Gaffen, 2001). Similarly, IL-15 uses the receptor IL-2R$\beta$ and IL-2R$\gamma_c$ chains for signalling, with a distinct third subunit, IL-15R$\zeta$ that acts as an affinity modulator. In order to explore whether the survival advantage mediated by IL-2 in transgenic lymphocyte cultures is also mediated by IL-15, an assay was conducted using IL-15 supplements. Spenic and bone marrow cells from line 26 mice were seeded in culture with or without IL-15 at 20 or 60 ng ml$^{-1}$ (Fig. 2b). Addition of IL-15 conferred a survival advantage to the splenocyte cultures and less so to the bone marrow cultures (data not shown) compared with cultures without cytokine supplement; however, only a marginal difference between transgenic and control cultures was apparent.

**Surviving cells include B cells**

In order to identify the surviving cell populations from the above IL-2 culture experiments, cells from all cultures in all groups were analysed by flow cytometry. Forward and side scatter plots did not reveal differences between transgenic and NSC splenocytes or bone marrow cells. In young mice prior to neoplasia, no differences between transgenic
(either line 26 or 59) and NSC were seen for surface immunoglobulin or ThB, CD23, CD38 or CD43 staining, from the start of culture up to the end point of survival for the NSC cultures (data not shown). Cultures surviving in IL-2 showed the same pattern of cell surface expression, regardless of transgene status, as exemplified by co-staining with anti-B220 (B-cell marker) and anti-CD3 (mature T cell marker) (Fig. 3a). No differences were seen in the initial proportions of B and T cells between transgenic and control mice (representative plots are shown in Fig. 3a for a line 59 culture sample, but all line 59, line 26 and NSC samples showed the same pattern). The freshly explanted splenic lymphocyte samples include three distinct populations: a B220$^{\text{high}}$ B cell population, an intermediate B220$^{\text{low}}$ B cell population and a B220$^{+}$/CD3$^{+}$ T cell population. In the bone marrow samples, two B cell populations were also noted (B220$^{\text{high}}$ and B220$^{\text{low}}$) with a B220/CD3 negative population that included immature T cells (data not shown). During culture, all surviving populations showed a similar progressive pattern, with the apparent loss of the CD3$^{+}$ population from the splenocytes (Fig. 3a, d16) and of the B220$^{-}$ population from the bone marrow cells within the first 2 weeks of culture. The distinct B220$^{+}$ populations merged into a diffuse B220$^{+}$ population by day 16, developing over time in culture into a largely B220$^{\text{low}}$ population (Fig. 3a, d28 and d36). This was the case for all transgene-positive samples from both lines (26 and 59), while transgene-negative control cultures were dead at the later time points, as described above. At the end of the study period, the surviving transgenic cell populations were B220$^{\text{low}}$/CD3$^{-}$. Secreted IgM could be detected in the medium throughout the culture period (including the end points) supporting the conclusion that B cells are amongst the surviving cells.

A further flow cytometry experiment was conducted in order to characterize the surviving B cell population in more detail. Freshly explanted spleen B cells (CD19$^{+}$) can be subdivided into distinct subpopulations on the basis of staining patterns, such that the self-renewing, minor B-1 subpopulation of B cells are B220$^{-}$/CD23$^{\text{low}}$/CD21$^{\text{low}}$/IgM$^{\text{high}}$/CD5$^{+}$ or $^{-}$, the major B-2 (or follicular) B cells are B220$^{+}$/CD23$^{+}$/CD21$^{+}$/CD43$^{+}$ and marginal-zone B cells are B220$^{+}$/CD23$^{+}$/CD21$^{+}$/CD43$^{+}$/IgM$^{\text{high}}$/CD5$^{+}$ or $^{-}$. These populations were then cultured separately with IL-2 and supplemented with IL-2. With a low yield of highly enriched cells, the small cultures showed poor viability from both transgenic and control mice, suggesting that either the B cells were not viable alone or the selection protocol was inhibitory to survival. To address these possibilities, a further experiment was conducted, pooling cells from a large number of 6-month-old animals from each line to ensure sufficiently dense and large volume cultures could be initiated to optimize survival potential. T cell populations (Thy1.2$^{+}$) from bone marrow and spleen cells from NSC, line 59 and line 26 mice were selected, yielding a T-cell-selected population (T; on average, the proportion of Thy1.2$^{+}$ cells increased from 38 to 85%) and a remaining population that was consequently enriched for non-T-cells (B$^{\text{plus}}$; on average, the proportion of CD19$^{+}$ cells increased by 32% and the T cell component approximately halved (0.54-fold reduction) compared with the preselected population). These populations were then cultured separately and supplemented with IL-2 along with reconstituted cultures comprising 40% T and 60% B$^{\text{plus}}$ (Fig. 3c). As these were pooled samples using only one representative of each genotype, survival differences cannot be accurately interpreted; however, all cultures from NSC mice died between 30 and 40 days of culture (Supplementary Fig. S1, available in JGV Online). The transgenic B cells showed extended survival compared with NSC (50 to 60 days). For each genotype, the T cell cultures showed poorer life spans compared with the B$^{\text{plus}}$ and reconstituted populations and line 26 T cells showed no survival advantage compared with NSC, while there was some suggestion that line 59 T cell cultures might show increased longevity (Supplementary Fig. S1). For each genotype, the reconstituted cultures showed a life span intermediate between that of the T and B$^{\text{plus}}$ cultures, indicating that reconstituting the T cell component does not provide a growth advantage to the B$^{\text{plus}}$ cultures, rather that this reflects simply a composite of their independent survival curves. Cells from each culture were examined by flow cytometry on a weekly basis to track the B and T cell components of the culture, first by co-staining with CD19 and CD3 (splenocytes) or CD19 and CD90/Thy-1 (bone marrow cells) and subsequently with IgM and CD3 (Fig. 3c). The selected T cell spleen populations from NSC and transgenic cultures revealed that the cells gradually lose CD3 positivity in culture. The subpopulations of non-T-cells in these...
cultures that remain over time indicate that they are not out-growing the cultures (still visible as separate populations at day 15 of culture and as IgM-positive subpopulations at the end of culture). Similarly, the staining pattern of the $B^{\text{plus}}$ cultures suggests that there is a gradual decline in CD19 staining of the surviving B cells (as for B220 above), rather than the emergence of a distinct, pre-existing CD19low population. The IgM staining at the end of culture confirms the (non-exclusive) presence of mature B cells. The reconstituted cultures reflect a simple additive combination of what is seen in the selected cultures.

**EBNA-1 enhances IL-2Rα/CD25 induction in culture**

The response to IL-2 is amplified in transgenic lymphocyte cultures compared with controls, while little difference was observed with IL-15. This could suggest that IL-2 receptor affinity, mediated by the unique component IL-2Rα/CD25, is modulated by EBNA-1. In order to explore if EBNA-1 affects IL-2Rα/CD25 expression in these transgenic cells, splenocytes and bone marrow B cells from both transgenic lines were analysed by flow cytometry. Using more than 18 pre-neoplastic transgene-positive and NSC pairs from each line, no significant difference was found either in the proportion of B cells positive for IL-2Rα/CD25 or in staining intensity between transgenic and controls (data not shown). In all cases, splenic and bone marrow B cells were largely negative for IL-2Rα/CD25 staining. To investigate if IL-2R expression can be induced in culture, transgenic and NSC splenocytes and bone marrow cells were analysed after 1 week in culture, using anti-B220 as a co-stain. Culture of mouse B cells in the presence of anti-immunoglobulin antibody (anti-μ) has been shown to induce the expression of IL-2Rα/CD25 (Moreau et al., 1995). We therefore compared IL-2R expression under four culture conditions: (i) no cytokine addition, (ii) addition of 60 ng IL-2 ml$^{-1}$, (iii) stimulation with anti-μ and (iv) stimulation with anti-μ plus addition of 60 ng IL-2 ml$^{-1}$. In all cases, no significant difference was observed in the degree of B cell staining for IL-2Rα/CD122 and IL-2Rα/CD132 between transgenic and NSC cultures (data not shown). However, stimulation with anti-μ (with or without IL-2) resulted in a pronounced induction of IL-2Rα/CD25 in line 59 transgenic splenic B cells (this was less apparent in controls; Fig. 4a), while a similar degree of induction was observed between transgenic and control bone marrow B cells (data not shown). Splenic B cells from line 26 mice showed comparable IL-2Rα/CD25 induction in response to anti-μ stimulation between transgenic and control cultures, this difference from line 59 could be due to the lower EBNA-1 expression levels in line 26. However, the line 26 transgenic cultures showed greater IL-2Rα/CD25 induction compared with controls in response to IL-2 (with or without anti-μ stimulation) (data not shown). As such, it was observed that the induction of IL-2Rα/CD25 expression in B cells by IL-2 or anti-μ was amplified in transgenic samples compared with controls.

**IL-2 and CD25 are induced in line 26 tumours**

In order to extend this observation, IL-2 and IL-2Rα/CD25 expression was examined in the B-cell lymphomas which arise in line 26 mice (Wilson et al., 1996). Four out of six splenic tumour biopsies examined showed B cells that were positive for CD25, while very few or no normal B cells from NSC showed CD25 expression (Fig. 4b). Moreover, 10 out of 12 serum samples examined from line 26 tumour-bearing mice showed high levels of IL-2. In contrast, IL-2 could not be detected in the serum of mice from lines 26 or 59 prior to tumour development, NSC mice or in tumour-bearing mice of the Eph-myc transgenic line (Adams et al., 1985) (Table 1).

**EBNA-1 transgenic lymphocyte cultures show enhanced proliferation**

All explanted lymphocyte cultures showed considerable cell death in the first days of culture (as shown in Figs 1 and 2).
In order to explore if a population of cells in the cultures was proliferating, a phenomenon masked in the total viable cell count by the cell death, aliquots were taken after one week of culture and labelled with $[^{3}\text{H}]$thymidine. In the absence of added cytokines, the transgenic splenocyte cultures showed a considerably higher rate of proliferation compared with NSC cultures, detected by $^{3}$H labelling values; there was three orders of magnitude difference for line 26 and one order of magnitude for line 59 (Fig. 5a, b). Bone marrow cultures in the absence of IL-2 were largely dead at this stage as shown in Fig. 2, and could not be assayed. In the presence of 60 ng IL-2 ml$^{-1}$, NSC cultures showed a pronounced induction of proliferation (by 2 to 3 orders of magnitude compared with no IL-2), which was almost as high as the transgenic splenic cultures with IL-2 (both lines; Fig. 5a, b). Bone marrow cultures in the presence of IL-2 showed three- to fourfold higher $[^{3}\text{H}]$thymidine labelling in the transgenic samples compared with controls for both transgenic lines.

Table 1. Presence of IL-2 in the serum of non-tumour- and tumour-bearing mice

IL-2 was assayed in serum, diluted 1:40, derived from non-tumour- and tumour-bearing (neoplastic) mice. The mean IL-2 concentration in the 10 IL-2-positive samples was 94.6 pg ml$^{-1}$ ($\pm$ SD, 36.9, $\pm$ SEM, 10.6).

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>Detectable IL-2</th>
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<tbody>
<tr>
<td>NSC</td>
<td>0/6</td>
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<tr>
<td>EμEBNA-1 line 26 pre-neoplastic</td>
<td>0/10</td>
</tr>
<tr>
<td>EμEBNA-1 line 59 pre-neoplastic</td>
<td>0/6</td>
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<tr>
<td>Eμc-myc neoplastic</td>
<td>0/4</td>
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<td>EμEBNA-1 line 26 neoplastic</td>
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The $y$ axes of (c) and (d) are scaled to the respective control (=100) for the purpose of comparison.
These data show that, 1 week after explantation, in the absence of cytokine supplement, splenocyte cultures from both transgenic lines showed higher proliferation rates compared with controls; however, this had little impact on the overall viability of the cultures (Fig. 1). Addition of IL-2 to the splenocyte cultures promoted proliferation in all cultures (transgenic and NSC) to a similar degree, which might suggest that it is cell survival that prolonged the viability of the transgenic cultures. However, the transgenic bone marrow cultures showed a higher proliferation rate compared with the NSC bone marrow cultures, suggesting that proliferation might initially contribute to the increased viability of these immature cell cultures.

**EBNA-1 is expressed in the surviving cells**

EBNA-1 is expressed in the spleen and not the thymus of line 26, while expression in the thymus was detected in line 59 (Wilson et al., 1996), suggesting that EBNA-1 expression is exclusive to B cells in line 26 but extends to T cells in line 59. In order to confirm this, splenocytes from line 26, 59 and NSC mice (three in each group) were immunoselected. A positively selected B cell population was obtained from splenocytes followed by a selected T cell population (using B220 Dynabeads and Thy1.2 Dynabeads sequentially, typically achieving 90% purity). Protein extracts from these populations were Western blotted to

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**Fig. 6.** EBNA-1 expression and activity. (a, b) Protein extracts (60 μg) were separated by 7.5% SDS-PAGE, Western blotted and probed with anti-EBNA-1 antisera (1H4) (Grasser et al., 1994). Raji (EBV positive), Bjab (EBV negative), A431 (EBV negative) and mB-cl (an EBNA-1 and LMP1 positive transgenic murine tumour B cell line 3959.48) were used as control cell lines. The 66 kDa Raji EBNA-1 (R) and the 88 kDa (B95-8 strain) transgenic EBNA-1 (tg) proteins are indicated with arrows. (a) Proteins were extracted from selected B cells from splenocytes (B220 enrichment) followed by selected T cells (Thy1.2 enrichment) from line 26, line 59 and NSC mice; each sample was pooled from three mice. (b) Proteins were extracted from splenocytes (S) and bone marrow (BM) cells of line 26, line 59 and NSC mice following 8 days of culture in 60 ng IL-2 ml^-1. (c) EBNA-1 specific DNA binding activity detected by EMSA of line 26 and 59 splenic tumour samples, with a 2697 tumour sample (EμEBNA-1.26 and Eμc-myc bitransgenic tumour) and mBcl cell line as positive controls and Bjab cell line as negative control. Probe alone with no extract (o) is shown. +, Excess (100x) competitor oligonucleotide was added. The EBNA-1 containing complex is indicated.
reveal clear EBNA-1 expression in the B cells from both lines. The level was higher in line 59 (as previously described), with a low level of expression in the T cells of line 59 (Fig. 6a). While the expression detected in line 59 in the T cell population may result from remaining B cells in the sample, no expression was detected in the similarly selected T cells of line 26. EBNA-1 could be detected in extracts from splenocyte and bone marrow cultures surviving in the presence of IL-2 from both lines 26 and 59 after 8 days of culture (Fig. 6b).

In accordance with the higher EBNA-1 expression level in line 59 B cells compared with line 26 B cells, EBNA-1-specific DNA binding was also higher in line 59 lymphoma samples compared with line 26 (Fig. 6c). Interestingly, this activity is considerably induced in an established B cell line which was derived from a line 59 lymphoma (mB-cl in Fig. 6) and also in a c-myc/EBNA-1.26 bi-transgenic tumour sample. The higher level and activity of EBNA-1 in line 59 samples compared with line 26 correlate with the extended life span of the cells in IL-2, which in all experiments was more pronounced in line 59 samples than line 26 samples.

**DISCUSSION**

EBNA-1 is the only viral protein consistently expressed in all EBV-associated tumour cells and is required for the maintenance of the viral genome. In order to explore whether EBNA-1 has the potential to play any additional role in tumorigenesis, separate from viral propagation, we examined the consequences of EBNA-1 expression in B cells in vivo. Data from two independently derived transgenic lines of mice have shown that the EBNA-1-expressing lymphocyte cultures (prior to any neoplastic change) show higher proliferation rates following explant compared with controls, even in the absence of cytokine supplement. These transgenic cultures also show prolonged survival compared with controls, dependent on the addition of IL-2 to the culture medium.

Importantly, the results are consistent for two EBNA-1-expressing transgenic mouse lines which have transgene insertion sites on different chromosomes; as such, the properties can be attributed to EBNA-1 expression rather than any additional insertion site effect in either line. These data indicate that EBNA-1 can provide proliferative and survival signals to lymphocytes, but the survival signals are indirect and context-dependent. While EBNA-1 expression does not appear to affect the cell type survival (where the controls survive they show the same phenotype as the transgenic cells), it does affect the duration of survival. The properties described correlate with EBNA-1 expression levels in the two lines, this being greater in line 59 tissues. This is in contrast to tumour incidence (more penetrating in line 26), which could reflect either the complexity of EBNA-1 action in vivo or that there is an additional tumorigenic factor in line 26, possibly mediated by genomic rearrangement at the transgene insertion site. Nevertheless, the enhanced cell survival described could contribute to the tumour phenotype observed in these lines and, indeed, CD25 B cell expression and IL-2 serum levels were induced in most lymphoma-bearing mice of line 26, which was not observed in Eμ-c-myc lymphoma-bearing transgenic mice. This would suggest that the IL-2/CD25 pathway is a factor in EBNA-1-mediated tumorigenesis in this system.

Extensive characterization of the progressive cell phenotype during prolonged culture has shown that, from explanted murine lymphocytes, irrespective of EBNA-1 transgenic status, the life span of both B cells and T cells can be extended with IL-2 and that the cells undergo a phenotypic drift, losing the T cell marker CD3 and the B cell markers CD19 and B220. However, this phenotypic drift does not shed light on the mechanism of survival extension mediated by EBNA-1 in the transgenic cultures. In the absence of an efficient means of prolonged culture of sufficient quantities (both in cell density and volume, as well as numbers of replicates) of highly purified selected cell subpopulations, it is not possible to determine if one population contributes to the survival of the other. However, the transgene is only expressed in the B cell compartment in line 26, while low-level expression in T cells is apparent in line 59. There is some evidence to suggest that the line 59 selected T cells might have an extended lifespan compared with NSC, which could be attributable to transgene expression. However, the possibility that B cell transgene expression could influence the cytokine milieu to affect T cell survival is a subject for further investigation.

IL-2 is produced primarily by activated T cells to trigger proliferation and survival of naive and recently activated T cells. Activated human and murine B cells can also proliferate in response to IL-2, while addition of gamma-interferon or stimulation with anti-μ with IL-2 leads to the maturation of B cells and immunoglobulin secretion (Moreau et al., 1995; Nakagawa et al., 1985; Zubler et al., 1984). IL-2 acts through its trimeric receptor (IL-2Rα, β and γc chains), the signalling components of which (β and γc chains) are shared by other cytokine receptors such as IL-15R (Gaffen, 2001). The unique IL-2Rα/CD25 receptor component modulates affinity for the ligand.

Expression of EBNA-1 could impact the IL-2 downstream pathways to enhance the action of IL-2; however, this seems unlikely, since supplementing the lymphocyte culture medium with IL-15 resulted in little difference between transgenic and control cultures. Therefore, the actions of EBNA-1 could either specifically augment or complement those of IL-2. One such mechanism would be to promote the expression or activity of IL-2Rα/CD25, and indeed lymphocyte survival in transgenic and control cultures was found to be IL-2 dose-dependent, with transgenic cultures showing prolonged survival, even at low doses, that were less effective in control cultures.
Furthermore, Kube et al. (1999) observed induction of IL-2Rα/CD25 upon EBNA-1 expression in the HD cell line L428. However, this did not lead to IL-2 responsiveness and, after prolonged cultivation, the CD25 induction was lost. Observation of CD25 expression in the transgenic lymphocyte explant cultures revealed enhanced induction of CD25 expression in the B-cell population compared with controls, only after IL-2 or anti-μ treatment.

Treatment of murine B cells with IL-2 and stimulation with anti-μ antibodies renders B cells responsive to IL-2 for proliferation and differentiation (Moreau et al., 1995). While the stimulation with anti-μ induces IL-2Rα/CD25 expression in resting B cells, it is not sufficient to induce IL-2 responsiveness without co-treatment with IL-2. EBNA-1 may similarly facilitate a response that is dependent on IL-2Rα/CD25 expression induction in the presence of IL-2. Unfortunately, testing any link between the tumorigenic potential of EBNA-1 and IL-2 in vivo using null mice is not viable, since IL-2-null mice die prematurely from inflammatory bowel disease and CD25-null mice show enlargements of lymphoid organs and develop a terminal inflammatory bowel disease (Sadlack et al., 1999; Willerford et al., 1995).

This poses a question: why might EBV have evolved a mechanism to utilize the IL-2 pathway in its lifecycle? In a virus-infected B cell expressing latent membrane protein (LMP)-2A, partial mimicry of B cell receptor signals can lead to cell survival, but block B cell activation (Caldwell et al., 1998, 2000; Miller et al., 1995). In a cell expressing LMP1, activation of CD40 signalling pathways could lead to proliferation, survival and/or differentiation to memory cells (Lam & Sugden, 2003). However, there is evidence for a critical role of IL-2 in the CD40-mediated activation of naïve B-cells. For primary T cells to act as competent helper cells to induce B-cell proliferation, they must express CD40L and secrete IL-2 to maximal levels (Johnson-Leger et al., 1998). Therefore, an enhanced B-cell response to IL-2, mediated by EBNA-1 that is independent of, or augments, antigen activation, could complement LMP1 activation of CD40 signalling pathways to allow the infected B cell to proliferate. Conversely, enhancement of the IL-2 response in a cell expressing LMP2A may serve to redirect the cell to memory-simulated survival.

In the absence of LMP1, expression of EBNA-1 and responsiveness to IL-2 could provide an alternative survival route under circumstances where there is an ongoing immune response and T cell IL-2 production. Thus, under circumstances of chronic infection, such as malaria, this could allow prolonged survival of an EBNA-1-expressing EBV-infected bystander B cell. In this context, one of the target genes upregulated by IL-2 signalling encodes the apoptosis inhibitor Bcl-xL (Cipres et al., 1999; Lord et al., 2000), expression of which would allow survival of Myc deregulated cells in the genesis of Burkitt’s lymphoma. To lend support to this hypothesis, bcl-xL expression was observed to be upregulated in EBNA-1-expressing transgenic B cells (Tsimbouri et al., 2002). Also, cooperation with Myc in B-cell lymphomagenesis was observed with EμEBNA-1.26 (Drotar et al., 2003). However, the increase in proliferation seen in transgenic cultures, even in the absence of IL-2 supplementation, suggests that there is a further activity of EBNA-1 that is yet to be defined.

EBNA-1 was found to be non-essential for the immortalization of B cells by EBV in culture, but it enhanced the process by several thousand fold (Humme et al., 2003). Humme et al. (2003) proposed that the latent viral replication and episomal maintenance functions of EBNA-1 enhanced the efficiency of LCL generation but it could not be ruled out that EBNA-1 might contribute otherwise to tumorigenesis. The finding that EBNA-1 contributes to the ongoing survival of EBV-positive BL cell lines suggests that EBNA-1 does have another function in addition to its viral genome maintenance activity (Kennedy et al., 2003). These data support this, as, using a model where viral genome propagation plays no role, we demonstrated that EBNA-1 can prolong lymphocyte survival, mediated at least in part in response to IL-2, which could have a significant impact in vivo. Our data cannot determine that this role is essential to EBV-induced tumorigenesis, especially under circumstances where several of the latent genes are expressed, and there are likely to be other routes to the same end. Nevertheless, the finding that serum concentrations of IL-2 are high in the majority of the transgenic tumours would imply a role for this cytokine in EBNA-1-mediated tumorigenesis. As such, the properties, namely enhanced proliferation and cell survival, that are conferred by EBNA-1 expression in B cells in this system indicate that EBNA-1 cannot be dismissed as a potential contributor to malignant disease processes.

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REFERENCES


