Analysis of the tumorigenic potential of common marmoset lymphoblastoid cells expressing a constitutively activated c-myc gene

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Summary

The respective roles of Epstein-Barr virus (EBV) and c-myc in the pathogenesis of endemic Burkitt’s lymphoma (BL) are unclear. In order to help resolve the question whether constitutive expression of the c-myc gene in an EBV-immortalised B cell is sufficient to induce a tumorigenic phenotype, B cells from a common marmoset (Callithrix jacchus) were immortalised with EBV, transfected with a constitutively activated c-myc gene and inoculated into the hypodermal tissue of nongrafted, athymic, nu/nu mice. Enhanced growth characteristics, in vitro and in vivo experiments demonstrated that this was not sufficient to induce a tumorigenic phenotype. This supports our previous findings with EBV-immortalised human B cells transfected with an activated c-myc gene (Hotchin et al., 1990).

Endemic BL is a monoclonal B cell tumour characterised by the presence of a human herpesvirus, Epstein-Barr virus (EBV) and a chromosome translocation involving the c-myc proto-oncogene on chromosome 8 and the IgH locus on chromosome 14, or, more rarely the Igκ or λ loci on chromosome 2 or 22 (Lenoir, 1986). The result of such a translocation is constitutive activation of the c-myc gene. It is widely accepted that other co-factors must be involved in the aetiology of endemic BL, a prime candidate being malaria which is holoendemic in regions where the endemic form of BL is found (Kafuko & Burkitt, 1970).

C-Myc is a nuclear protein with homology to a number of sequence-specific DNA-binding proteins (Cole, 1990). Recent data has indicated that c-Myc forms a heterodimeric complex with a protein called Max, and that this complex acts a sequence-specific transcriptional activating factor (Blackwood & Eisenman, 1991; Amati et al., 1992; Kretzner et al., 1992). Until recently the role of c-myc in both normal and malignant cells was unclear. Myc is clearly important in cellular proliferation, being expressed at low or undetectable levels in resting or quiescent cells, but induced to high levels in mitotically activated cells (Kelly et al., 1983). Furthermore anti-sense transcripts have been used to demonstrate that c-myc is required for cell proliferation (Prochownik et al., 1988). More recently, it has been shown that, in the presence of high levels of c-Myc, cells are unable to withdraw from the cell cycle, and when deprived of nutrients undergo programmed cell death (apoptosis) (Evan et al., 1992). On the basis of these data Evan et al. (1992) propose a model for myc function whereby c-myc provides a fail-safe mechanism to prevent uncontrolled proliferation and acts as a regulator of cell fate.

Deregulated c-myc expression is found in a number of mammanlian tumours (Spencer & Groudine, 1991) indicating the potential importance of this gene in tumorigenesis. Early experiments, introducing exogenous activated c-myc genes into fibroblasts, resulted in an immortalised, but not fully transformed, phenotype (Land et al., 1983; Mougneau et al., 1984; Land et al., 1986). In transgenic mice, linking c-myc to the lgr promoter resulted in the development of B cell lymphomas (Adams et al., 1985). The monoclonality of these tumours, however, suggested that other factors in addition to activation of c-myc were necessary for development of these tumours. This was supported by data from co-transfection experiments demonstrating cooperation between myc and ras resulting in a transformed phenotype (Land et al., 1986).

More recently it has been shown that c-Myc induced apoptosis can be overcome by constitutive expression of the bcl-2 proto-oncogene (Fandini et al., 1992; Bissonnette et al., 1992) which is known to inhibit apoptosis in B-cells (Vaux et al., 1988; Hockenbury et al., 1990). Interestingly, whilst expression of bcl-2 could rescue cells from apoptosis and allow indefinite proliferation in the absence of mitogenic stimuli, morphological transformation was not evident (Fandini et al., 1992).

The majority of EBV infections are subclinical and occur early in life, with over 90% of the adult population demonstrating evidence of past infection (Henlé & Henle, 1979). If primary infection is delayed past the age of 12 years then a common consequence of infection with EBV is infectious mononucleosis (IM), a usually benign, self-limiting lymphoproliferative disease. EBV is also associated with a number of other pathological conditions, including nasopharyngeal carcinoma (NPC), oral hairy leukoplaikia and lymphoproliferative disease in patients with severe T-cell immunosuppression. In common with other herpesviruses, EBV persists for life following primary infection, and is tightly controlled by the cell mediated immune response (Richardson, 1986).

The role of EBV in development of BL is unclear. EBV is able to immortalise B cells in vitro to form lymphoblastoid cell lines (LCL) which express a number of latency associated associated proteins – EB nuclear antigens (EBNA1-6), the latent membrane protein (LMP) and two terminal proteins (TP1 and TP2) (reviewed in Kieff & Liebowitz, 1990). However, the two viral proteins implicated in this process, EBNA-2 (Hammerschmidt & Sudgen, 1989) and LMP (Wang et al., 1985; Baichwal & Sudgen, 1985) are not expressed in BL (Rowe et al., 1987a). LMP has also been shown to upregulate expression of bcl-2 and prevent apoptosis in BL cell lines (Henderson et al., 1991). However, Bcl-2 is unlikely to be involved in development of BL as it is expressed only at low or undetectable levels in BL cells which most resemble the in vivo phenotype (Henderson et al., 1991).

Lombardi et al. (1987) reported that transfection of an activated c-myc gene into EBV immortalised B cell lines was sufficient to induce a transformed phenotype. A subsequent study transfected the same plasmid constructs into a different LCL found that, whilst the cells expressing a con-
ststitutively activated c-myc gene had altered surface phenotype and a reduced serum dependency, they were not transformed as determined by growth in semi-solid media and tumour formation in nude mice (Hotchin et al., 1990).

In order to examine further the role of EBV and c-myc in tumour development, common marmosets (Callithrix jacchus) were used as hosts for immortalised autologous B cells containing a constitutively expressed c-myc gene. Inoculation of the common marmoset with EBV results in apparently asymptomatic infection as demonstrated by the presence of IgG class antibodies to the viral capsid antigen (VCA) which remain detectable for life (Wedderburn et al., 1984; N.W. unpublished observations). In addition heterophile antibodies and IgG antibodies against the viral early antigen (EA) complex have also been detected in some common marmosets infected with EBV (Wedderburn et al., 1984). Both of these markers are typically found in IM in humans and, at least in terms of the serological response to EBV, infection of common marmosets mimics the asymptomatic infection seen in humans (Niederman et al., 1970; Henle & Henle, 1979). Unlike cotton-top marmosets (Saguinus oedipus oedipus), common marmosets do not develop lymphoproliferative or malignant lesions in response to inoculation with EBV (Miller, 1979; Wedderburn et al., 1984), thus the common marmoset represents a better model than cotton-top marmosets for studying, in vivo, the role of c-myc and EBV in the pathogenesis of BL.

Materials and methods

Experimental animals

Triplet common marmosets, two females (244 and 245) and one male (246), were used in these experiments. They were born and raised in a colony to which there have been no further additions since 1970.

Cell culture

M245 LCL is an EBV immortalised B cell line derived by infecting cells from the peripheral blood of marmoset number 245 with the M81 strain of EBV (Desgranges et al., 1976) which was isolated from a common marmoset LCL originally transformed by a strain of EBV derived from a nasopharyngeal carcinoma (de The et al., 1970). Cells were cultured at 37°C in a 5% CO2 incubator using RPMI 1640 containing 2 mM L-glutamine, 100 IU penicillin, 10 μg/ml streptomycin and 10% foetal calf serum (FCS). For transfections the amount of hygromycin B (Calbiochem) required to kill non-transfected cells (100 μg/ml−1) was determined by titration.

Plasmid DNA constructions

pHEBoSVmycl2,3 contains all three exons of the c-myc gene under control of the SV40 early region promoter (Lombardi et al., 1987) inserted into pHEBo, a plasmid which permits episomal replication in EBV infected B cells and selection with the antibiotic hygromycin B (Sugden et al., 1985). pHEBoSVmycl2,3 was a gift from Dr R. Dalla-Favera (New York University School of Medicine).

Liposome mediated transfection

Liposome mediated transfection of M245 cells was carried out using a commercially supplied reagent (Lipofectin, BRL). Cells were adhered to 35 mm cell culture dishes coated with 4.5 μl of a 10 mg ml−1 solution of ‘Cell-Tak’ (BioPolymers Inc.) by incubating 4 x 10⁵ cells in 2 ml ‘Opti-MEM 1’ medium (BRL) for 30 min at 37°C. After removing any non-adherent cells 1 μg of plasmid DNA was added to 0.5 ml of OpiMEM I and which was mixed with 0.5 ml OpiMEM I containing 10 μg of ‘Lipofectin’ before adding to the adherent cells. After a 5 h incubation at 37°C the cells were removed from the plastic by incubation with 0.05% trypsin, 0.02% EDTA, washed and resuspended in 10 ml normal culture medium. After 48 h hygromycin B was added to 100 μg ml−1.

Serology

Serum samples were prepared from clotted peripheral blood taken at regular intervals under anaesthesia from each animal, and stored at −20°C until required. Antibody titres against the EB viral capsid antigen (VCA) and early antigen

Figure 1 Detection of plasmid-derived c-myc transcripts in M245-mycl23 using polymerase chain reaction (PCR). cDNAs were generated from total cellular RNA isolated from M245 and M245-mycl23 cell lines. Primers located in the SV40 early region promoter and exon II of c-myc e, were annealed to the cDNAs and sequences between the two primers amplified by PCR. The PCR products were electrophoresed on an agarose gel, transferred to a nylon membrane and probed with a 32P-labelled probe for c-myc. a, ethidium bromide stained gel prior to transfer. b, nylon membrane probed for c-myc sequences with 32P-labelled probe. Track 1, M245; track 2, M245-mycl23. Positions of molecular weight markers are indicated (M). Sizes of markers, in kb from the top of the gel are: 1.35, 1.08, 0.87, 0.60, 0.31, 0.28/0.27, 0.23, 0.19.
Results

Establishment of c-myc transfected marmoset LCL

M245 LCL was transfected with pHEBo-SVmyc123 using Lipofectin and transfected cells selected with hygromycin B (100 µg·ml⁻¹). One drug resistant cell line was established (M245-myc123). Attempts to obtain a pHEBoSV transfected control cell line proved unsuccessful and the parental M245 LCL was used as a control. To establish that the plasmid derived c-myc gene was being expressed in M245-myc123 cells, cDNAs were generated from total RNA using reverse transcriptase. Plasmid derived transcripts were detected using PCR to amplify sequences encompassed by primers in the SV40 early region promoter/enhancer element and in the second exon of the c-myc gene (Figure 1c). Results (Figure 1a and b) clearly demonstrate the presence of plasmid derived transcripts in M245-myc123 but not in the control M245 cell line. Use of primers within the SV40 promoter/enhancer element and exon 2 of the c-myc gene allowed us to exclude the possibility of amplifying contaminating plasmid DNA sequences. Immunoblotting of protein samples extracted from M245 LCL and M245-myc123 using a monoclonal antibody against c-Myc demonstrated considerably higher levels of c-Myc protein in the myc-transfected cell line compared to the control cell line (Figure 2). Both control and transfected cell lines expressed the full range of EBV latency associated proteins (EBNA 1-6 and LMP, data not shown).

Serum dependency

Growth in reduced serum concentrations was assessed by culturing cells in medium containing 1% FCS for 48 h and measuring uptake of tritiated thymidine following a 4 h pulse. Figure 3 demonstrates the results from three replicate
Figure 3  Proliferation of M245 and M245-myc123 in low serum concentrations was assessed by culturing cells for 2 days in medium containing 1% foetal calf serum and measuring incorporation of tritiated thymidine during a 4 h period. Results are the mean and standard error of three separate experiments.

Experiments. As can be clearly seen the myc-transfected cells incorporated significantly more thymidine than control cells.

**Tumorigenic potential of cell lines**

The tumorigenic potential of M245 LCL and M245-myc123 was assessed by colony formation in soft agar. Whilst the control BL cell line Raji readily formed colonies in soft agar neither the control or myc-transfected cell lines formed colonies.

**Inoculation of common marmosets with M245 and M245-myc123**

The c-myc transfected cell line, M245-myc123, was inoculated into marmosets 245 and 246. Each animal was inoculated with $5 \times 10^7$ cells intravenously (i.v.) and $5 \times 10^5$ cells intraperitoneally (i.p.) under anaesthesia. As a control an equivalent number of cells from the M245 LCL were inoculated via the same routes into marmoset 244. Marmosets from multiple births are haematologically chimeric as a result of anastomoses between placenta (Hetherington et al., 1981), and are thus tolerant to each other haemopoietic cells.

**EBV serology**

Antibody titres to EBV antigens, as determined by indirect immunofluorescence, are indicated in Table I. All three marmosets were EBV seronegative prior to inoculation, indicating that they had not previously been infected with EBV. All developed anti-VCA IgM responses with 7 days of inoculation and IgG class antibodies to VCA were detectable 14–21 days post inoculation. IgM antibody titres fell below detectable levels within 28 days, but anti-VCA IgG remained detectable in all animals throughout the experiment. Neither IgG class antibodies to EA or anti-VCA IgA were detectable at any stage.

**Haematology**

Haematological data for the three animals is summarised in Table II. No significant increase in total white cell count (WCC) was observed in the control marmoset (244) over a period of 24 weeks following inoculation. On morphological grounds the haematological picture of marmoset 244 remained relatively stable. The only unusual feature was the transient appearance of atypical lymphoblastoid cells 70 days post inoculation which at their peak represented 15% of the total WCC. These atypical cells did not persist in the circulation and had virtually disappeared by day 84. In contrast, marmoset 245 - inoculated with M245-myc123 - developed a large number of atypical, lymphoblastoid cells soon after inoculation. These comprised 49% of total lymphocytes on day 63, and still represented 13% of the total lymphocyte count on day 168. Figure 4 shows the appearance of these atypical cells. Despite this abnormality the total WCC was not dramatically raised, although a 2-fold increase was observed soon after inoculation, and the count did not return to pre-inoculation levels until day 84. In the other marmoset inoculated with M245-myc123 cells the haematological picture was similar to that seen in the control animal, with only low numbers of atypical cells detected.

**Immunocytochemistry**

Cytospin preparations and thin blood films from peripheral blood obtained 84 days post inoculation from the three animals were stained for the presence of the EBNA complex using ACIF. Positive staining was detected in control Raji cells, but no EBNA positive cells were detected from any of the three animals.

**Tumorigenic potential of the cell lines in the host animals**

No evidence for malignant disease was observed in any of the three marmosets, all of which remained well throughout the experiment. No lymph nodes were palpable and significant changes in weight were not observed. The marmosets were subjected to post mortem examination 11 months after inoculation. No gross abnormalities were observed, however marmoset 245 had enlarged mesenteric lymph nodes com-

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**Table I**  Reciprocal antibody titres to EBV viral capsid antigen (VCA) and early antigen (EA) complexes. Serum samples were taken at regular time points and antibody titres determined by indirect immunofluorescence. NT – not tested; p.i. – post inoculation; < – less than.

### (A) Marmoset 244

| Days p.i. | VCAlgG | VCAlgA | VCAlgM | EA lgG |
|----------|--------|--------|--------|--------|
| -34      | <8     | NT     | <8     | <8     |
| 7        | <8     | <8     | 128    | <8     |
| 14       | 8      | <8     | 64     | <8     |
| 21       | 8      | <8     | 8      | <8     |
| 28       | 16     | <8     | 8      | <8     |
| 42       | 32     | <8     | <8     | <8     |
| 168      | 16     | NT     | NT     | <8     |

### (B) Marmoset 245

| Days p.i. | VCAlgG | VCAlgA | VCAlgM | EA lgG |
|----------|--------|--------|--------|--------|
| -34      | <8     | NT     | NT     | <8     |
| 7        | <8     | <8     | 128    | <8     |
| 14       | <8     | <8     | 32     | <8     |
| 21       | 8      | <8     | 16     | <8     |
| 28       | 8      | <8     | <8     | <8     |
| 42       | 16     | <8     | <8     | <8     |
| 168      | 32     | NT     | NT     | <8     |

### (C) Marmoset 246

| Days p.i. | VCAlgG | VCAlgA | VCAlgM | EA lgG |
|----------|--------|--------|--------|--------|
| 0        | <8     | NT     | NT     | <8     |
| 7        | <8     | <8     | 8      | <8     |
| 14       | 8      | <8     | 16     | <8     |
| 21       | 16     | <8     | <8     | <8     |
| 35       | 32     | <8     | <8     | <8     |
Table II Haematological data. Peripheral blood was taken at regular intervals and total white cell counts (WCC) performed. Thin blood films were also prepared, fixed in methanol and stained with Giemsa. PMN – polymorphonuclear cells; Lymph. – lymphocytes; Mono. – monocytes; atypical MN – atypical mononuclear cells (lymphoblastoid appearance); ND – not done

(A) Marmoset 244

| Days p.i. | WCC | PMN | Lymph | Mono. | Atypical MN |
|-----------|-----|-----|-------|-------|-------------|
| – 34      | 4.3 | 69  | 31    | 0     | 0           |
| 0         | ND  | 65  | 31    | 4     | 0           |
| 7         | 3.2 | 74  | 23    | 3     | 0           |
| 14        | 4.0 | 60  | 31    | 9     | 0           |
| 21        | 3.4 | 69  | 30    | 1     | 0           |
| 28        | 2.0 | 50  | 46    | 4     | 0           |
| 42        | 3.0 | 57  | 41    | 0     | 2           |
| 56        | 3.2 | 54  | 42    | 3     | 1           |
| 63        | ND  | 70  | 27    | 3     | 0           |
| 70        | 2.0 | 64  | 18    | 1     | 15          |
| 84        | 2.9 | 65  | 29    | 2     | 3           |
| 111       | 3.1 | 78  | 15    | 1     | 2           |
| 137       | ND  | 81  | 17    | 2     | 0           |
| 158       | ND  | 72  | 22    | 6     | 0           |
| 179       | ND  | 54  | 36    | 8     | 2           |

(B) Marmoset 245

| Days p.i. | WCC | PMN | Lymph | Mono. | Atypical MN |
|-----------|-----|-----|-------|-------|-------------|
| – 34      | 4.0 | 46  | 52    | 2     | 0           |
| 0         | ND  | 52  | 44    | 4     | 0           |
| 7         | 8.4 | 26  | 61    | 6     | 7           |
| 14        | 6.2 | 38  | 41    | 15    | 6           |
| 21        | 6.6 | 29  | 56    | 1     | 14          |
| 28        | 5.2 | 39  | 50    | 1     | 9           |
| 42        | 9.2 | 39  | 52    | 0     | 10          |
| 56        | 6.0 | 41  | 38    | 1     | 20          |
| 63        | ND  | 32  | 18    | 1     | 49          |
| 70        | 7.2 | 34  | 22    | 1     | 43          |
| 84        | 5.2 | 32  | 41    | 2     | 25          |
| 111       | 4.9 | 28  | 49    | 0     | 23          |
| 137       | ND  | 54  | 29    | 5     | 12          |
| 158       | ND  | 30  | 47    | 10    | 13          |
| 179       | ND  | 40  | 43    | 4     | 13          |

(C) Marmoset 246

| Days p.i. | WCC | PMN | Lymph | Mono. | Atypical MN |
|-----------|-----|-----|-------|-------|-------------|
| – 146     | 5.0 | 69  | 31    | 0     | 0           |
| 0         | 4.9 | 46  | 43    | 2     | 9           |
| 7         | 6.6 | 73  | 22    | 1     | 4           |
| 14        | 3.1 | 54  | 41    | 0     | 5           |
| 21        | 3.8 | 55  | 42    | 1     | 3           |
| 35        | 6.0 | 53  | 39    | 3     | 5           |
| 81        | ND  | 67  | 30    | 0     | 3           |
| 105       | ND  | 52  | 43    | 3     | 2           |
| 126       | ND  | 55  | 44    | 0     | 1           |

Discussion

It has previously been proposed that constitutive expression of a c-myc gene in an EBV immortalised B cell is sufficient to induce a tumorigenic phenotype (Lombardi et al., 1987). Our previous work has indicated that this is not necessarily the case, and this study further supports our basic observation that constitutive expression of c-myc in a lymphoblastoid cell is not sufficient to induce a tumorigenic phenotype (Hotchin et al., 1990). In agreement with previously published reports (Lombardi et al., 1987; Hotchin et al., 1990) we found that the BCL expressing a constitutively active c-myc gene had a reduced serum dependency when compared to its control cell line. These enhanced growth characteristics, however, were insufficient to result in a tumorigenic phenotype, as evidenced by growth in semi-solid medium or tumour formation in the host animal. As we have previously stated there are a number of possible explanations for this anomaly (Hotchin et al., 1990). It may be that there is a threshold of c-Myc expression required for a transformed phenotype and our cell lines might express lower amounts of c-Myc relative to those used by Lombardi et al. This could be resolved by direct comparison of the cell lines. Alternatively, it may be that a further event, such as activation of ras, may have occurred in the lines used by Lombardi et al., resulting in a tumorigenic phenotype. We also suggested that the resistance of our cell lines to tumorigenic change might be a consequence of our use of a clonal population of EBV immortalised B cells for our transfection experiments (Hotchin et al., 1990). The cell lines used in this study were not clonal in origin, nor were they subjected to clonal selection following transfection, thus we feel that the differences in tumorigenicity are unlikely to be a consequence of using a clone of B cells intrinsically more resistant to malignant transformation.

Haematological abnormalities were observed in one of the two animals inoculated with the mvc-transfected cell line. Large numbers of atypical mononuclear cells were observed in the circulation of marmoset 245. We were unable to ascertain the identity of these cells using standard immunocytochemical techniques utilising monoclonal antibodies specific for human T and B cells (data not shown). However, it is
unlikely that these cells were derived from the inoculated cells since no circulating EBNA-positive cells were detected 84 days post-inoculation when the atypical cells represented 25% of the total lymphocyte count of marmoset 245. It is possible that the abnormal cells were a consequence of a pre-existing haematological disorder and cytogenetic studies have revealed a reciprocal chromosome translocation involving chromosomes 2 and 9 (J.A.B., unpublished observations). It is not known whether this translocation was present prior to the start of this experiment but it was not present in either of the other marmosets, nor in either of the cell lines used for inoculation. It seems unlikely, however, that this translocation was a consequence of inoculation of the myc-transfected cell line.

The absence of EBNA-positive circulating B cells in any of the animals suggests that the inoculated cells were eliminated by the cell mediated immune system, presumably as a consequence of the cell lines expressing the EBV antigens known to be recognised by EBV-specific cytotoxic T lymphocytes (CTL), namely EBNA-2, EBNA-3, EBNA-6 and LMP (Thorley-Lawson & Israelsohn, 1987; Moss et al., 1988; Murray et al., 1990; Burrows et al., 1990a, 1990b). This implies that constitutive high level expression of c-myc is insufficient to induce a phenotype capable of evading immunosurveillance either by down-regulation of viral proteins or other molecules involved in interactions with CTL. It would be interesting to attempt further experiments in which marmosets inoculated with myc-transfected cells were concurrently either immunosuppressed with cyclosporin A or infected with malaria. It is tempting to speculate that concurrent inoculation of myc-transfected cells and immunosuppression might result in the appearance of tumours similar to the EBV-associated lymphomas seen in humans with profound T cell dysfunction (Thomas et al., 1991). If, as seems likely, the T cell dysfunction caused by malaria infection is not sustained following acute malaria (Whittle et al., 1984) then one would not expect such tumours to arise following malarial infection and inoculation of myc-transfected cells into marmosets.

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