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Mutations in the Epstein-Barr virus latent membrane protein-1 (BNLF-1) gene in spontaneous lymphoblastoid cell lines: effect on in vitro transformation associated parameters and tumorigenicity in SCID and nude mice

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Abstract

***Aims*—(1) To study the frequency of putative malignancy associated point mutations and a 30 base pair (bp) deletion in exon 3 of the C-terminus of the Epstein-Barr virus (EBV) encoded latent membrane protein (LMP)-1 (BNLF-1) gene in wild type EBV strains. (2) To assess the influence of these mutations on the tumorigenicity of lymphoblastoid cell lines (LCL).**

***Methods*—Eight spontaneous EBV (wild type) infected LCL were established from seven subjects. Deletions and single base mutations in the C-terminus of the BNLF-1 gene were demonstrated using bi-directional solid phase dideoxy sequencing following PCR amplification of viral DNA from the LCL. Tumorigenicity of the LCL was assessed in SCID and nude mice. Serum dependent growth and ability to form colonies in soft agarose were assessed for representative LCL.**

***Results*—All LCL showed sequence differences compared with the prototypic EBV strain B95-8. The 30 bp deletion could be detected in three of eight LCL and a 69 bp deletion (including the 30 bp deletion) was identified in an additional LCL. A range of single base mutations (including those described previously in association with EBV related neoplasias) was also seen in some of the LCL. In transformation studies, the genetic variations did not seem to influence the in vitro behaviour of the LCL. In the tumorigenicity studies, the presence of the 30 bp deletion had no influence on the behaviour of the LCL which were, as expected, tumorigenic in SCID mice but not in nude mice. In contrast, the LCL carrying the 69 bp deletion was tumorigenic in both SCID and nude mice.**

***Conclusions*—Genetic changes described previously in the C-terminus of the LMP-1 gene in various malignancy derived EBV strains are also present frequently in wild type viruses and do not simply define tumour specific EBV strains. Changes within this region may, however, still be important for the tumorigenicity of LMP-1 and thus play a role in EBV oncogenesis.**

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Epstein-Barr virus (EBV) is a human herpes virus that causes infectious mononucleosis[1] and is associated with various malignancies, including nasopharyngeal carcinoma, Burkitt's lymphoma, Hodgkin's disease, peripheral T cell lymphomas, and lymphoproliferative disorders in immunosuppressed patients.[2–7]

In vitro, EBV transformed lymphoblastoid cell lines (LCL) can be established by spontaneous outgrowth of EBV infected B cells from the peripheral blood of seropositive subjects.[8] LCL consistently display a restricted pattern of EBV gene products (recently designated latency type III), characterised by the expression of EBV nuclear antigen (EBNA)-1, EBNA-2, EBNA-3a, b and c, and EBNA-LP, latent membrane protein (LMP)-1 and LMP2a/b, and EBV small nuclear RNAs (EBERs)-1/2.[9] LMP-1 plays an important part in EBV induced cell transformation. Among other things, it is essential for the transformation of primary B cells,[10] it can transform rodent fibroblasts and human keratinocytes,[11 12] inhibit human epithelial cell differentiation,[13] and it is toxic to cells when expressed at high concentrations.[14 15] In transgenic mice LMP-1 induces hyperplastic dermatitis and abnormal keratin expression.[16] LMP-1 induces DNA synthesis[17] and upregulates expression of several lymphocytic activation markers and adhesion molecules.[18]

Recently, Hu *et al*[19] demonstrated several mutations in the gene, BNLF-1, encoding LMP-1 in nude mouse propagated Chinese nasopharyngeal carcinoma (CAO) cells compared with the prototype EBV strain B95-8 (ECACC no. 85011419). Subsequently, it has been suggested that these BNLF-1 gene mutations characterise an EBV strain in Asia, which is associated preferentially with nasopharyngeal carcinoma.[20] Tumorigenicity studies on SCID and nude mice indicated that the CAO LMP-1 was more tumorigenic than B95-8-LMP-1.[20 21]

Analysis of the CAO BNLF-1 gene has revealed a 30 base pair (bp) deletion and seven single base mutations in the C-terminal region, between the coding triplets for amino acids 322 and 366 of exon 3.[19] In a recent study of EBV positive lymphomas we found the 30 bp

deletion and six of the seven single base mutations in all peripheral T cell lymphomas from Malaysian patients, in about 60% of peripheral T cell lymphomas from Danish patients and in about 30% of cases of Hodgkin's disease and infectious mononucleosis.[22] The mutations were detected in both EBV subtypes A and B, and the single base mutations and the 30 bp deletion occurred independently in some cases. In addition, we detected a 12 bp deletion consisting of 12 of the nucleotides of the 30 bp deletion in one case of Hodgkin's disease. These findings indicate that mutations in the C-terminal part of the BNLF-1 gene are not associated specifically with either Asian nasopharyngeal carcinoma or EBV subtype A, although they do seem to be more common in Asia than in the West. Rather, the 30 bp deletion and some of the sites of single base mutation may be hot spots which have mutated independently throughout the evolution of EBV strains. Similar findings have been reported independently from other laboratories.[23 24]

The apparent convergent evolution of EBV strains of both subtype A and B from different geographical locations suggests that these mutations impart some advantage to the virus. In vitro studies by Moorthy and Thorley-Lawson showed that the C-terminal part of LMP-1 is essential for transformation.[15 25] Furthermore, their results indicated that the absence of the amino acid sequence between positions 334 and 364 generated a toxic LMP-1 protein, suggesting that this amino acid sequence is essential for the normal function of the protein. The 30 bp deletion is located within this sequence and may therefore affect the function of the LMP-1 protein. This is supported by recent findings from Li *et al.*[26] Their transfection study on Balb/3T3 cells showed that introduction of the 30 bp deletion into the B95.8 BNLF-1 gene made these cells tumorigenic in nude mice. In the present study we have sequenced the C-terminal part of the EBV-BNLF-1 gene in eight spontaneously established LCL and identified wild type EBV strains that display various mutations within this region. The effect of these mutations on LCL transformation related parameters in vitro and LCL tumorigenicity in SCID and nude mice in vivo was investigated.

Methods

ESTABLISHMENT OF LCL

As part of a separate study, LCL were established with cells from six patients with multiple sclerosis and from one blood donor. Two separate LCL were established (with a 42 day interval) from cells from one of the patients. In each case, 50 ml of venous blood was drawn into Venoject tubes. Heparinised blood was diluted 1 in 2 in phosphate buffered saline (PBS)/heparin (PBS pH 7.4, 20 IU/ml heparin), before separating the mononuclear cells by Ficoll-Isopaque density gradient centrifugation. The mononuclear cell layer was isolated and washed in PBS and subsequently in RPMI 1640 (BioWhittaker, Walkersville, Maryland, USA). The cells were then counted and seeded at densities between 2×10^6/ml and 4×10^6/ml in 5 ml growth medium, which, on day 3, was adjusted to 10 ml. Growth medium consisted of RPMI 1640 supplemented with 200 IU/ml penicillin (Leo, Ballerup, Denmark), 0.2 mg/ml streptomycin (Sigma, St Louis, Missouri, USA), 0.29 mg/ml glutamin (Sigma), 0.01M HEPES buffer (BioWhittaker), and 10% heat inactivated human serum in Falcon Primaria bottles (50 ml). For each patient there were at least two mononuclear cell cultures, with and without 0.1 µg/ml cyclosporin A (Sandoz, Basel, Switzerland). The cyclosporin A was maintained in the medium for five weeks by regular refeeding. Half of the supernatant medium was changed three times weekly until a LCL was established or all cells were dead. LCL appeared after 60 to 180 days. Cultures were then split every two to three days and reseeded at a concentration of 5×10^5/ml.

GENETIC FINGERPRINTING

To test for cross contamination of the LCL, typing of DNA from each LCL culture was performed. The DNA was purified according to standard methods from mononuclear cells isolated on the day of blood sampling and from LCL. Restriction fragment length polymorphisms (RFLPs) of variable numbers of tandem repeat (VNTR) regions were investigated.[27-30] HLA-DQα typing was performed on PCR amplified DNA with the AmpliType kit (Roche, USA).

CYTOGENETICS

Apart from the following modifications, flow cytometry analysis was performed as described previously.[31] After washing twice in PBS, the cells were resuspended in 2 ml staining solution containing 10 mM Tris-HCl, 80 mM KCl, 20 mM NaCl, 3.4 mM sodium citrate, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM Spermine, 0.5 mM Spermidine, 0.1% Nonidet P40, pH 7.6, and propidiurn iodide (PI) to a final concentration of 100 µl PI/ml. The staining/lysis time was 10–15 minutes. A normal diploid reference was superimposed on the expected normal diploid peak in the histogram. The DNA profile was analysed on a FACStar Plus analyser (Becton Dickinson, Burlingame, California, USA).

TUMORIGENICITY

Female SCID and nu/nu mice of NMRI background were obtained from Bomholtgaard Animal Breeding and Research Centre, Ry, Denmark, and kept under conventional conditions during the experiments. The mice were seven to eight weeks old, with five to 10 mice of each kind being used for each cell line. Suspensions of 5.5×10^6 cells in 200 µl 0.9% NaCl were inoculated subcutaneously in the left flank. The mice were inspected weekly for the appearance and progressive growth of tumours. Tumour tissue was snap frozen and stored at −80°C. Animals without tumours were kept for 26 weeks.

IMMUNOHISTOLOGY

Cell pellets from cell cultures and tumour tissue from SCID and nude mice were snap frozen. Sections were cut and stained using APAAP immunohistology. The LCL from both cultures and tumours were characterised as B cells using monoclonal antibodies directed against CD19, CD20 and CD21. The sections were also stained for activation markers intracellular adhesion molecule-1 (ICAM-1) (CD54), LFA-3 (CD58) and CD23, and for bcl-2. Expression of EBV gene products was demonstrated using the monoclonal antibodies PE2 (EBNA-2), CS.1-4 (LMP-1) and BZ1 (BZLF-1 protein), as described previously.[32]

SAMPLE PREPARATION FOR PCR

Five 10 μm frozen sections were cut from the mouse tumours and placed in an Eppendorf tube using disposable Pasteur pipettes. Before and after cutting sections for PCR, histological control sections were cut and stained with haematoxylin to demonstrate the presence of tumour cells. Between cases the cryostat was cleaned with 70% alcohol and for each case a new knife was used. For every tumour two tubes with sections from a pellet consisting only of Tissue-Tek cryostat embedding medium (Miles, Elkhart, Indiana, USA) were cut as negative controls of the sectioning procedure. For PCR on cell lines, 10^6 cells were spun down directly from cultures. Both sections and cell pellets were digested in 250 μl proteinase K (200 μg/ml) for 48 hours at 56°C. The proteinase was inactivated by heating to 98°C for 15 minutes. The remains of the tissue was spun down briefly and the supernatant was used for PCR.

PCR

PCR was carried out in an automated thermal cycler (Perkin Elmer, Cetus, Norwalk, USA). All primers were synthesised by DNA Technology (Aarhus, Denmark). For EBNA-2 typing a single step PCR procedure was used. Primers flanking a 115 bp deletion in the U2 region encoding EBNA-2A gave rise to a 378 bp product from EBNA-2A EBV strains and a 483 bp product from EBNA-2B strains.[33] EBV cell lines B95.8 and AG876 carrying EBNA-2A and EBNA-2B, respectively, were used as controls.[34] To test for deletions in the C-terminus of the BNLF-1 gene, a PCR product was amplified from nucleotide positions 168390 to 168074, according to Hudson *et al.*[35] Hodgkin's disease cases and the cell line AG 876 previously shown to contain the BNLF-1 30 bp deletion, and Hodgkin's disease cases and the cell line B95.8 shown not to contain deletions, were used as positive and negative controls, respectively.[22] PCR was carried out in a 50 μl reaction mixture (3.75 mM $MgCl_2$ and 5 units of AmpliTaq polymerase Stoffel fragment (Perkin Elmer Cetus)). Primers and PCR conditions have been described in detail elsewhere.[22 36] The size of the PCR product was verified after electrophoresis in Visigel separation matrix (Stratagene, La Jolla, California, USA) stained with ethidium bromide.

BI-DIRECTIONAL SOLID PHASE DIDEOXY SEQUENCING

Sequence analysis was performed to demonstrate the size and the location of deletions, and to detect single base mutations, as described recently.[22] Briefly, in all cases two parallel PCRs were performed, one for sequencing the sense strand and one for sequencing the antisense strand. The PCR product for sequencing the sense strand was produced with a non-biotinylated sense primer and a 5' biotinylated antisense primer. The product for sequencing the antisense strand was produced with a non-biotinylated antisense primer and a 5' biotinylated sense primer (the same primers as used to amplify the PCR product). After PCR the amplified fragments were captured from 35–50 μl of the reaction mix (the amount needed for sequencing was estimated by electrophoresis), with 30 μl Streptavidin conjugated magnetic beads (Dynabeads, Dynal, Norway), according to the manufacturer's instructions. After several washing steps the double stranded PCR product was denatured by incubating with 20 μl 0.1 M NaOH for four minutes at room temperature. The supernatant containing the PCR product generated by the non-biotinylated primer was removed. The Dynabeads/single stranded DNA complex was washed once in 250 mM Tris (pH 8)/0.1% Tween 20, once in TE buffer and once in double distilled water, and resuspended in 10 μl double distilled water. The sequencing reaction was carried out according to the manufacturer's instructions (PRISM Sequenase Terminator Single-Stranded DNA Sequencing Kit, Applied Biosystems Inc., Foster City, California, USA), using fluorescence labelled dideoxynucleotide terminators as described.[22] The gel was electrophoresed for 14 hours on a semiautomated ABI 373A sequencer (Applied Biosystems), and the results were analysed using the Seg Ed software program (Applied Biosystems).

ANALYSIS OF SERUM DEPENDENT GROWTH

Representative LCL with a 30 bp deletion (MS1845), with a 69 bp deletion (MS1859) and with no deletion (MS1851 and MS1858) in the C-terminal region of the LMP-1 molecule were used for analysis of growth and survival at different serum concentrations. Cells were seeded in triplicate at a starting density of 0.4×10^6 cells per ml (day 1) in 10 ml growth medium with either 0.1%, 1.0% or 10% human serum. The number of viable cells was counted daily using the trypan blue exclusion test.

COLONY FORMATION IN SOFT AGAROSE

Seaplaque agarose (BioWhittaker) was dissolved in distilled water. A base layer of 0.3% agarose in 1.5 ml RPMI 1640 supplemented with 200 IU/ml penicillin (Leo), 0.2 mg/ml streptomycin (Sigma), 0.29 mg/ml glutamin (Sigma), 0.01 M HEPES buffer (BioWhittaker), and 10% heat inactivated human serum was poured into six-well multidishes (Nunc, Roskilde, Denmark).

	322+	323	324	325	326	327	328+	329+	330+	331+	332	333	334+	335+	336	337	338+	339	340	341	342–	343	344
B95.8	CAA	TTG	ACG	GAA	GAG	GTT	GAA	AAC	AAA	GGA	GGT	GAC	CAG	GGC	CCG	CCT	TTG	ATG	ACA	GAC	GGA	GGC	GGC
CAO	**AAT**	TTG	ACG	GAA	GAG	GTT	**GCA**	AAC	AAA	GGA	GGT	GAC	**CGG**	GGC	CCG	CCT	**TCG**	ATG	ACA	GAC	**GGT**	*GGC*	*GGC*
1851	CAA	TTG	ACG	GAA	GAG	GTT	**C**AA	AAC	AAA	GGA	GGT	GAC	CAG	GGC	CCG	CCT	TTG	ATG	ACA	GAC	GGA	GGC	GGC
1874	CAA	TTG	ACG	GAA	GAG	GTT	**C**AA	AAC	AAA	GGA	GGT	GAC	CAG	GGC	CCG	CCT	TTG	ATG	ACA	GAC	GGA	GGC	GGC
1844	CAA	TTG	ACG	GAA	GAG	GTT	**C**AA	AAC	AAA	GGA	GGT	GAC	CAG	GGC	CCG	CCT	TTG	ATG	ACA	GAC	GGA	GGC	GGC
1858	**G**AA	TTG	ACG	GAA	GAG	GTT	GAA	AAC	AAA	GGA	GGT	GAC	**CGG**	GGC	CCG	CCT	**TCG**	ATG	ACA	GAC	**GGT**	GGC	GGC
1847	**AAT**	TTG	ACG	GAA	GAG	GTT	GAA	AAC	A**C**A	GGA	GGT	GAC	**CGG**	GGC	CCG	CCT	**TCG**	ATG	ACA	GAC	**GGT**	*GGC*	*GGC*
1845	**AAT**	TTG	ACG	GAA	GAG	GTT	GAA	AAC	AAA	GGA	GGT	GAC	**CGG**	**A**GC	CCG	CCT	**TCG**	ATG	ACA	GAC	**GGT**	*GGC*	*GGC*
1533	**AAT**	TTG	ACG	GAA	GAG	GTT	GAA	AAC	A**C**A	GGA	GGT	GAC	**CGG**	GGC	CCG	CCT	**TCG**	ATG	ACA	GAC	**GGT**	*GGC*	*GGC*
1859	**G**AA	TTG	ACG	GAA	GAG	GTT	GAA	A**G**C	AAA	**A**GA	*GGT*	*GA*											

	345	346	347	348	349	350	351	352+	353	354	355	356–	357	358	359	360	361–	362	363	364	365	366+
B95.8	GGT	CAT	AGT	CAT	GAT	TCC	GGC	CAT	GGC	GGC	GGT	GAT	CCA	CAC	CTT	CCT	ACG	CTG	CTT	TTG	GGT	TCT
CAO	*GGT*											GAT	CCA	CAC	CTT	CCT	ACG	CTG	CTT	TTG	GGT	**ACT**
1851	GGT	CAT	AGT	CAT	GAT	TCC	GGC	CAT	GGC	GGC	GGT	GA**C**	CCA	CAC	CTT	CCT	ACG	CTG	CTT	TTG	GGT	**ACT**
1874	GGT	CAT	AGT	CAT	GAT	TCC	GGC	CAT	GGC	GGC	GGT	GA**C**	CCA	CAC	CTT	CCT	ACG	CTG	CTT	TTG	GGT	**ACT**
1844	GGT	CAT	AGT	CAT	GAT	TCC	GGC	CAT	GGC	GGC	GGT	GA**C**	CCA	CAC	CTT	CCT	ACG	CTG	CTT	TTG	GGT	**ACT**
1858	GGT	CAT	AGT	CAT	GAT	TCC	GGC	C**G**T	GGC	GGC	GGT	GAT	CCA	CAC	CTT	CCT	AC**A**	CTG	CTT	TTG	GGT	**ACT**
1847	*GGT*											GAT	CCA	CAC	CTT	CCT	ACG	CTG	CTT	TTG	GGT	**ACT**
1845	*GGT*											GAT	CCA	CAC	CTT	CCT	ACG	CTG	CTT	TTG	GGT	**ACT**
1533	*GGT*											GAT	CCA	CAC	CTT	CCT	ACG	CTG	CTT	TTG	GGT	**ACT**
1859												T	CCA	CAC	CTT	CCT	AC**A**	CTG	CTT	TTG	GGT	**G**CT

Figure 1 Sequence analysis of the C-terminal region of the EBV BNLF-1 gene which encodes LMP-1. Single base mutations identical with those found in the CAO BNLF-1 sequence are underlined and in bold. Single base mutations differing from both the B95.8 and CAO BNLF-1 gene are underlined. Amino acid changes identical with those found in the CAO LMP-1 protein are indicated by framing the coding triplet. The 5 bp and the 11 bp repeat sequences which could be implicated in the generation of the 30 bp and 69 bp deletions, respectively, are in italics. + indicates that the single base mutation affects the amino acid sequence; – indicates that the single base mutation does not affect the amino acid sequence.

The top layer was either 0.2%, 0.3%, 0.45%, or 0.6% agarose in 1.5 ml of the same medium as used for the base layer; 3000 cells were added per well from one of the following cell lines: MS1845, MS1851, MS1858 or MS1859. After setting, the agarose was covered with 1 ml medium. All culture dishes were incubated at 37°C in 5% CO_2. Three independent experiments were carried out and each cell line was plated in triplicate. A colony was defined as a cluster consisting of more than 10 cells.

Results

PCR

EBNA-2 type A was detected in all eight LCL and in the EBV subtype A control cell line B95.8. EBNA-2 B was only found in the EBV subtype B control cell line AG 876. Amplification of the C-terminal region of the BNLF-1 gene indicated the presence of a deletion in four of eight LCL. In three cases (MS1845, MS1847 and MS1533) gel electrophoresis indicated that the deletion was approximately 30 bp. In the fourth case (MS1859) it was larger. PCR control experiments on tumours from SCID and nude mice gave results identical with those found in the respective inoculated LCL.

SEQUENCE ANALYSIS

The results are summarised in fig 1. In the three cases with electrophoretical evidence of a deletion around 30 bp, sequence analysis confirmed the size to be 30 bp. Examination of the sequence environment surrounding the 30 bp deletion revealed two 9 bp repeats (GGCG-GCGGT) coding for three Gly residues (amino acids 343 to 345 and amino acids 353 to 355). We have proposed previously that the 30 bp deletion can be generated by slipped mispairing of direct repeats.[22] The sequence analysis suggests that this has probably been preceded by a single base mutation in amino acid 342 (A to T) giving two 10 bp repeats (TGGCGGCGGT; third base of the coding triplet of amino acids 342 to 345 and third base of the coding triplet of amino acids 352 to 355), one of which is included in the 30 bp deletion (fig 1). Indeed, one of the LCL without the deletion (MS1858) has this mutation at amino acid 342. In addition, this cell line has a mutation in the coding triplet for amino acid 352 (A to G), giving two 11 bp repeats adjacent to the 30 bp deletion, a change that would further increase the likelihood of a deletion occurring by slipped mispairing. Thus, there is circumstantial evidence that both of these mutations may be present prior to the deletion of the 30 bp sequence.

In the MS1859 LCL with electrophoretical evidence of a deletion larger than 30 bp, sequence analysis revealed a 69 bp deletion comprising the sequence of the 30 bp deletion and a further 39 bp. The sequence environment of the 69 bp deletion contains two five nucleotide repeats (GGTGA), one of which is included in the 69 bp deletion (fig 1).

Six of the seven single base mutations detected in the CAO BNLF-1 gene between amino acids 322 and 388 were found in all

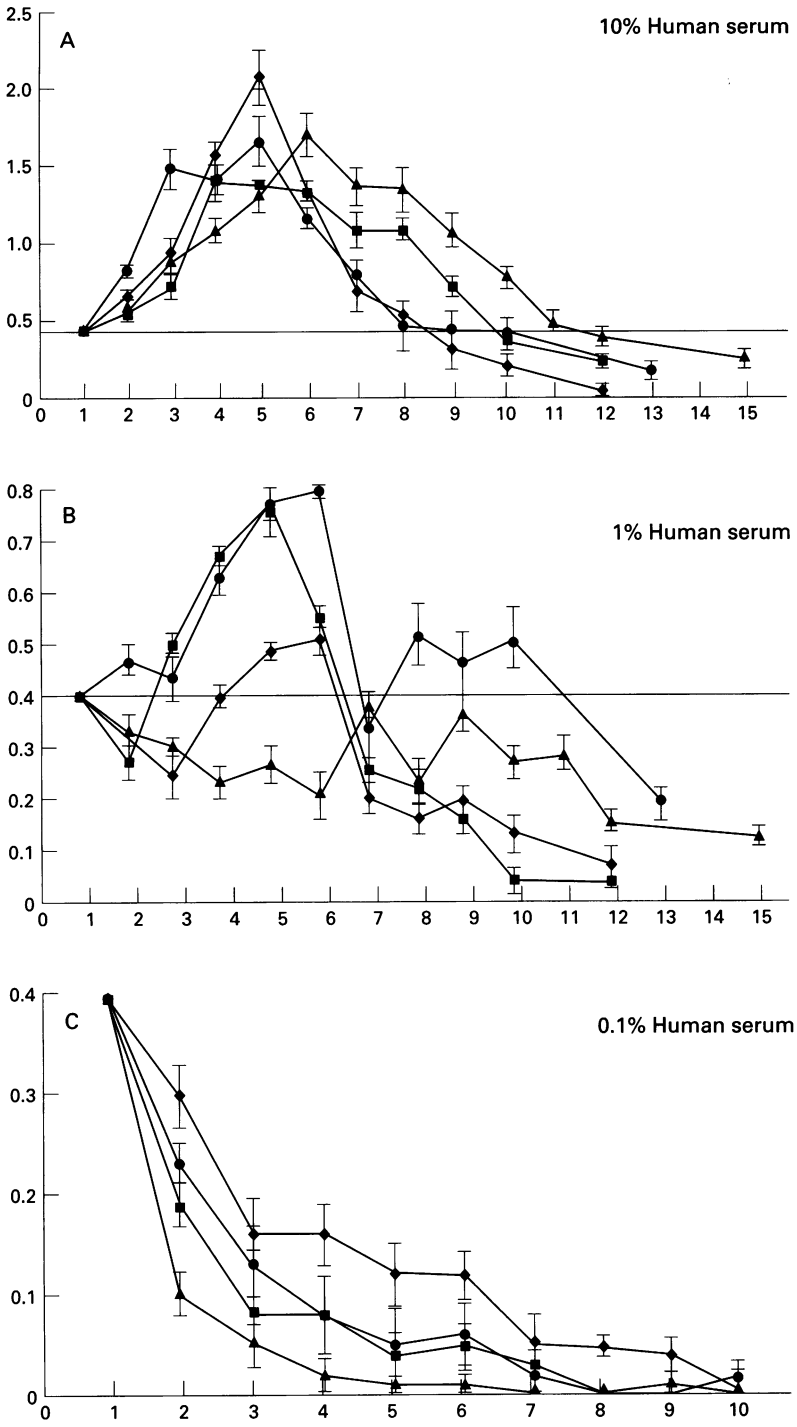

Figure 2 The ability of the cell lines MS1845, MS1851, MS1858, and MS1859 to survive in low serum concentrations was analysed by daily counts of viable cells. The serum concentrations (0.1 %, 1.0% and 10% human serum, respectively) are indicated on the graphs. The y-axis indicates the number of live cells/ml divided by 10^6 and the x-axis the number of days in culture. Each LCL was cultured in triplicate. The curves represent the mean (SD). The labelling of the curves are as follows: ■, MS1845; ●, MS1851; ♦, MS1858; and ▲, MS1859. The horizontal line at 0.4 × 10^6 live cells/ml indicates the cell concentration at day 1.

three cases containing the 30 bp deletion, the mutation affecting amino acid 328 being the one absent in all cases (fig 1). Two of these LCL (MS1847 and MS1533) had an additional mutation affecting amino acid 330 (A to C: Lys to Thr) and the third (MS1845), a mutation affecting amino acid 335 (G to A: Gly to Ser) (fig 1). The case with the 69 bp deletion (MS1859) contained none of the single base mutations found in this region of the CAO BNLF-1 gene. However, single base mutations were demonstrated in the coding triplets of amino acids 322 (C to G: Gln to Glu), 329 (A to G: Asn to Ser), 331 (G to A: Gly to Arg), 356 (T to C: no amino acid change), 361 (G to A: no amino acid change), and 366 (T to G: Ser to Ala) (fig 1).

Three of the LCL without deletions (MS1851, MS1874 and MS1844) showed a nucleotide sequence identical with B95.8 except for amino acid 328 (G to C: Glu to Gln) and amino acid 366 (T to A: Ser to Thr). The fourth cell line (MS1858) contained mutations at amino acids 322 (C to G: Gln to Glu) and 352 (A to G: His to Arg) (fig 1), together with the single base mutations affecting amino acids 334, 338, 342, and 366 detected in the CAO BNLF- 1 gene and in the three LCL with the 30 bp deletion. In the sequence from amino acids 366 to 388 no single base mutations were detected in any of the LCL (data not shown).

GENETIC FINGERPRINTING

In all eight LCL individual genetic fingerprinting patterns were demonstrated excluding cross contamination with cells from flask to flask. Six of the eight virus isolates contained individual BNLF-1 sequences (see earlier) essentially ruling out virus cross contamination. The LCL MS1844 and MS1851 were identical in the sequenced part of the BNLF-1 gene, raising the possibility of identical EBV strains. These LCL were established independently and cultured at separate locations, strongly arguing against cross contamination.

TIME REQUIRED FOR LCL ESTABLISHMENT

Transformation of B lymphocytes leads to phenotypic changes and immortalisation. Development of LCL was characterised by free floating spherical or oval clusters of more or less tightly packed cells increasing in number over time. The time interval from seeding the mononuclear cell cultures to the appearence of LCL varied between 60 and 180 days. There was no correlation between the time taken to establish a LCL and the presence of the 30 bp or the 69 bp deletion in the BNLF-1 gene.

SERUM DEPENDENT GROWTH

The number of live cells as a function of days in culture is shown in fig 2. There was no significant difference between the serum dependent growth of the cell lines. However, there was a tendency for a more serum dependent growth for the cell line MS1859 (containing the 69 bp deletion), most clearly seen in the experiment with 1% human serum in the culture medium. The number of live cells in the MS1859 culture decreased after day 0 compared with the other cell lines which proliferated until about day 7. The same tendency was seen for MS1859 with 0.1% human serum where the cells died very quickly and with 10% human serum where the cell line proliferated more slowly and reached the maximum number of live cells later than for the other LCL.

CLONING IN SOFT AGAROSE

The ability to form colonies in soft agarose with low agarose concentrations (0.2% and 0.3%) was not associated with the presence of

Table 1 Incidence of tumour formation by inoculation of lymphoblastoid cell lines subcutaneously in SCID and nude mice

Mouse type	Cell line	Tumour incidence
SCID mice	MS1845	3 of 7
	MS1851	4 of 5
	MS1858	4 of 5
	MS1859	6 of 10
Nude mice	MS1845	0 of 5
	MS1851	0 of 5
	MS1858	0 of 5
	MS1859	3 of 5

mutations. Only the number of live and dead cells in the culture flasks counted before seeding in agarose influenced colony formation. A relatively high number of live cells (about 1×10^6 cells/ml) and a fraction of dead cells under 0.1 increased colony formation.

TUMORIGENICITY

The incidence of tumour formation in SCID and nude mice is shown in table 1. Tumours were generated in SCID mice by all four cell lines tested (MS1845, MS1859, MS1851, and MS1858). Invasive growth into the skin resulting in necrotic ulcers was evident in two of four mice with tumour inoculated with the LCL MS1851 and MS1858, two of three mice with tumour inoculated with MS1845 and in five of six mice inoculated with MS1859. Tumours were generated in three of five nude mice inoculated with the LCL MS1859 (69 bp deletion) and grew non-invasively up to a size of 1 cm^3 over the course of three to four weeks. Thereafter, the tumours regressed leaving a tumour of only 0.5 cm^3 in two of the mice after 26 weeks, at which time the mice were sacrificed. In the third mouse no residual tumour was found. None of the nude mice inoculated with the other cell lines showed evidence of tumour formation over the 26 weeks of follow up.

CYTOGENETICS

The four cell lines used for tumorigenicity studies (MS1845, MS1859, MS1851, and MS1858) were all shown to be normal diploid cell lines by flow cytometry.

IMMUNOHISTOLOGY

All cell lines displayed characteristic LCL immunophenotype. No differences in the expression of the adhesion molecules ICAM-1 and LFA-3 or the activation marker CD23 could be demonstrated between cell lines with and without mutation of the BNLF-1 C-terminus. Comparable concentrations of EBV gene products EBNA-2, LMP-1 and BZLF-1 were detected in all LCL. The LMP-1 staining pattern indicated cytoplasmic and membrane staining with focal positivity, suggesting patching in the membrane. No differences in staining patterns between cell lines with and without mutations of the BNLF-1 C-terminus could be detected. Bcl-2 was expressed by all cell lines at comparable levels. SCID mice tumours displayed a staining pattern similar to LCL, except for CD21 and CD23 which were slightly down regulated in all tumours from SCID mice. In two tumours from nude mice EBNA-2 expression was demonstrated in the nuclei of tumour cells, confirming the presence of EBV infected lymphocytes rather than a non-specific reaction.

Discussion

Recent studies have shown that single base mutations and the 30 bp deletion detected in the C-terminus of the CAO BNLF-1 gene can be detected frequently in EBV associated malignancy in vivo.[22 23 36 37] The CAO BNLF-1 isolate shows increased tumorigenicity in SCID and nude mice compared with prototypic EBV strains, and it has been suggested that mutant LMP-1 is preferentially associated with aggressive forms of EBV malignancy.[36] The present study shows that similar genetic changes occur frequently in wild type EBV strains present in spontaneously arising LCL and do not by themselves characterise a tumour specific genotype. Clearly, care must be taken in interpreting the results of this study. Only viruses carrying LMP-1 capable of supporting transformation will be present in LCL. This selection bias means that the variants we have studied may not be completely representative of wild type viruses as a whole.

Recombinant EBV molecular genetic experiments with specifically mutated LMP-1 genes have demonstrated that LMP-1 is essential for LCL outgrowth.[10] Our results indicate that neither the 30 bp nor the 69 bp deletions are essential for LMP-1 mediated primary B cell transformation. Furthermore, comparison of transformation related parameters indicated that the deletions did not affect the in vitro behaviour of the LCL significantly. However, there was a tendency for the LCL with the EBV strain containing the 69 bp deletion to be more serum dependent than the other LCL.

It is tempting to ascribe the increased tumorigenicity of MS1859 to the 69 bp LMP-1 deletion. Clearly, however, this may be because of changes elsewhere in the viral genome not identified by our analysis, and formal proof will require further transfection studies.

In vitro evidence that the 30 bp deletion may still play a role in tumorigenesis has come from a recent study by Li *et al.*[26] They found differences in the behaviour of Balb/3T3 cells when transfected with B95.8 BNLF-1 gene in which the 30 bp sequence had been deleted, compared with the prototypic B95.8 BNLF-1 gene. The transfected cells became tumorigenic in nude mice, the transforming capacity of the virus increased, and the gene was toxic to the cells at lower expression levels compared with the wild type B95.8 BNLF-1 gene. The apparent discrepancies between this study and our findings may be partly due to differences in the behaviour of the BNLF-1 gene in rodent compared with human cell lines, as has been described in other circumstances.[38]

Alternatively, failure to detect an effect of the 30 bp deletion may be because of a lack of sensitivity in our assay. In any event, the results of our study and those of Li *et al*[26] suggest that the

sequences of the 30 bp and 69 bp deletions may have an important role in LMP-1 protein function.

Recent studies by Moorthy and Thorley-Lawson may offer an explanation for the apparent increased tumorigenicity of EBV variants with the 30 and 69 bp deletions in Balb/3T3 cells and in our LCL, respectively. They showed that deleting 23 (Δ 364–386) or 81 amino acids (Δ 306–386) from the C-terminus resulted in a LMP-1 protein that was non-transforming, but turned over normally in Rat-1 fibroblasts. In contrast, deleting 64 (Δ 323–386) or 53 (Δ 334–386) amino acids from the C-terminus generated toxic LMP-1 mutants, suggesting that the toxicity was caused by the absence of the amino acid sequence 334–364.[15] The 69 bp deletion described by us constitutes about two thirds of this amino acid sequence. Thus, the toxicity in Rat-1 cells and the increased tumorigenicity of LCL may be caused by similar changes in function of the LMP-1 protein. The 30 bp deletion variant has lost about one third of the 334–364 amino acid sequence, and this may account for its increased toxicity in Balb/3T3 cells[26] when driven by a weaker promoter compared with the B95.8 BNLF-1 gene. Trivedi *et al*[39] have shown that the LMP-1 protein encoded by the CAO BNLF-1 gene (which contains the 30 bp deletion) is less immunogenic than B95.8 LMP-1. This offers a possible explanation for the increased tumorigenicity of variants lacking the 30 bp and 69 bp sequences, although it cannot account for the increased transforming capacity of the 30 bp deletion variant found by Li *et al* compared with B95.8.[26] LMP-1 has been shown to induce the expression of bcl-2 in B cells and this response was found to be delayed relative to NF-κB activation, suggesting that NF-κB might mediate this response.[40] The 30 bp and the 69 bp deletions are located close to or possibly overlap the NF-κB activation region of LMP-1,[38] suggesting that the bcl-2 response of LMP-1 expression could be affected by the deletions. However, we found no immunohistological evidence for this.

The 69 bp deletion is rare in vivo, having been demonstrated previously in only one AIDS related lymphoma, in one case of chronic lymphoproliferative disease in a child, and in a single case of Hodgkin's disease in a Liberian patient but in none of some 100 European Hodgkin's disease cases investigated.[22 36 37] We have previously proposed that the 30 bp deletion can be generated by slipped mispairing of direct repeats.[22] If a similar mechanism is responsible for the 69 bp deletion, then the relative rarity of this variation compared with the 30 bp deletion may simply be explained by the size of the deletion and the direct repeats. The direct repeats present in the sequence environment of the 30 bp and the 69 bp deletions are of 11 bp and 5 bp, respectively. The frequency of slipped mispairing has been shown to be proportional both to the length of the direct repeat motif and to the extent of homology between the direct repeats, but inversely proportional to the distance between them.[41] As a consequence of this the 69 bp deletion should be generated much less frequently than the 30 bp deletion.

In conclusion, these results and our recently published data on Hodgkin's disease, peripheral T cell lymphoma and infectious mononucleosis show that mutations in the C-terminal region of the BNLF-1 gene occur frequently in both malignant and benign EBV infected cells and are more widespread among wild type EBV strains than originally thought.[22] While these mutations clearly do not simply define tumour specific EBV strains, our study suggests that the LMP-1 amino acid sequence 334–356 (or part of this sequence) is of importance for the tumorigenic effects of LMP-1, and that changes in the function of LMP1 may result in altered tumorigenicity of EBV infected LCL. Studies on larger numbers of LCL from normal EBV seropositive subjects are needed to investigate which mutations occur regularly in the BNLF-1 gene. Further mutational studies in vitro are needed to define the effect of the 69 bp deletion described in this study.

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