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Expression of Epstein–Barr virus BMRF-2 and BDLF-3 genes in hairy leukoplakia

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The high level of Epstein–Barr virus (EBV) replication found in hairy leukoplakia (HL) provides a unique opportunity to study EBV expression in the oral epithelium. Screening of a cDNA library from an HL biopsy revealed expression of two genes not previously described *in vivo*: BMRF-2 and BDLF-3. Sequence analysis of the cDNAs demonstrated several nucleotide changes from the B95-8 sequence. In all six different HL strains studied, only one amino acid change was found in BMRF-2 relative to B95-8 and two amino acid changes were found in the BDLF-3 ORF. mRNA expression of both genes was localized to the lower prickle cell layer of the tongue epithelium. BMRF-2 protein expression was primarily detected in the cell nuclei of the upper prickle cell layer; immunoelectron microscopy revealed that BMRF-2 was associated with the nuclear chromatin. BDLF-3 protein expression was observed in the perinuclear space and cytoplasm of the prickle cells. BDLF-3 has recently been identified as a virion-associated protein, but the functions of BMRF-2 and BDLF-3 have not been elucidated.

Introduction

Hairy leukoplakia (HL) is a white lesion typically found on the lateral border of the tongue of immunosuppressed individuals, primarily those with human immunodeficiency virus (HIV) infection (Greenspan *et al.*, 1985). HL is associated with high levels of Epstein–Barr virus (EBV) replication. The histopathology of the lesion includes thickening of the epithelium, hyperparakeratosis and 'ballooning' of cells in the prickle cell layer. The lesion contains large numbers of EBV particles in the nuclei of the upper cell layers of the oral epithelium (Rabanus *et al.*, 1991).

EBV genes from different stages of the virus cycle have been reported to be expressed in HL. These include: immediate early and early genes such as the major viral transactivator BZLF-1 (Young *et al.*, 1991) and EA-D (BMRF-1); late genes such as VCA (viral capsid antigen) and gp350/220 (the major envelope glycoprotein) (Rabanus *et al.*, 1991); and genes associated with virus latency and transformation such as Epstein–Barr nuclear antigen (EBNA)-2 and latent membrane protein (LMP)-1 (Walling & Raab-Traub, 1994; Palefsky *et al.*, 1996). The expression of other genes such as BDRF-1 (a putative scaffold protein), BCRF-1 (viral IL-10 homologue), and BARF-0 (function unknown) (Lau *et al.*, 1993) has also been reported in HL, but the full repertoire of EBV gene expression and its role in the pathogenesis of HL have not been elucidated. To further study the expression of EBV genes in HL, we constructed a cDNA library from an HL biopsy and detected expression of two EBV genes that have not been previously described in HL: BMRF-2 and BDLF-3.

The BMRF-2 ORF encodes a protein with multiple hydrophobic domains surrounding a central hydrophilic region with an arginine, glycine, aspartic acid (RGD) motif and potential phosphorylation sites for a number of protein kinases including cAMP/cGMP-dependent protein kinases, casein kinase II, protein kinase C and tyrosine kinase. Homologues for BMRF-2 have been identified in herpesvirus saimiri, Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) and equine herpesvirus using the software from the Wisconsin Package version 9.0, Genetics Computer Group, Madison, Wisconsin, USA. However, knowledge of the expression, regulation and function of the BMRF-2 protein is very limited. Previous work has suggested that BMRF-2 may be expressed as part of a bicistronic message with BMRF-1 (Pfitzner *et al.*, 1987). In B-lymphocyte cell lines, a 1·5 kb transcript has been mapped to the BMRF-2 ORF collinear with a 2·6 kb BMRF-1 transcript (Sample *et al.*, 1986). A cDNA clone expressing

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BMRF-2 was also identified from a B-lymphocyte library following induction of lytic replication (Pfitzner *et al.*, 1987). A protein complex of 53/55 kDa was identified in B-lymphocytes using an antibody to a portion of the BMRF-2 ORF (Modrow *et al.*, 1992) and the protein was localized to the nuclear and membrane fractions in these cells. To date, BMRF-2 expression has not been described in either EBV-associated lymphoid malignancies *in vivo* or in epithelial lesions. In this report, we describe the expression and localization of BMRF-2 in HL.

The BDLF-3 ORF encodes a late viral glycoprotein with nine potential *N*-linked glycosylation sites and a transmembrane domain (Nolan & Morgan, 1995). BDLF-3 has recently been reported to be a virion-associated protein (Kurilla *et al.*, 1995). Like BMRF-2, the expression and function of BDLF-3 have not been previously described in EBV-associated lesions *in vivo* and in this paper we describe the expression and localization of BDLF-3 in HL.

Methods

■ **Cell culture.** Lymphoblastoid cell lines (LCLs) were maintained in RPMI 1640 medium supplemented with 10% newborn calf serum. All media were purchased from the UCSF Tissue Culture Facility (San Francisco, USA). B95-8 is an LCL from marmoset lymphocytes transformed with a human EBV strain from infectious mononucleosis (Baer *et al.*, 1984). P3HR-1 is an LCL from Burkitt's lymphoma which spontaneously produces EBV particles (Hinuma *et al.*, 1967).

■ **Biopsies and scrapings.** Tongue biopsies were obtained from volunteers after giving informed consent in a protocol approved by the Committee on Human Research, University of California, San Francisco, USA. A biopsy from an HL lesion diagnosed as described previously (Greenspan *et al.*, 1992) was obtained from an HIV-positive subject for the construction of a cDNA library. To perform DNA and RNA hybridization *in situ*, HL biopsies were taken from the lateral border of the tongue of six HIV-positive subjects. As controls, tongue biopsies consisting of hyperkeratotic lesions other than HL were obtained from six other subjects, two of whom were HIV-positive. Normal tongue tissue biopsies were also obtained from five HIV-negative subjects. All the tissues were fixed in buffered formalin immediately after collection, paraffin-embedded and 7 μm sections were cut with RNase-free blades.

To obtain nucleotide sequence confirmation, five HL scrapings (SC) from different subjects were collected by scraping the lateral border of HL lesions with the blunt edge of a scalpel (Palefsky *et al.*, 1996). EBV sequences from an LCL established from an HL strain (R5-97) were also studied (Palefsky *et al.*, 1996). SCs and LCL cells were digested overnight with 200 μg/ml proteinase K at 50 °C in preparation for PCR amplification.

■ **Generation and screening of the HL cDNA library.** The cDNA library was constructed as described previously (Palefsky *et al.*, 1996). Briefly, total RNA was isolated from an HL biopsy by homogenization of the tissue in 4 M guanidinium thiocyanate and ultracentrifugation was carried out through a cushion of 5 M CsCl. mRNA was isolated with oligo(dT)–cellulose type 7 (Pharmacia LKB) and copied into cDNA with M-MuLV reverse transcriptase (Stratagene). Double-stranded cDNA was ligated into lambda using the Zap cDNA synthesis kit (Stratagene). The ligated cDNA was packaged into Gigapack II Gold packaging extracts and plated according to the manufacturer's instructions (Stratagene). To select for EBV genes, approximately 10^5 clones of the cDNA library were plated on soft agar. Plaque lifts were hybridized with a cocktail of six probes generated from cosmid clones, kindly provided by G. W. Bornkamm (Institut fur Klinische Molekularbiologie und Tumourgenetik, GSF Munchen, Germany). The cosmid probes spanned all but the *Bam*HI C segment of the EBV genome from the M-ABA strain (Polack *et al.*, 1984). The cosmid clones were labelled with [α-^{32}P]dCTP (Amersham) utilizing the random primer DNA labelling system (Gibco BRL Life Technologies). Positive plaques were isolated and re-hybridized individually with each of the six EBV cosmid clones from the cocktail. The second round of positive plaques were excised in BluescriptII SK(+/−) phagemid (Stratagene) for further identification.

■ **DNA sequencing.** Nucleotide sequence analysis of the BMRF-2 and BDLF-3 cDNAs was performed using Sequenase version 2.0 (Amersham). DNA samples from five HL scrapings, one LCL (R5-97) and B95-8 were generated by PCR, and purified using the Wizard DNA Purification System (Promega). Nucleotide sequencing was performed using the *fmol* DNA sequencing system (Promega). The sequences of the cDNA clones, scrapings and lymphocytes were compared to that of the EBV prototype strain B95-8 through GenBank using the Wisconsin Package version 8.01-UNIX. The following oligonucleotides were synthesized as primers for nucleotide sequence determination: BDLF-3 middle 5′ TTTGGAGGACAGCGTGGG 3′; BMRF-2 5′ CCAGGCAGAAGCAGAAGC 3′; BMRF-2 middle 5′ GGCTTTGGGCTCTGGAAG 3′; BMRF-2-1 5′ CTGGCGGCGGCGCTTAGCCT 3′; 2-5.1 5′ CCGTGCTGTTTATCTCA 3′; 2-5.1A 5′ TTCACCTCCGCCACCTC 3′; 2-5.2 5′ GGGCTGTGTCT*TCTGTC 3′ (*mismatch noted); 2-5.3 5′ TGCCCCCTGTCTCTTTC 3′; 2–5.4 5′ CGGAGACGCAGCATTTT 3′.

■ **RNA *in situ* hybridization.** Using riboprobes for BMRF-2 and BDLF-3, RNA *in situ* hybridization was performed on HL, non-HL hyperkeratosis and normal tongue tissues, using the RNA Colour kit (Amersham). The sense and antisense riboprobes were synthesized from T3 and T7 RNA promoters, respectively, which flanked the cDNA clones corresponding to these genes (see Fig. 1*a*, *b*). To synthesize antisense riboprobes, the cDNA was linearized with *Sma*I for BMRF-2 (Fig. 1*a*) and with *Xho*I for BDLF-3 (Fig. 1*b*). The riboprobes were synthesized using fluorescein-labelled ribonucleotides according to the manufacturer's instructions (Amersham). EBV DNA *in situ* hybridization of these tissues was performed with a riboprobe generated from the T7 RNA promoter in the *Bam*HI W clone (kindly provided by Nancy Raab-Traub, University of North Carolina, Chapel Hill, NC, USA).

In situ hybridization was performed on microscope slides containing 7 μm sections of paraffin-embedded tissue. The samples were deparaffinized with xylene, rehydrated through a gradient of alcohol concentrations and treated with 50 μg/ml proteinase K (Boehringer Mannheim) for 10 min at room temperature. The tissues were hybridized with fluorescein-labelled probes overnight at 55 °C, treated with 10 μg/ml RNase (Boehringer Mannheim) for 30 min at 37 °C to degrade the excess probe, washed with Tris-buffered saline (TBS1) (100 mM Tris pH 7·6, 400 mM NaCl) and incubated with an alkaline phosphatase-labelled anti-fluorescein isothiocyanate (FITC) antibody overnight at 4 °C. The samples were then washed with TBS1 and developed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro-blue tetrazolium chloride (NBT) substrates for alkaline phosphatase according to the manufacturer's instructions (Amersham).

■ **Northern blot analysis.** Northern blot analysis of RNA extracted from a pool of three HL biopsies was performed to examine the BMRF-2 transcript in clinical tissues. For comparison, whole-cell RNA was harvested from P3HR-1 cells 48 h after induction with 30 μg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma). RNA was isolated using guanidinium isothiocyanate as described previously (Chirgwin *et al.*, 1979). Briefly, after washing cells with PBS, cells were lysed in

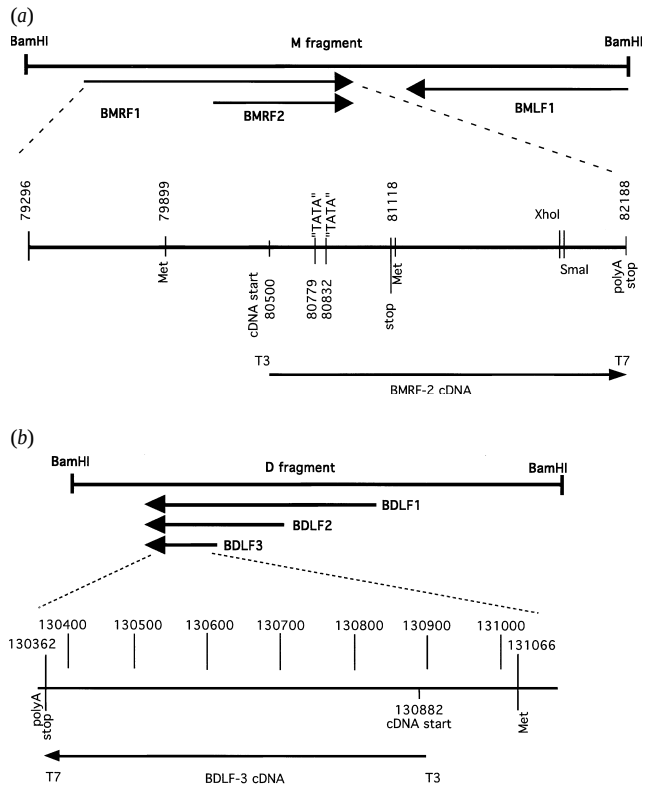

Fig. 1. Transcripts from the *Bam*HI M and D fragments of EBV. (*a*) Schematic drawing of two right frame transcripts and one left frame transcript from the *Bam*HI M fragment shown as arrows (top). The cDNA clone isolated from the HL library is depicted at the bottom as a long arrow containing the full BMRF-2 transcript and part of BMRF-1. The T3 and T7 RNA promoters that flank the cDNA are noted. The predicted BMRF-2 TATA boxes, polyadenylation site and in-frame stop codons are noted. Numbers correspond to the coordinates from the published sequence of the B95-8 EBV lymphocyte strain. (*b*) Schematic drawing showing the structure of the *Bam*HI D fragment and the three left frame transcripts depicted as arrows (top). Indicated at the bottom is the cDNA clone from the HL library (long arrow) and the T3 and T7 RNA promoters used to generate riboprobes for *in situ* hybridization. The initiation site (Met) for BDLF-3 and the polyadenylation site shared by the three left frame transcripts are noted.

guanidinium solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0·5% *N*-lauroylsarcosine, 0·15% mercaptoethanol) and centrifuged through a 5·7 M CsCl cushion. Total RNA (10 μg) was separated on a 1% agarose–6% formaldehyde gel, transferred to a GeneScreenPlus membrane (DuPont/NEN) and hybridized with an [α-^{32}P]dCTP-labelled *Stu*I–*Xho*I fragment spanning the BMRF-2 region (Fig. 1*a*). As a control for RNA quantity, the membrane was hybridized with an [α-^{32}P]dCTP-labelled β-actin probe.

■ **Antisera.** A peptide was synthesized that corresponded to a region of the BMRF-2 protein that was predicted to be immunogenic using the Chou–Fasman program (RRSIFCARGDHSVASLK) (Chou & Fasman, 1978). The peptide was linked to keyhole limpet haemocyanin and used to generate the polyclonal rabbit antiserum α-BMRF-2 (Berkeley Antibody Company). Total protein concentrations of the α-BMRF-2 antibody and the pre-immune serum were 80 and 70 μg/ml, respectively. Pre-immune and α-BMRF-2 antibodies were precipitated with ammonium sulphate and α-BMRF-2 was purified through a BSA/BMRF-2 peptide-bound affinity column Affi-Gel 15 (Bio-Rad) as described previously (Palefsky *et al.*, 1991). The antibodies were tested by ELISA. Polyclonal rabbit antiserum against BDLF-3 (V8 Bl2), at a concentration of 4·7 mg/ml, was kindly provided by Lindsey Hutt-Fletcher (University of Missouri, Kansas City, MO, USA).

■ **ELISA and Western blot analysis.** Indirect ELISA was performed to determine the anti-peptide antibody titre. Peptide was coated onto Immulon 1 plates (Dynatech Laboratories) at a concentration of 50 μg/ml and incubated overnight at 4 °C. Serum dilutions were added to the plate after blocking with 10 mg/ml BSA in PBS and incubating for 1 h at 37 °C according to methods previously described (Coligan *et al.*, 1992). The enzyme substrate *p*-nitrophenyl phosphate was purchased from Sigma. ELISAs were read at 405 nm on a microplate reader (Molecular Devices).

Protein samples for Western blot analysis were obtained from P3HR-1 cells either uninduced or induced with 30 μg/ml PMA and harvested at 48 h. Cells were lysed in Triton lysis buffer (25 mM Tris–phosphate, 1% Triton X-100, 2 mM DTT, 10% glycerol) and mixed with an equal volume of Laemmli buffer. Samples were loaded onto a 12% acrylamide gel and subjected to polyacrylamide gel electrophoresis. Gels were transferred to PVDF membranes (Amersham) using a Bio-Rad transfer tank. Following transfer, the membranes were blocked for 1 h with PBS containing 5% skim milk and 0·1% Tween. Primary antibody α-BMRF-2 was diluted 1:200 in PBS–0·1% Tween 20, 1% skim milk and incubated for 1 h at room temperature. Blots were washed twice for 5 min with PBS–0·1% Tween. Secondary antibody, goat anti-rabbit conjugated to alkaline phosphatase (CALTAG Laboratories), was diluted 1:3000 in PBS–0·1% Tween and incubated for 1 h at room temperature. Blots were washed twice for 5 min with PBS–0·1% Tween and developed in Western Blue stabilized substrate for alkaline phosphatase (Promega).

■ **Immunohistochemistry.** BMRF-2 and BDLF-3 gene expression was examined in paraffin sections of tongue tissues. Immunostaining was performed using the Vectastain kit (Vector Laboratories). The samples were deparaffinized with xylene cyanol, dehydrated through a gradient of alcohol concentrations, and microwaved in 0·1 M citrate buffer for 15 min for antigen retrieval. All subsequent steps were performed at room temperature. The tissues were incubated in Tris-buffered saline (TBS2) (100 mM Tris, 0·9% sodium chloride, pH 7·5) buffer plus 1% BSA for 10 min. The primary antibody (either α-BMRF-2 or α-BDLF-3) was added at a dilution of 1:1000 and samples were incubated overnight at 4 °C. The samples were washed with TBS2 plus 0·1% Tween, incubated with a 1:5000 dilution of the secondary antibody (goat anti-rabbit avidin-conjugated) for 30 min, washed and incubated with avidin–biotin–alkaline phosphatase for 60 min. The tissues were then washed and developed with NBT and BCIP as described by the manufacturer (Vector Laboratories).

■ **Immunoelectron microscopy.** To determine the localization of BMRF-2 in the cell at an ultrastructural level, we performed immunogold labelling on two HL biopsies. The tissues were fixed in a mixture of 1% paraformaldehyde and 3% glutaraldehyde in 0·067 M sodium cacodylate buffer (Karnovsky fixative) for 1 h at room temperature; samples were then dehydrated in a series of ethanol gradients while lowering the temperature to −30 °C. The tissues were embedded in Lowicryl K4M (Chemische Werke, Waldkreiburg, Germany) and exposed to ultraviolet light as described (Greenspan *et al.*, 1989). Immunogold labelling was performed as described previously (Gelderblom *et al.*, 1985). All procedures were conducted at room temperature. Briefly, the tissue sections were placed in Formvar-coated nickel grids, floated on PBS containing 1·2 mg/ml bovine trypsin type XII-S (Sigma) for 5 min, washed in PBS and incubated in 0·25% BSA (Sigma) in PBS to block non-specific binding sites. Sections were incubated with 1:5, 1:10 and 1:20 dilutions of affinity-purified α-BMRF-2 antibody or with 1:5, 1:10 and 1:20 dilutions of ammonium sulphate-precipitated pre-immune rabbit

serum. Both the affinity-purified α-BMRF-2 and the pre-immune sera were used at an initial protein concentration of 8 μg/ml in PBS. After rinsing with PBS, the sections were incubated for 1 h with anti-rabbit IgG conjugated with 10 nm colloidal gold particles (Janssen). The grids were washed five times each with PBS and water, fixed and post-stained as described above; examination was carried out with the transmission electron microscope JEOL-1200 EX at 80 kV.

Results

Identification of the BMRF-2 and BDLF-3 cDNA clones

In an initial screening of 10^5 plaques from the cDNA library, 2% of the cDNA clones hybridized to the EBV probes. The genes detected with the highest frequency were BZLF-1 (ZEBRA), BLLF-1 (gp350/220) and BNLF-1 (LMP-1), followed by BDLF-3, BMRF-1 and BMRF-2.

The complete BMRF-2 cDNA began at 80500 bp and the partial BDLF-3 cDNA began at 130882 bp, which correspond to the B95-8 map positions (Fig. 1*a*, *b*). The nucleotide sequences of the BMRF-2 and BDLF-3 cDNAs were analysed for comparison with the published sequence of B95-8. To determine whether the cDNA nucleotide changes were representative of HL strains, we determined the nucleotide

Table 1. Sequence variation of BMRF-2 and BDLF-3 in HL

ND, Not determined. SC, HL scraping.

bp	B95-8 DNA	HL cDNA	SC 1599	SC 1977	SC 2225	SC 2266	SC 2275	R5-97	HL library	Amino acid change
Sequence variation of BMRF-2										
81654	T	C								None
81708	C			T						None
81717	A	G								None
81780	C	A					A/C			None
81834	A	G								None
82067	C	T								Ala to Ser
Sequence variation of BDLF-3										
130875	T	C	C	C	C	C	C	C	C	None
130857	A	G			G		G/A		G	None
130856	A				T					Thr to Ser
130850	A	C			C		C/A		C	Ile to Leu
130848	T	C	C	C	C	C	C	C	C	None
130819	T	C	C	C	C	C	C	C	C	Phe to Ser*
130794	C	T			T		T		T	None
130774	C							A		Ser to Tyr
130667	T	A	A	A	A	A	A	A	A	Ser to Thr*
130661	A	G			G		G		G	Thr to Ala
130644	T	G			G		G/T		G	None
130494	G	A	ND	ND	ND	ND	ND	ND	ND	None
130482	G	A	ND	ND	ND	ND	ND	ND	ND	None
130461	G	A	ND	ND	ND	ND	ND	ND	ND	None

* Amino acid changes found in all HL samples.

Table 2. *In situ* hybridization for BMRF-2 and BDLF-3 expression

Number of patients	HIV status	Tongue lesion	Positive hybridization	
			BMRF-2	BDLF-3
4	+	HL	4/4	4/4
2	+	HL and *Candida*	2/2	2/2
2	−	Hyperkeratosis	0/4	0/4
4	−	Hyperkeratosis and *Candida*	0/4	0/4
1	−	Fissured tongue	0/1	0/1
5	−	Normal tongue	0/5	0/5

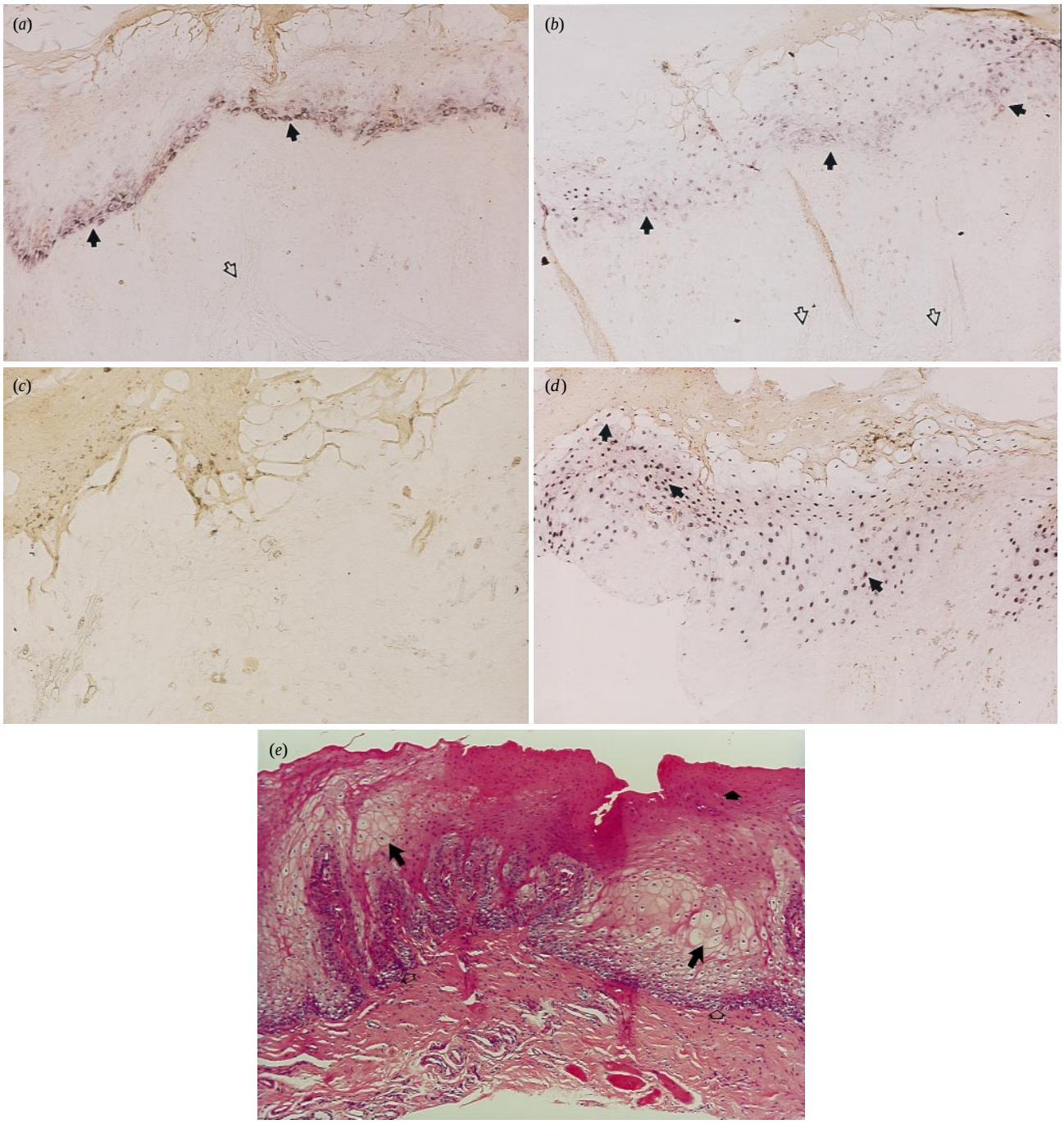

Fig. 2. *In situ* hybridization of paraffin sections of HL tongue biopsies. Assays were performed using fluorescein-labelled antisense or sense riboprobes synthesized from the T7 or T3 promoter and detected with alkaline phosphatase-labelled antibody to fluorescein. Magnification ×25 unless otherwise indicated. (*a*) mRNA *in situ* hybridization to antisense probe for BMRF-2 expression using a BMRF-1–BMRF-2 cDNA linearized with *Sma*I (Fig. 1*a*). (*b*) mRNA *in situ* hybridization to antisense probe for BDLF-3 expression using BDLF-3 cDNA linearized with *Eco*RI. Arrows indicate areas of mRNA expression and open arrows indicate the location of the basal cell layer. (*c*) mRNA *in situ* hybridization to BMRF-1–BMRF-2 cDNA using a sense probe linearized with *Xho*I (magnification ×50). (*d*) EBV DNA *in situ* hybridization using a *Bam*HI W probe linearized with *Eco*RI. Arrows indicate viral DNA expression. (*e*) Haematoxylin and eosin stained section. Open arrows indicate basal layer, large arrows indicate ballooning cells and the small arrow indicates hyperparakeratosis.

sequence of other HL samples. We also sequenced B95-8 DNA from the BMRF-2 and BDLF-3 regions prepared in the same manner as the HL samples as a control to determine that there were no artefacts introduced by PCR. The results are shown in Table 1. The HL-derived BMRF-2 cDNA had five nucleotide changes, with one change at position 82067 resulting in an

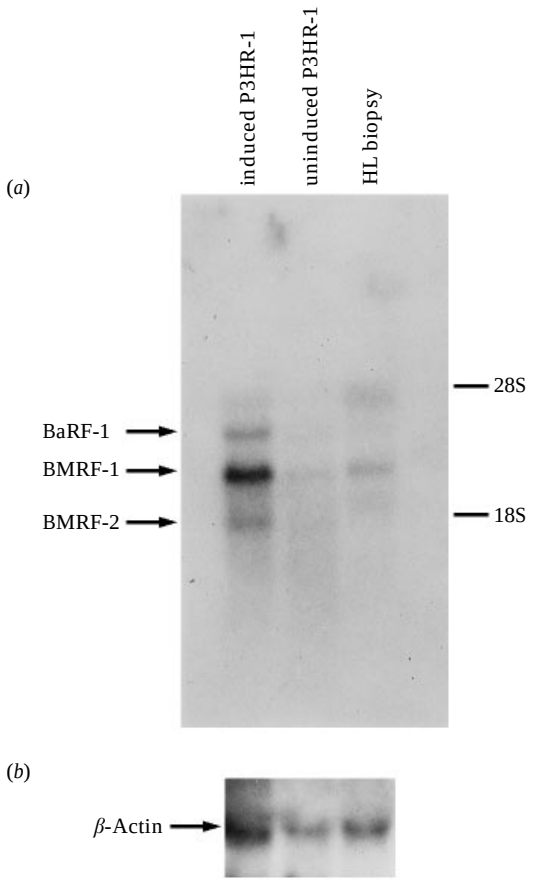

Fig. 3. RNA blot hybridization of transcripts from HL biopsies and P3HR-1 lymphocytes. P3HR-1 cells were induced with PMA (30 μg/ml) for 48 h. Total RNA (10 μg) was loaded in each lane and separated on a 1% agarose–6% formaldehyde gel, transferred to a nylon membrane and hybridized with an [α-^{32}P]dCTP-labelled BMRF-2 DNA fragment (Fig. 3*a*) or [α-^{32}P]dCTP-labelled β-actin clone (Fig. 3*b*). The arrows to the left indicate the transcripts detected by the labelled BMRF-2 DNA probe. The 28S and 18S ribosomal RNA bands are noted to the right. Lane 1, PMA-induced P3HR-1; lane 2, uninduced P3HR-1; and lane 3, HL biopsy pool.

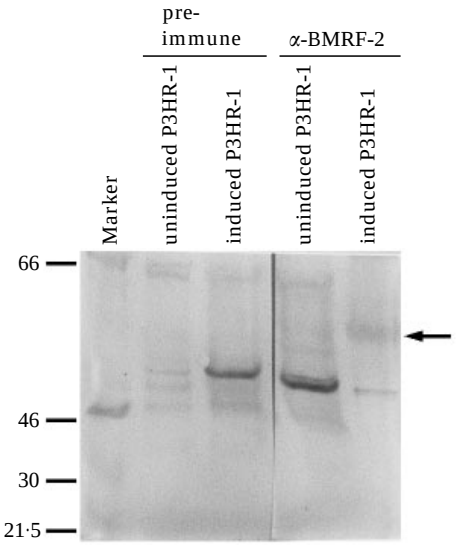

Fig. 4. Western blot analysis of α-BMRF-2 antibody. P3HR-1 cells were either induced with PMA or uninduced and harvested into lysis buffer. A 10 μl sample was mixed with Laemmli buffer and loaded onto a 12% polyacrylamide gel for electrophoresis. Samples were transferred onto PVDF membranes and Western blotting was performed using pre-immune or α-BMRF-2 antiserum at a dilution of 1:200. Lane 1, molecular mass markers; lane 2, uninduced P3HR-1; lane 3, induced P3HR-1; lane 4, uninduced P3HR-1; and lane 5, induced P3HR-1. Pre-immune serum was used in lanes 2 and 3; α-BMRF-2 serum was used in lanes 4 and 5.

alteration in one amino acid. Three additional HL scrapings (SC 1977, SC 2225 and SC 2275) were analysed, but these scrapings had fewer nucleotide changes than the BMRF-2 cDNA and no amino acid changes. BMRF-2 derived from B95-8 did not differ from the published sequence.

Because the BDLF-3 cDNA showed more sequence variation than BMRF-2, we determined the nucleotide sequence of a second sample from the library, five additional HL scrapings (SC 1599, SC 1977, SC 2225, SC 2266 and SC 2275) and one LCL derived from an HL strain (R5-97). The results are shown in Table 1. All HL strains and the LCL had the same nucleotide changes at four positions, 130875, 130848, 130819 and 130667. Two of these changes (positions 130819 and 130667) encoded amino acid substitutions that were conserved in all HL strains and the LCL. Single nucleotide changes were noted in SC 2225 and R5-97 which resulted in amino acid substitutions, but were not found in the other HL strains. SC 2275 was previously shown to have both EBNA-1 and -2, and thus contains at least two strains of EBV (Palefsky *et al.*, 1996). Two different preparations of the SC 2275 template consistently gave two bands of equal intensity at the nucleotide positions noted (Table 1). BDLF-3 derived from B95-8 did not differ from the published sequence.

Expression of BMRF-2 and BDLF-3 in HL biopsies

To confirm the expression and localization of the products of these two genes in HL, we performed RNA *in situ* hybridization on paraffin-embedded HL biopsies and immunohistochemistry with antibodies to BMRF-2 and BDLF-3. The results of the *in situ* hybridization studies and patient profiles are summarized in Table 2. High levels of mRNA expression were seen with the antisense probes to BMRF-2 and BDLF-3 in a relatively narrow portion of the epithelium, beginning in the lower prickle cell layers as indicated by arrows (Fig. 2*a*, *b*). The basal cell layer and the upper hyperkeratotic layers did not show hybridization. No cytoplasmic hybridization was observed in the negative control (sense probes for BMRF-2 or BDLF-3, respectively; see Fig. 2*c*). In some samples nuclear hybridization with both probes was observed, possibly due to the presence of single DNA strands of replicating virus. Consistent with this, cytoplasmic hybridization was not detected after RNase treatment (data not shown), but nuclear hybridization was observed. In contrast, DNA *in situ* hybridization indicated the presence of nuclear viral DNA throughout the cell layers with stronger staining above the line of mRNA

cytoplasmic expression and throughout the hyperkeratotic layer (Fig. 2*d*). A section from an HL biopsy stained with haematoxylin and eosin shows the histology of the lesion, including ballooning cells and hyperparakeratosis (Fig. 2*e*).

Due to collinearity of EBV transcripts, we cannot rule out the notion that riboprobes are also detecting the upstream transcripts such as BDLF-1 and BDLF-2 in the case of BDLF-3 and BMRF-1 in the case of BMRF-2. To address this concern, we examined the HL lesion by immunohistochemistry with antibodies to either BDLF-3 or BMRF-2 (see below).

Northern blot analysis of BMRF-2 expression

Northern blot analysis of P3HR-1 cells induced with PMA for 48 h revealed three major bands when probed with a ^{32}P-labelled BMRF-2 probe (Fig. 3*a*). The bands were approximately 3·5, 2·6 and 1·5 kb in size, corresponding to BARF-1, BMRF-1 and BMRF-2, respectively. The transcripts are collinear and may share the same polyadenylation signal downstream of BMRF-2 (Fig. 1*a*). Uninduced P3HR-1 showed low levels of the 2·6 kb transcript (Fig. 3*a*). A pool of three HL biopsy tissues revealed three bands of approximately 3·5, 2·6 and 2·0 kb (Fig. 3*a*). In both epithelial cells and B-lymphocytes the predominant message found was the 2·6 kb transcript. The same samples were probed with β-actin as a control for RNA quality (Fig. 3*b*).

Characterization of the α-BMRF-2 antibody

ELISA analysis of the α-BMRF-2 antiserum and the affinity-purified α-BMRF-2 antiserum indicated that the antibody reacted specifically with the BMRF-2 peptide at a concentration of 1 : 1000 when compared to the pre-immune serum from the same rabbit (data not shown). Western blot analysis showed a diffuse band of approximately 55 kDa in the PMA-induced P3HR-1 cells that was not present in uninduced cells or with pre-immune serum (Fig. 4).

Expression of BMRF-2 and BDLF-3 proteins in HL

Immunohistochemistry of paraffin-embedded HL tissues with α-BMRF-2 serum showed localization of BMRF-2 in the cell nucleus (Fig. 5*a*). Staining was observed primarily in cell layers just above those expressing the largest quantities of mRNA. In contrast to the BMRF-2 protein localization, BDLF-3 expression was observed in the cell cytoplasm and in the perinuclear compartment (Fig. 5*b*). Staining was not observed with pre-immune rabbit serum (Fig. 5*c*). Specific staining was not observed with the α-BMRF-2 serum in a tongue biopsy obtained from a normal individual without HL (Fig. 5*d*) or in the adjacent normal tissue of the same patient (Fig. 5*a*).

Immunoelectron microscopy

Immunogold labelling was performed on HL biopsies with affinity-purified α-BMRF-2 and pre-immune rabbit serum. Antibodies against BMRF-2 demonstrated positive labelling with gold particles in areas of marginated chromatin in the nucleus of the differentiated keratinocyte and did not label the viral particles or the cell membrane (Fig. 6*a*). Normal tongue tissue was not labelled with the α-BMRF-2 antibody (data not shown). The pre-immune rabbit serum was negative in both the HL tissue and the normal tongue tissue (Fig. 6*b*).

Discussion

The EBV genome as it exists in the latent B-lymphocyte has been well studied and much is known about the functions of the limited repertoire of viral genes expressed during the latent cycle of the virus. Less is currently known about EBV genes expressed during the lytic phase of its life cycle. Since EBV replication is a prominent feature of HL, this lesion provides an excellent model for the study of lytic phase viral genes. In these studies, we describe the expression in an HL cDNA library of two genes (BMRF-2 and BDLF-3) whose expression has not previously been described *in vivo*.

mRNA expression of these genes in HL was found to be closely linked to epithelial cell differentiation as indicated by the specific localization of expression in the lower prickle cell layer. In contrast, EBV DNA hybridization was observed predominantly in more mature cell layers, suggesting that expression of these genes precedes high-level virus replication. Expression of the BZLF-1 transactivator protein was shown to be differentiation-associated (Young *et al.*, 1991). These findings are consistent with RNA *in situ* hybridization of other EBV genes, including BZLF-1 and EBNA-2, which were observed only in the prickle cell layer and not in the basal cell layer (Niedobitek *et al.*, 1991; Thomas *et al.*, 1991).

In HL, the 2·0 kb transcript corresponding to BMRF-2 appeared slightly larger than the 1·5 kb BMRF-2 transcript in P3HR-1 cells; this may be due to more extensive polyadenylation. The 1·5 kb band has been previously described in B-cells (Sample *et al.*, 1986). BMRF-2 is collinear with BMRF-1 (see Fig. 1*a*) and BDLF-3 is collinear with BDLF-1 and BDLF-2 (see Fig. 1*b*). Since RNA *in situ* will also detect the upstream transcripts, we performed immunohistochemistry with specific antibody to detect only the BMRF-2 and BDLF-3 proteins.

Upon infection with EBV, marked changes are visible in the nuclear matrix of epithelial cells, which may be sites of transcriptional activity or virus replication and assembly (Rabanus *et al.*, 1991). A variety of cell types infected with other herpesviruses including herpes simplex virus type 1 and cytomegalovirus show significant changes in the nuclear matrix, including margination of chromatin (Puvion-Dutilleul & Besse, 1994; Teot *et al.*, 1993). The BMRF-2 protein, which is conserved among the gammaherpesviruses, was found to be associated with chromatin in the nucleus of the epithelial cells, suggesting a possible role for this gene in virus transcription, replication or virion assembly.

The translated nucleotide sequence of the BMRF-2 ORF revealed an RGD motif in this protein not found within the other gammaherpesvirus BMRF-2 ORF homologues. The

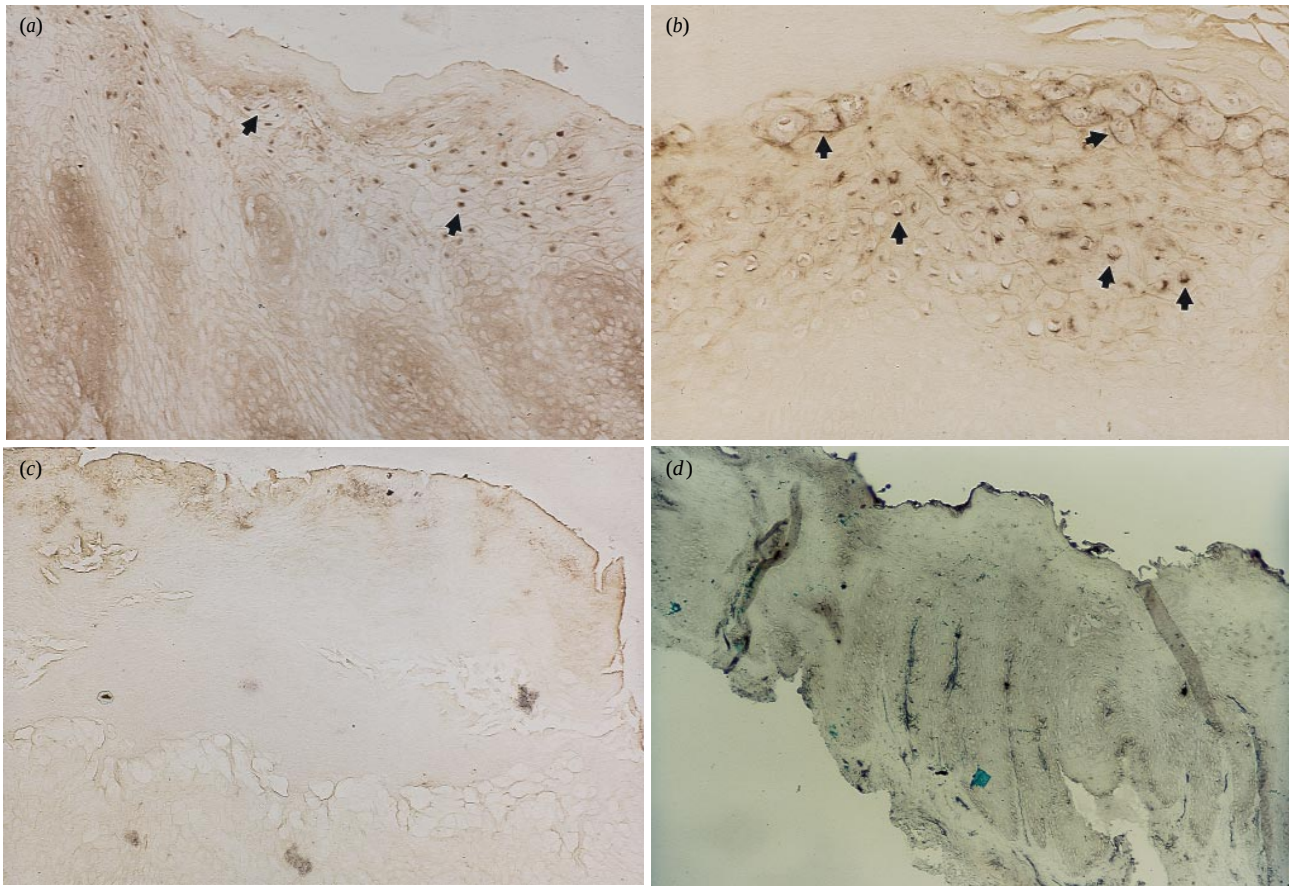

Fig. 5. Immunohistochemistry staining of HL tongue biopsies. Protein expression was detected using the Vectastain kit with alkaline phosphatase detection. Rabbit antiserum against (*a*) BMRF-2 synthetic peptide (magnification × 25) and (*b*) BDLF-3 (magnification × 50) was used. Arrows point to representative cells expressing protein. No protein expression was detected with the pre-immune rabbit antiserum (*c*; magnification × 50) or in normal tongue biopsy stained with α-BMRF-2 serum (*d*; magnification × 25).

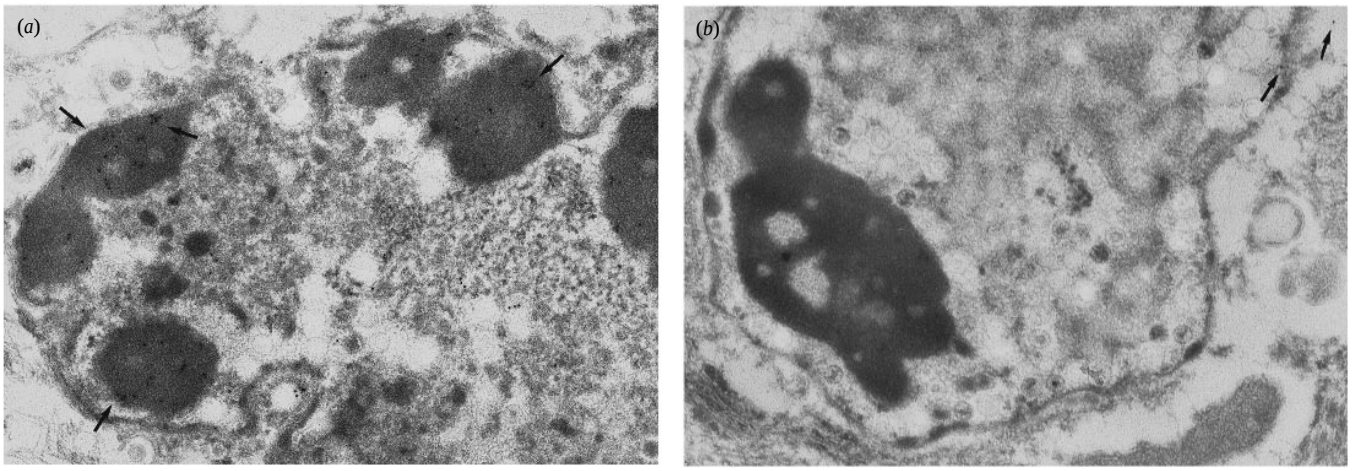

Fig. 6. Transmission electron micrographs of immunogold labelling of keratinocytes from an HL biopsy. (*a*) α-BMRF-2 labelling of areas of dense chromatin in the cell nucleus (arrows). (*b*) Pre-immune rabbit serum showing few non-specific binding of gold particles (arrows). Original magnification × 25000.

RGD motif was found in all HL samples and B95-8. RGD motifs are important for cellular attachment via integrins and cell penetration by some viruses such as adenovirus (Cuzange *et al*., 1994; Goldman & Wilson, 1995; Mathias *et al*., 1994) and coxsackie virus (Liebermann *et al*., 1991; Roivainen *et al*., 1991). RGD motifs have also been implicated in signal transduction through binding to integrin and activation of MAP kinases (Chen *et al*., 1994). These data suggest the possibility that BMRF-2 may also play a role in cell entry by EBV, and consistent with this, BMRF-2 has been reported in the cytoplasmic membrane in B-lymphocytes (Modrow *et al*., 1992). BMRF-2 has also been reported in the nucleus (Modrow *et al*., 1992), which is consistent with our findings, and it is of interest that gp110, a late EBV glycoprotein essential for virus entry or assembly (Herrold *et al*., 1996), is also found in the nucleus (Gong & Kieff, 1990).

In contrast to the BMRF-2 protein, the pattern of expression of BDLF-3 was consistent with that of a glycoprotein trafficking from the endoplasmic reticulum and Golgi compartments to the cell membrane. Recent reports showed that BDLF-3 is incorporated into the viral particle (Kurilla *et al*., 1995). Sequence analysis of the BDLF-3 cDNA showed multiple nucleotide changes. Two amino acid changes were found in seven different HL samples relative to B95-8. These changes may represent substitutions that confer a selective advantage to EBV in the epithelium. Alternatively, the high levels of replication of EBV found in HL may account for increased nucleotide changes. Studies are in progress to further elucidate the functions of BMRF-2 and BDLF-3 in the pathogenesis of EBV infection of the epithelium.

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