

Expression of Epstein-Barr virus (EBV) transcripts encoding homologues to important human proteins in diverse EBV associated diseases

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Abstract

Aims—To examine the expression of Epstein-Barr virus (EBV) transcripts encoding proteins homologous to important human proteins in diverse EBV associated diseases. The proteins were: BHRF1 (homologous to Bcl-2), BDLF2 (homologous to cyclin B1), BARF1 (homologous to intercellular cell adhesion molecule 1 (ICAM-1)), and BCRF1 (viral IL-10 (vIL-10), homologous to human IL-10 (hIL-10)).

Methods—Six cases of oral hairy leukoplakia, seven of Hodgkin's disease, eight of T cell non-Hodgkin's lymphoma, and nine of nasopharyngeal carcinoma were examined at the mRNA level using either the reverse transcriptase polymerase chain reaction (RT-PCR) or nucleic acid sequence based amplification (NASBA). Different primer sets allowed the differentiation by RT-PCR of the latent (Cp/Wp driven) and lytic (Hp driven) transcripts of BHRF1. A specific NASBA reaction was developed for the detection of vIL-10 and BDLF2 transcripts and this was tested initially on cell lines and later on clinical samples.

Results—vIL-10 and BDLF2 were expressed almost exclusively in oral hairy leukoplakia, whereas BARF1 transcripts were present in all cases of nasopharyngeal carcinoma, with weak expression in one oral hairy leukoplakia and isolated cases of lymphoid malignancy. Both BHRF1 transcripts were detected across the range of tissues tested, but strong expression of lytic BHRF1 transcripts was seen only in oral hairy leukoplakia.

Conclusions—vIL-10 and BDLF2 transcripts are expressed during productive EBV infection and are unlikely to be important in the pathogenesis of EBV associated malignancies. BARF1 appears to be expressed preferentially during viral

latency and is more closely associated with malignant rather than benign epithelial proliferations. The alternative transcripts derived from the BHRF1 open reading frame may have very different roles during latent or productive infection.

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Keywords: Epstein-Barr virus associated diseases; interleukin 10; bcl-2; cyclin B1; intercellular cell adhesion molecule 1

Epstein-Barr virus (EBV) is a ubiquitous γ -herpesvirus that infects ~ 95% of the human population. In vivo, both B lymphocytes and stratified squamous epithelium are target tissues, the former being a site of latent (non-productive) infection and the latter allowing virus replication to occur.¹ EBV has been associated with lymphoid malignancies (such as Burkitt's lymphoma, Hodgkin's disease, and lymphoid proliferations in the immunosuppressed) and epithelial malignancies (such as nasopharyngeal carcinoma, gastric adenocarcinomas, and oral hairy leukoplakia).¹ Of the ~ 100 proteins encoded by EBV,² four have been identified that are partially homologous to important human proteins involved in apoptosis inhibition, cell cycle regulation, and immunosuppression (table 1).³⁻⁹ The gene encoding the first protein of interest, BHRF1, shows partial sequence homology to the human bcl-2 proto-oncogene and both have been shown to protect human B lymphocytes from apoptosis.⁷ The second gene, BARF1, encodes a 33 kDa protein that is ~ 23.5% homologous to intercellular adhesion molecule 1 (ICAM-1), with which it shares a transmembrane sequence.⁹ Moreover, the BARF1 protein shares homology with the human colony stimulating factor 1 receptor (hCSF-1R), and can neutralise its proliferative effects.⁸ The third gene of interest is BCRF1, which encodes a protein that is homologous to human interleukin 10 (hIL-10).³ This protein is termed viral IL-10 (vIL-10) and it shares most of the immunosuppressive activities of hIL-10.⁴ Based on amino acid sequence alignment, homology between the BDLF2 protein (encoded by the fourth gene of interest) and human cyclin B has been suggested (B Griffin, personal communication, June 1998), although functional homology between these two proteins has yet to be confirmed.

Transcription of the abovementioned EBV genes can be investigated using sensitive

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Table 1 Overview of homology of EBV genes

Viral gene	Human homologue	Homology at the amino acid level	Functional homology	Reference
BCRF1	Interleukin 10	84%	Yes	3, 4, 5, 6
BDLF2	Cyclin B1	28% in 68 amino acid domain	Unknown	B Griffin (personal communication)
BHRF1	bcl-2 proto-oncogene	25% in 150 amino acid domain	Yes	7
BARF1	hCSF-1 receptor	38% in 13 amino acid domain	Yes	8
	ICAM-1 (CD54)	23.5%	Unknown	9

hCSF-1, human colony stimulating factor 1; ICAM-1, intercellular adhesion molecule 1.

Table 2 Clinical material and summary of results

Diagnosis	Body site	vIL-10	BDLF2	BHRF1				
				H2	Y2	BARF1	BZLF1	BARF0
<i>Hodgkin's disease</i>								
1	ns	Cervical ln	-	-	-	-	+/-	+
2	ns	Cervical ln	-	-	-	-	-	+
3	ns	Unknown	-	-	+/-	-	+/-	+
4	ns	Unknown	-	-	+/-	+	+	+
5	ns	Cervical ln	-	-	+/-	-	-	+
6	ns	Cervical ln	-	-	-	-	+/-	+
7	ns	Unknown	-	-	-	-	+	+
<i>T cell non-Hodgkin's lymphoma</i>								
1	pl. small	Nose	+/-	-	-	-	-	+
2	pl. m/l	Nose	-	-	+/-	-	-	+/-
3	ALCL	Groin ln	-	-	-	-	-	+
4	pl. small	Nose	-	-	-	-	+/-	+
5	pl. m/l	Intestine	-	-	+/-	-	-	+/-
6	pl. m/l	Nose	-	-	+/-	+/-	+/-	+
7	pl. small	Axillary ln	-	-	+/-	-	-	+
8	pl. m/l	Nose	-	-	-	-	-	+
<i>Nasopharyngeal carcinoma</i>								
1	Undiff.	Nasopharynx	-	-	-	+	+/-	-
2	Undiff.	Nasopharynx	-	-	-	-	+/-	-
3	Undiff.	Nasopharynx	-	-	-	-	+/-	-
4	Undiff.	Nasopharynx	-	-	-	-	+/-	-
5	Undiff.	Nasopharynx	-	-	-	+	+	-
6	Undiff.	Nasopharynx	-	-	+/-	+	+	-
7	Undiff.	Nasopharynx	-	-	+/-	-	+	-
8	Undiff.	Nasopharynx	-	-	-	+/-	+	-
9	Undiff.	Nasopharynx	-	-	-	-	+/-	-
<i>Oral hairy leukoplakia</i>								
1	HIV assoc.	Tongue	++	+	++	++	-	ND
2	HIV assoc.	Tongue	+/-	+	++	-	+/-	++
3	HIV assoc.	Tongue	+	+/-	++	-	-	ND
4	HIV assoc.	Tongue	+	+	++	-	-	ND
5	HIV assoc.	Tongue	+/-	+	++	+	-	ND
6	HIV assoc.	Tongue	+	+	++	+	-	++

vIL-10, viral interleukin 10; ns, nodular sclerosis; pl., pleomorphic; m/l, medium/large; ALCL, anaplastic large cell lymphoma; ln, lymph node; undiff., undifferentiated; ND, not determined; HIV assoc., HIV associated; +/-, weak signal (clearly visible only after overnight exposure); +, clear signal after four hours' exposure; ++, strong signal, comparable to or stronger than positive controls.

reverse transcriptase polymerase chain reaction (RT-PCR) assays, which are specific for particular alternative transcripts through the use of different primer sets.¹⁰⁻¹² In addition, an alternative RNA specific amplification assay (nucleic acid sequence based amplification (NASBA))¹⁵ has been shown to amplify EBV mRNA specifically, even in the presence of DNA and without knowledge of splice sites.¹⁴ This technique is especially useful for the detection of BARF1, BCRF1, and BDLF2 transcripts, which are all unspliced.

To gain insight into the role of EBV in the pathogenesis of its associated diseases, and to see whether differences between these diseases exist, we chose to study the expression of these four genes at the mRNA level in a variety of conditions. The tissues used in our study were chosen to represent examples of lymphoid malignancies (Hodgkin's disease and T cell non-Hodgkin's lymphoma), and benign (oral hairy leukoplakia) and malignant (nasopharyngeal carcinoma) epithelial proliferations associated with EBV. Moreover, these diseases represent different phases of EBV infection: Hodgkin's disease, T cell non-Hodgkin's lymphoma, and nasopharyngeal carcinoma all contain predominantly latently infected neoplastic cells, showing a latency type II pattern of gene expression,¹⁵⁻¹⁷ with occasional cells showing expression of immediate early proteins like BZLF1¹⁸; whereas in oral hairy leukoplakia, lytic replication of the virus has been demonstrated.

Materials and methods

CELL LINES

Ramos is a cell line derived from an EBV negative Burkitt's lymphoma, Raji is derived from an EBV positive Burkitt's lymphoma, and JY is generated from EBV transformed peripheral blood lymphocytes. Where stimulated cells were used, stimulation to productive EBV infection was achieved using tetra phorbol acetate (TPA) (final concentration 30 ng/ml) and sodium butyrate (final concentration 5 mM). The optimal stimulation time was determined to be 72 hours by subjecting equal quantities of cell derived RNA to NASBA amplification (see below) after stimulation for 24, 48, and 72 hours (results not shown). EBV negative Louckes cells transfected with a BARF1 expression construct¹⁹ were kindly provided by T Ooka (Laboratoire de Virologie Moleculaire, CNRS, Lyon, France).

CLINICAL MATERIAL

Table 2 gives details of the clinical tissues used. Snap frozen material was obtained from the department of pathology, University Hospital Vrije Universiteit, Amsterdam, The Netherlands, except for the nasopharyngeal carcinoma tissue, which was obtained from the department of pathology, Queen Mary Hospital, Hong Kong. The following tissues were used: EBV positive Hodgkin's disease (n = 7), oral hairy leukoplakia (n = 6), nasopharyngeal carcinoma (n = 9), and T cell non-Hodgkin's lymphoma (n = 8). For Hodgkin's disease,

Table 3 Sequences of oligonucleotide primers and probes

Target	Oligo	Sequence	EBV genomic coordinates
vIL-10	1.1	tggagcgaaggttagtggtc	9676-9695
	1.2	tacctggcactgagtggtgag	9720-9739
	2.1	T7*-agaattggatcatttctgacaggg	9921-9898
	2.2	T7-agacatgtctcttggcttcagggtc	9983-9957
	Probe	cagaccaatgtgacaattttcccaaatgt	9745-9774
BDLF2	1.1	ctacctccacgacttcacc	132229-132210
	1.2	aagtctttataaggctccggc	132277-132256
	2.1	T7*-aggccatggtgtcatccatc	132020-132039
	2.2	T7-agagagagagtaggtccgagg	132669-132689
	Probe	ccaatggggaggagagacaaagaccaata	132139-132110
BHRF1†	Antisense	ttctctctgctgactgccca	54480-54461
	Sense	gtcaaggtttcgtctgtgtg	53830-53849
Hp driven Cp/Wp driven	Sense	tacgcattagagaccatttgagcc	47897-47921
	Probe	atgcacacgactgtcccgtatacac	54435-54411
BARF1	1.1	caggrtcatcgctcagctcc	165509-165528
	2.2	T7-agcatggagatgttggcagc	165798-165780
	Probe	ctggtttaactgggcccaggagaggagca	165644-165673
BZLF1†	Antisense	cggggataatggagtcacatcc	102250-102273
	Sense	cgacacggaaaccacaacagc	102684-102663
	Probe	gcttggccacatctgcttcaacagg	102300-102276
BARF0†	Antisense	agagaccaggctgtaaaaca	157154-157173
	Sense	aaccagcttctctccagag	159194-159175
	Probe	aagacgttggaggcagctg	157359-157378

*T7 RNA polymerase promoter sequence: 5'-aat tct aat acg act cac tat agg g-3'.

†BHRF1, BZLF1, and BARF0 specific oligonucleotides have been published previously.^{15 21}

vIL-10, viral interleukin 10.

nasopharyngeal carcinoma, and T cell non-Hodgkin's lymphoma tissues, the presence of EBV in the tumour cells was confirmed using EBV encoded RNA in situ hybridisation (RISH), as described previously.²⁰

All samples of Hodgkin's disease also showed latent membrane protein 1 (LMP1) positivity in the Reed-Sternberg cells after immunohistochemical staining using the S12 monoclonal antibody (Organon Teknika, Boxtel, The Netherlands) and/or the CS1-4 mixture of monoclonal antibodies (Dako, Glostrup, Denmark).

RNA ISOLATION

For RNA isolation before amplification, 12 frozen sections (5 µm thick) were cut (15 × 10 µm sections for the nasopharyngeal carcinomas), of which the first and last were used for haematoxylin and eosin staining to confirm the presence of tumour, and the remaining 10 sections were retained in an Eppendorf tube for RNA isolation by means of the RNazol (Cinna Biotecx, Houston, Texas, USA) method, according to the manufacturer's instructions. After extraction, the RNA was stored in isopropanol at -80°C. The concentration and purity ratio of the RNA were determined spectrophotometrically and the integrity of the RNA was determined by gel electrophoresis, with visibility of 18S/28S ribosomal RNA being used as an index of good RNA quality.¹²

RT-PCR

Table 3 lists all RT-PCR primers and probes.^{15 21} Both BHRF1 transcripts were evaluated by RT-PCR, as was BZLF1 mRNA expression. Because the BZLF1 protein is an immediate early transcription factor,²² BZLF1 transcripts were used to identify cases that might have switched from latent to lytic infection. RT-PCR was performed according to a published protocol for small biopsies.¹² In brief, 1 µg total RNA was incubated for five minutes at 65°C in the presence of antisense

primers in a total volume of 5 µl. Reverse transcription was then carried out at 42°C for two hours in 20 µl volumes containing 50 mM Tris/HCl, pH 8.3, 60 mM KCl, 3 mM MgCl₂, dNTPs (1 mM each), 10 mM dithiothreitol (DTT), 1 U avian myeloblastosis virus reverse transcriptase (AMV-RT) (Promega, Madison, Wisconsin, USA), and 10 U RNase inhibitor (Promega). As an additional check for EBV specific mRNA detection, most of the tissues used in our study were subjected to RT-PCR for BARF0 mRNA, known to be expressed in all types of latency.²³

Forty cycles of PCR were carried out (one minute denaturation at 94°C, one minute primer annealing at 55°C, and one minute primer elongation at 72°C) in a final volume of 50 µl containing 100 mM Tris/HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 µM of each dNTP, 25 pmol each of the sense and antisense primers, and 1 U Taq polymerase (AmpliTag; Perkin-Elmer, Norwalk, Connecticut, USA). PCR products were separated by gel electrophoresis, transferred to nylon filters (Qiabran; Qiagen, Santa Clarita, California, USA) by alkaline Southern blotting, and hybridised to specific γ^{32} P-ATP end labelled oligonucleotide probes.

NASBA REACTIONS

NASBA is an isothermal amplification method that specifically amplifies RNA templates independent of the presence of DNA.^{13 24} A BARF1 specific NASBA has been developed recently.¹⁴ For vIL-10 and BDLF2, two sense and two antisense primers were selected, the latter bearing a T7 RNA polymerase recognition sequence at the 5' end. vIL-10 primers were chosen to include the area homologous to hIL-10, but in such a way that they did not cross-hybridise to the human transcript. Table 3 gives the sequences of NASBA probes and primers. Optimal primer combinations were determined using 10 ng and 100 ng total RNA derived from stimulated and unstimulated JY and Raji cell lines (1.1/2.2 for vIL-10 and 1.1/2.1 for BDLF2; table 3), and these combinations alone were studied further.

Because KCl is known to influence the efficacy of primer annealing, a concentration series varying from 30 to 90 mM KCl was tested, each NASBA reaction being identical in every other respect. The optimal KCl concentration for the BDLF2 NASBA was determined to be 40 mM and that for vIL-10 60 mM.

We also anticipated difficulties with amplification because of folding of the RNA, especially with BDLF2, based on its tightly folded two dimensional structure and its G:C rich sequences as determined by RNA fold computer analysis. Attempts to overcome this by prolongation of the 65°C heating step resulted in complete loss of signal (data not shown). However, the osmolyte N,N,N-trimethylglycine (betaine) was shown to improve amplification of G:C rich DNA sequences.²⁵ For this reason, and because it can confer protection to enzymes against a variety of denaturing stresses, we included this reagent (Sigma-

Aldrich, Milwaukee, Wisconsin, USA) in our NASBA reactions. Using a betaine concentration series varying from 0 to 300 mM (final NASBA concentration), the optimum concentration of betaine was determined to be 225 mM for BDLF2 and 300 mM for vIL-10.

NASBA reactions for BARF1, vIL-10, and BDLF2 were carried out as described previously.^{13 14 26} After centrifugation of the isopropanol RNA precipitates, they were washed with 70% ethanol and dissolved in 5 µl of RNase free water. Primers were added to the buffer mix (200 mM Tris, pH 8.5, 60 mM MgCl₂, 20 mM DTT, 5 mM of each dNTP, 10 mM riboATP (rATP), rUTP, rCTP, 7.5 mM rGTP, and 2.5 mM inosine trisphosphate (ITP)) and appropriate volumes (see above) of betaine and KCl were added to a volume of 15 µl. The samples were heated to 65°C for five minutes and allowed to cool to 41°C before the addition of 5 µl of enzyme mix (6.5 mM sorbitol, 3.4 µg bovine serum albumin (BSA), 0.08 U RNase H, 32.0 U T7 RNA polymerase, 6.44 U AMV-RT (all from Pharmacia, Piscataway, New Jersey, USA)). The reaction was allowed to proceed for 90 minutes at 41°C. NASBA products were evaluated by gel electrophoresis under RNase free conditions using 1.5% agarose in Tris borate EDTA electrophoresis buffer (TBE), after which they were transferred by capillary blotting in 10× saline sodium citrate (SSC) to nylon filters (Qiabran; Qiagen) and hybridised to specific γ^{32} P-ATP end labelled oligonucleotide probes.

Results

EVALUATION OF CLINICAL MATERIAL

To avoid false negative results caused by poor quality of the input material, several good quality controls were performed before the clinical samples were used in the NASBA. To check the integrity and purity of the isolated RNA, spectrophotometric analyses of the purity ratios and electrophoresis analysis for the presence of ribosomal RNA (rRNA) bands were performed. As an additional check for the integrity of the RNA, and to exclude the presence of factors that might inhibit subsequent enzymatic reactions, RT-PCR for the U1A "housekeeping" gene²⁷ was performed. Moreover, BARF0 RT-PCR was performed to determine whether amplifiable EBV mRNA was present in the samples because BARF0 is expressed in all EBV positive disorders.²³ All samples included in our study showed clearly visible rRNA bands at the gel level and gave clear signals after U1A RT-PCR. All cases of Hodgkin's disease, T cell non-Hodgkin's lymphoma, and nasopharyngeal carcinoma, in addition to the two cases of oral hairy leukoplakia tested, showed clear positive signals for BARF0. Both U1A and BARF0 RT-PCR products were visible at the gel level; hybridisation with radioactive oligonucleotide probes revealed no additional positive samples.

Furthermore, because it is known to be an immediate early transcription factor, BZLF1 expression was used as an indicator of cases which may have undergone the switch from latent to productive viral infection. After

hybridisation of the blotted RT-PCR products to radioactive oligonucleotide probes, a clear signal was seen in three Hodgkin's disease samples and one T cell non-Hodgkin's lymphoma; a weak signal could be detected in one Hodgkin's disease sample and three T cell non-Hodgkin's lymphoma samples. However, synthesis of the BZLF1 protein could not be confirmed by immunohistochemistry, either in neoplastic cells or in infiltrating lymphocytes using the BZ1 monoclonal antibody (Dako). Both samples of oral hairy leukoplakia tested showed a strong BZLF1 RT-PCR signal, whereas none of the nasopharyngeal carcinoma samples did.

GENE EXPRESSION IN CLINICAL TISSUES

RT-PCR

BHRF-1: this Bcl-2 homologue can be encoded by a latent (containing exon Y2) and a lytic transcript, the former driven by the Cp/Wp promoter and the latter by its own Hp promoter.²⁸ By using different primer sets (table 3) we were able to discriminate between the two possible transcripts. Strong signals for the Hp transcript were seen in all six oral hairy leukoplakia samples at the gel level, but after hybridisation of the blotted RT-PCR products to radioactive oligonucleotide probes, weak signals could be seen across the range of other tissues tested: three of the seven Hodgkin's disease samples, two of the nine nasopharyngeal carcinoma samples, and four of the eight T cell non-Hodgkin's lymphoma biopsies. Clear signals for the latent (Y2) transcript were detected in three cases each of oral hairy leukoplakia and nasopharyngeal carcinoma and one case of Hodgkin's disease; after hybridisation of the RT-PCR products to a radioactive oligonucleotide probe, a single T cell non-Hodgkin's lymphoma sample and also one case of nasopharyngeal carcinoma showed weak Y2 signals.

NASBA

It was difficult to interpret NASBA products at the gel level. Usually, signals of approximately the expected size were seen in all samples tested including negative controls. However, after blotting of the NASBA products and hybridisation to radioactively labelled oligonucleotide probes, the specificity was enhanced considerably. Therefore, NASBA results discussed below are results after radioactive hybridisation.

BARF1: all nine nasopharyngeal carcinoma samples were positive for BARF1 RNA, four clearly positive and the remainder showing

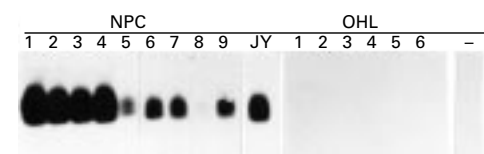


Figure 1 BARF1 nucleic acid sequence based amplification (NASBA) (after electrophoresis, blotting, and radioactive hybridisation) of nasopharyngeal carcinoma (NPC) and oral hairy leukoplakia (OHL) samples using 100 ng RNA/reaction. Positive control (JY), 100 ng RNA from tetra phorbol acetate/butyrate stimulated JY cells; dash, negative (water) control.

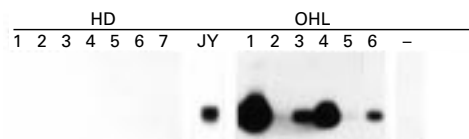


Figure 2 Viral interleukin 10 (vIL-10) nucleic acid sequence based amplification (NASBA) (after electrophoresis, blotting, and radioactive hybridisation) of Hodgkin's disease (HD) and oral hairy leukoplakia (OHL) samples using 100 ng RNA/reaction. Positive control (JY), 100 ng RNA from tetra phorbol acetate/butyrate stimulated JY cells; dash, negative (water) control.

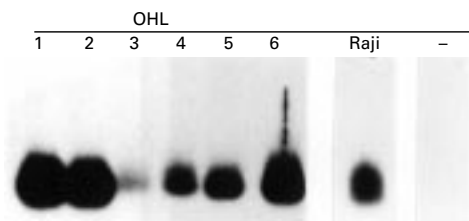


Figure 3 BDLF2 nucleic acid sequence based amplification (NASBA) analysis of oral hairy leukoplakia (OHL) samples (after electrophoresis, blotting, and radioactive hybridisation) using 100 ng RNA/reaction. Positive control (Raji), 100 ng RNA from tetra phorbol acetate/butyrate stimulated Raji cells; dash, negative (water) control.

weak signals (fig 1). Weak positivity was also seen in two of the eight T cell non-Hodgkin's lymphoma samples and three of the seven cases of Hodgkin's disease.

vIL-10: a positive signal for vIL-10 was obtained in all six oral hairy leukoplakia samples, many of which showed clear, strong signals. A very weak signal was seen in one only of the eight T cell non-Hodgkin's lymphoma samples tested. No Hodgkin's disease or nasopharyngeal carcinoma samples showed detectable vIL-10 transcription (fig 2).

BDLF2: all six oral hairy leukoplakia samples were clearly positive for BDLF2 RNA (fig 3) but a positive signal was not detected in the T cell non-Hodgkin's lymphoma, nasopharyngeal carcinoma, or Hodgkin's disease samples tested.

Discussion

In our study we have examined the expression of four EBV genes homologous to important human proteins in diverse EBV associated diseases. We have found differences in the expression of these genes between epithelial and lymphoid diseases, between disorders representing latent and lytic EBV infections, and between malignant and benign disorders. These three aspects will be discussed separately. It is important to mention that detection of mRNA transcripts is not always related to detectable protein synthesis. For example, this has been noted for BHRF1 in post-transplant lymphoproliferative disorders.²⁹ However, such discrepancies might also be caused by low level protein synthesis and/or insensitivity of the antibodies used for protein detection. Eventually, all transcription studies need confirmation both morphologically and at the protein level.

A number of important differences in gene expression could be seen when the lymphoid

malignancies (Hodgkin's disease and T cell non-Hodgkin's lymphoma) were compared with nasopharyngeal carcinoma or oral hairy leukoplakia, both representative of EBV associated epithelial proliferations. First, although weak BRF1 expression could be found in occasional Hodgkin's disease and T cell non-Hodgkin's lymphoma samples, it was present in all cases of nasopharyngeal carcinoma. BRF1 is known to be expressed in epithelial EBV associated malignancies,^{14 30} and this is supported by the virtual absence of BRF1 mRNA in benign epithelial proliferations (oral hairy leukoplakia).

Typically, nasopharyngeal carcinoma shows a dense infiltrate of reactive T lymphocytes and yet the tumour cells are not eradicated effectively. BRF1 expression in the neoplastic cells might be involved in this phenomenon. BRF1 might be able to induce local immune suppression in many different ways, being an antagonist of the hCSF-1R, which is involved in various immune response mechanisms.⁸ In addition, because BRF1 is partially homologous to ICAM-1,⁹ it might occupy ICAM-1 receptors on T lymphocytes without leading to the proper stimuli necessary for T cell activation.

By contrast, weak BRF1 positivity in two T cell non-Hodgkin's lymphoma samples and three cases of Hodgkin's disease could be explained by the presence of infiltrating reactive B cells, although expression of BRF1 in lymphoid cells has been shown to be low.^{14 30} In the absence of a morphological technique (such as immunohistochemistry or in situ hybridisation) it is not possible to be certain whether expression occurs in tumour cells or the reactive infiltrate.

An even more striking difference was seen in the case of BDLF2: transcripts were clearly detected in all six oral hairy leukoplakia samples but in no lymphoid tumour samples. Furthermore, it is interesting to note that the strongest signals for BHRF1 (especially the Hp driven lytic transcript) were also seen in oral hairy leukoplakia, and this gene has been shown to interfere with epithelial differentiation.³¹

Except for weak expression in one case of T cell non-Hodgkin's lymphoma, vIL-10 was not detected in any of the lymphoid malignancies, but was present in every oral hairy leukoplakia sample. It is tempting to speculate that the remarkable lack of inflammatory infiltrate seen in all cases of oral hairy leukoplakia is (at least in part) a result of modification of cell mediated immunity by vIL-10, and this is consistent with the observation that HIV positive men with normal CD4 counts,³² and also those rare cases of oral hairy leukoplakia occurring in immune competent individuals,³³ also show a paucity of inflammatory cells. By contrast, vIL-10 mRNA was not detected in the nine nasopharyngeal carcinoma samples. This is an exciting observation because nasopharyngeal carcinoma classically shows a prominent surrounding reactive lymphocytic infiltrate, and the immunosuppressive functions of vIL-10 would be inconsistent with this.

Because EBV is known to have both a latent and a lytic (productive) life cycle, we used the BZLF1 transcript as an indicator of cases that might have switched from latent to productive infection. Although it is known to be an immediate early transcription factor, the relation between BZLF1 transcription and productive infection is not always straightforward, because two cases of nasopharyngeal carcinoma and one case of Hodgkin's disease showed signals for the lytic (H2) BHRF1 transcript and one case of T cell non-Hodgkin's lymphoma showed signals for vIL-10 without detectable BZLF1. Although known to be involved in the initiation of replicative EBV infection,³⁴ these results are consistent with the notion that it is not essential for maintenance of the lytic state.

In spite of these limitations, oral hairy leukoplakia (a classic example of productive EBV infection *in vivo*³⁵) showed the strongest signals for the lytic (H2) BHRF1 transcript, vIL-10, and BDLF2. vIL-10 is already widely considered to be a late gene product³⁶ and our results are entirely consistent with this. Almost nothing is known about BDLF2, but its detection in oral hairy leukoplakia and not in the other diseases characterised by latent infection lends support to our belief that BDLF2 is also a late gene expressed during the lytic cycle.

Although nasopharyngeal carcinoma and oral hairy leukoplakia are both epithelial EBV associated diseases, BARF1 expression could be seen in all nasopharyngeal carcinoma samples tested but only weakly in one case of oral hairy leukoplakia. There is evidence that BARF1 is expressed before the onset of viral replication,⁹ and because nasopharyngeal carcinoma is characterised by latent viral infection, whereas oral hairy leukoplakia represents replicative infection, our results support the notion that BARF1 is expressed predominantly during latency.

The oncogenic and transforming capacity of BARF1 has been discussed previously,^{19, 37} as has its preferential expression during viral latency. Its consistent expression in all cases of nasopharyngeal carcinoma, but weak expression in only a single benign oral hairy leukoplakia sample, underlines its close association with malignant rather than benign epithelial proliferations.

The anti-apoptosis and tumour promoting functions of BHRF1 are also well known,⁷ and its expression across the range of lymphoid and epithelial diseases is likely to be important in tumorigenesis. The latent transcript of the BHRF1 open reading frame could be detected in three cases of nasopharyngeal carcinoma and one Hodgkin's disease sample, in addition to three oral hairy leukoplakia samples. Homologous to the bcl-2 proto-oncogene, a previous study²¹ reports the presence of the latent transcript in seven of nine EBV associated B cell lymphomas. By contrast, clear and strong signals for the lytic (H2) BHRF1 transcript were seen only in benign oral hairy leukoplakia, scattered weak signals being present in the other diseases. However, the presence of these transcripts or the encoded protein in neoplastic cells should be confirmed morphologically.

Both our results and previous findings raise the possibility that the two transcripts might have different functions: the importance of the latent transcript may lie in its expansion of cell survival, allowing oncogenic "hits" to accumulate; whereas the lytic transcript, through the inhibition of apoptosis, might allow the generation of a maximal number of virions.

In conclusion, the almost exclusive expression of both vIL-10 and BDLF2 in oral hairy leukoplakia but not in the remaining EBV associated diseases is likely to be attributable to their preferential expression during productive infection. However, this pattern of expression might also suggest that vIL-10 and BDLF2 are unlikely to be important in the maintenance of EBV associated malignancies, but rather may fulfil a role in protecting the cell from immune elimination and programmed cell death by host derived cell cycle regulatory factors. Furthermore, the almost exclusive expression of BARF1 in nasopharyngeal carcinomas and not in benign epithelial (oral hairy leukoplakia) or lymphoid (Hodgkin's disease and T cell non-Hodgkin's lymphoma) disorders suggests an important role for this protein in malignant transformation of epithelial cells.

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