Epstein–Barr virus infection in oral hairy leukoplakia: virus replication in the absence of a detectable latent phase

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Epstein–Barr virus (EBV) infects both B lymphocytes and oropharyngeal epithelium, and it has been argued that the true reservoir of virus persistence in vivo is the self-renewing basal epithelial compartment. The identification of oral hairy leukoplakia (HL) of AIDS patients as a clinically apparent focus of EBV replication in lingual epithelium therefore provides a means of studying the EBV–epithelial cell interaction in situ. Replicative EBV DNA and productive cycle antigens are restricted to the upper, more differentiated epithelial layers in HL, and here we have applied highly sensitive in situ hybridization and immunohistological methods to examine the lower basal/suprabasal layers for evidence of latent EBV infection. We could not detect EBV DNA in these layers using an in situ DNA hybridization protocol which, on reference B cell lines, detected 1 viral genome/cell. Likewise, using sensitive in situ RNA hybridization for both the small non-polyadenylated EBER RNAs (abundant transcripts seen in all known forms of EBV latency) and the latent membrane protein (LMP) mRNA (the most abundant viral mRNA in B lymphoblastoid cell lines), the basal/suprabasal cells in HL were consistently negative; immunohistological staining with specific monoclonal antibodies also gave no evidence of latently infected LMP-positive cells. When the biopsy extracts were analysed by immunoblotting with selected human antisera, in addition to abundant productive cycle antigens, a band of constant size (66K) was observed which also reacted with immunopurified antibodies monospecific for one of the latency-associated nuclear antigens, EBNA 1; the cellular origin of this EBNA 1 could not be ascertained, but it is possible that in HL the protein is expressed during the productive cycle. The absence of demonstrable EBV latency in the basal/suprabasal cells of HL suggests that this is purely a virus replicative lesion which is sustained by continual re-infection of the maturing epithelium, not by the maturation of latently infected cells from the basal compartment.

Introduction

Epstein–Barr virus (EBV) is a human herpesvirus infecting more than 90% of the population world-wide and persisting for life in the infected host. The best characterized target cell for EBV infection both in vivo and in vitro is the B lymphocyte; thus, when peripheral blood lymphocytes from virus carriers are placed in culture, EBV-immortalized B lymphoblastoid cell lines (LCLs) can be established by spontaneous outgrowth (Pope, 1967; Nilsson et al., 1971). Similarly, immortalization of B lymphocytes from previously uninfected individuals can be achieved by experimental EBV infection in vitro. Although a small subset of cells in LCLs may sustain EBV replication, the vast majority of cells remains non-productively (i.e. latently) infected. These latently infected B cells constitutively express a subset of viral genes encoding six EBV-encoded nuclear antigens (EBNA 1, 2, 3a, 3b, 3c and LP) and two membrane proteins, latent membrane protein (LMP) and terminal protein or LMP 2 (reviewed by Kieff & Liebowitz, 1990). The most abundant virus-encoded RNA species in LCLs, however, are small non-polyadenylated nuclear RNAs (EBERs) which are not translated into proteins (Howe & Steitz, 1986; Sample & Kieff, 1990). Other examples of latent EBV infection are provided by endemic Burkitt’s lymphoma (BL) and undifferentiated nasopharyngeal carcinoma (NPC) (Epstein & Achong, 1979; Klein, 1979), both tumours in which malignant cells are consistently EBV genome-positive. However,
the expression of latent viral antigens in these lesions is restricted to EBNA 1 in BL (Rowe et al., 1987a) and to EBNA 1 usually accompanied by LMP in NPCs (Young et al., 1988; Fahraeus et al. 1988). In addition, the EBERs are transcribed in both BL and NPC, apparently to similar levels as in LCLs (Howe & Steitz, 1986; Rowe et al., 1987a; Gilligan et al., 1990). Thus, the EBERs appear to be consistent markers of latent EBV infection irrespective of the precise form of latency adopted.

Although EBV displays a strong tropism for B lymphocytes in vitro, the virus is clearly also capable of infecting epithelial cells in vivo. This was first apparent from the virus' association with undifferentiated NPC (Klein, 1979), but subsequently EBV has been observed replicating in normal oropharyngeal epithelial cells as these are shed into buccal fluid during the terminal stages of their differentiation (Lemon et al., 1977; Sixbey et al., 1984). This raises the possibility that EBV actually persists in the epithelial compartment and that virus replication at this site is responsible for continual secondary infection of the circulating B lymphocyte pool (Rickinson et al., 1985; Allday & Crawford, 1988). It was envisaged that the virus would be carried as a latent infection in the self-renewing basal layer of normal oropharyngeal epithelium, and that emigrating cells would enter the productive cycle once they reached the upper keratinizing layers (Sixbey, 1989). At that time, however, there was no system available for testing this hypothesis.

Oral hairy leukoplakia (HL) is a recently described epithelial lesion of the oral mucosa associated predominantly with human immunodeficiency virus (HIV) infection (Greenspan et al., 1984). It presents macroscopically as a raised white lesion, usually at the lateral edge of the tongue, and is characterized histologically by intracellular oedema of the spinous cells, alterations of the nuclei and superficial hyperparakeratosis (Greenspan et al., 1984; Löning et al., 1987; Fowler et al., 1989). Although these morphological alterations are reminiscent of those induced by human papillomavirus (HPV) infections (Greenspan et al., 1984), it is now clear that HL is a focus of EBV replication, with viral DNA and productive cycle antigens easily detectable in the more differentiated layers of the lesion (Greenspan et al., 1985; Löning et al., 1987; De Souza et al., 1989; Young et al., 1991). HL therefore provides the most accessible means of studying the interaction between EBV and squamous epithelium, the implicit assumption being that this lesion represents an exaggeration of the epithelial infection present in healthy virus carriers. In this study, we have investigated the distribution of EBV DNA and the expression of the viral genome in HL lesions with special emphasis on the basal/suprabasal layers of the epithelium. We have employed highly sensitive in situ hybridization techniques for the detection of EBV DNA and latent RNAs, as well as immunohistology and immunoblotting to detect virus-encoded proteins.

**Methods**

**Tissues and cell lines.** In situ hybridization and immunohistology were carried out on sections of HL biopsies obtained from six HIV-seropositive patients; the biopsies were immediately snap-frozen in liquid nitrogen and stored at −70 °C until required. As controls, a microscopically normal buccal mucosa specimen from one of the above HL patients, and autopsy specimens from the lateral margin of the tongue and the buccal mucosa of an immunologically normal donor were also snap-frozen in liquid nitrogen. Immunoblotting studies were carried out on 20 additional snap-frozen HL samples and six normal tongue mucosa specimens. Reference cell lines used in this work included the EBV-positive transplantable NPC cell line C15, kindly provided by Dr P. Busson and passaged in nude mice (Busson et al., 1988; Young et al., 1988), the EBV-transformed LCLs B95.8 and X50-7, the EBV-positive BL cell line Akata, the P3HR1 EBV-converted BL cell line AW Ramos (kindly provided by Dr G. Klein, Stockholm, Sweden; Klein et al., 1975) and the EBV-negative BL cell lines BL31 and BL41 (see Rowe et al., 1987a).

**Plasmids and probes.** The plasmid pBS-W, harbouring the 3-kb BamHI W fragment of EBV strain M-ABA was a kind gift from Dr G. W. Bornkamm, Munich, Germany (Polack et al., 1984). A plasmid with specificity for HPV-11, kindly provided by Dr H. zur Hausen, Heidelberg, Germany, served as a negative control probe. Total plasmid DNA was labelled with [35S]dCTP (>1000 Ci/mmol; Amersham) by nick translation to a specific activity of 3 × 10⁹ to 5 × 10¹⁰ d.p.m./μg.

For the preparation of RNA probes, cDNA fragments were subcloned into the plasmid pBluescript KS containing promoters for T7 and T3 RNA polymerases. The plasmid pBSL13 contains the LMP open reading frame from a partial M13 digest of genomic viral DNA inserted at the BamHI site. The pBSW plasmid contains the BamHI W fragment of EBV subcloned at the BamHI site. The plasmids pBSJJ1 and pBS JJ2 contain EBER 1- and EBER 2-specific fragments, respectively. These fragments were derived from plasmids pJJ1 and pJJ2 (Jat & Arrand, 1982), kindly provided by Dr J. Arrand, Manchester, U.K., and subcloned into the BamHI and EcoRI and EcoRI and HindIII sites, respectively. After linearization with the appropriate restriction enzyme, 35S-labelled antisense (complementary to mRNA) or sense (anti-complementary, negative control) run-off transcripts were generated using either T3 or T7 RNA polymerases (Bethesda Research Laboratories (BRL)) as described previously (Melton et al., 1984; Milani et al., 1989). The length of the RNA probes was adjusted to about 100 to 200 bases by controlled alkaline hydrolysis. For in situ hybridization experiments, the antisense probes obtained from plasmids pBSJJ1 and pBSJJ2 were mixed to increase sensitivity. The sense probes derived from the same plasmids were also mixed. An oligonucleotide consisting of 25 T residues and an N-hydroxysuccinimide ester (Bethesda Research Laboratories (BRL)) was synthesized on a Biotech Instruments BT8500 DNA synthesizer using phosphoramidite chemistry (Dr J. Fox, Alta Bioscience) and labelled with biotin using a caproylamidobiotin-N-hydroxysuccinimide ester (BRL).

In situ hybridization. In situ DNA–DNA hybridization was carried out as described previously (Niedobitek et al., 1989). In brief, frozen sections or cytospin preparations were fixed in 4% paraformaldehyde/PBS, incubated sequentially in 0.2 M HCl and 0.125 mg/ml pronase (Boehringer Mannheim), acetylated in 0.1 M-triethanolamine pH 8.0,
0.25% (v/v) acetic anhydride, dehydrated through graded ethanols and air-dried. Hybridization mixture (25 µl) containing 50% deionized formamide, 2.5 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 10% dextran sulphate, 100 µg/ml carrier DNA, 10 mM-DTT and 1 ng of labelled probe were applied to a section. Probe and tissue DNA were denatured simultaneously for 2 min on a 95°C heat block. Hybridization was carried out at 37°C overnight. Subsequently, the slides were washed in 50% formamide, 2.5 × SSC, 10 mM-DTT at 37°C for 4 h, rinsed in 2 × SSC, dehydrated through graded ethanols, air-dried and dipped into Ilford G5 nuclear emulsion. The slides were exposed for between 3 and 12 days at 4°C, developed, fixed and counterstained with haematoxylin and eosin (H & E). In some experiments, HL sections were u.v.-irradiated with a dose sufficient for virus inactivation prior to DNA–DNA hybridization.

In situ RNA–RNA hybridization was performed as described previously (Milani et al., 1989). Fixation and treatment of sections with HCl and pronase was as described above, followed by post-fixation in 4% paraformaldehyde/PBS, acetylation and dehydration as above. Hybridization mixture (25 µl) containing 50% deionized formamide, 2.5 × SSC, 10% dextran sulphate, 0.2 µg/ml yeast RNA and 2 × 10^5 to 5 × 10^6 c.p.m. of labelled probe was applied per section. Hybridization was performed to assess the presence of polyadenylated mRNAs (Pringle et al., 1987). The expression of productive cycle antigens was determined by in situ hybridization of the AW Ramos cell line; this P3HR-1-converted subline of the EBV-negative BL cell line Ramos contains one copy of the viral genome per cell (Andersson & Lindahl, 1976). Upon hybridization to the EBV-specific probe, AW Ramos cells showed small clusters of grains over the nuclei of epithelial cells in the upper layers of the epithelium, indicative of virus replication and productive infection (Fig. 1b). The presence of latent EBV genomes in every cell of the transplantable NPC cell line C15 was also easily detected (data not shown). When analysed using these same conditions, all six HL specimens showed a much more intense EBV-specific signal over the nuclei of epithelial cells in the upper layers of the epithelium, indicative of EBV gene transcription characterized previously.

Antisera and monoclonal antibodies (MABs). Immunohistological analysis of frozen sections was performed using the EBV-specific MABs PE2, which recognizes both the EBNA 2A and EBNA 2B proteins (Young et al., 1989a), BZ1, directed against the immediate early BZLF-1 protein (Young et al., 1991), and a pool of MABs, CS1-4, specific for LMP (Rowe et al., 1987b). MAB Ki-67, directed against a proliferation-associated cell nuclear antigen (Gerdes et al., 1983), was kindly provided by Dr H. Stein, Berlin, Germany. The expression of EBNA2s was determined by immunoblotting with human sera, RA39, which has strong reactivity against EBNA 1, 2a, 3a, 3b, 3c and LP, and Mo, which is preferentially reactive against EBNA 1 (Young et al., 1989a, 1989b), as well as an EBNA 1 monospecific antibody preparation affinity-purified on a Gly-Ala peptide column (Rowe et al., 1991). An EBNA-LP monospecific reagent was prepared similarly by affinity purification of a human serum, LF, on a β-galactosidase fusion protein containing BamH1 W sequences as previously described (Wang et al., 1987). The expression of productive cycle antigens was determined by immunoblotting with the human serum EE, which is predominantly reactive against BZLF-1, the 45K to 60K early antigen (EA-D) complex and the viral capsid antigen (VCA) complex (Young et al., 1988).

Immunoblotting. Protein extracts from 20 HL specimens from 18 HIV-seropositive patients, six normal tongue specimens and the BL41 and B95.8 cell lines were separated by discontinuous PAGE and blotted onto nitrocellulose membranes as previously described (Young et al., 1988). Owing to the small size of the biopsy specimens, none of these cases was also available for in situ hybridization or immunohistology work.

Immunohistology. Immunohistological staining of acetone-fixed frozen sections was performed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique as described (Cordell et al., 1984). The APAAP complex was kindly provided by Dr H. Stein.

Results

In situ DNA–DNA hybridization

The sensitivity of our in situ hybridization technique for detection of viral DNA was assessed first using cytospin preparations of the AW Ramos cell line; this P3HR-1-converted subline of the EBV-negative BL cell line Ramos contains one copy of the viral genome per cell (Andersson & Lindahl, 1976). Upon hybridization to the EBV-specific probe, AW Ramos cells showed small clusters of grains over the nuclei (Fig. 1a) which could be detected after 2 to 3 days exposure of the autoradiograph. Low background distribution of grains was observed after parallel hybridization with the control, HPV-11. The presence of latent EBV genomes in every cell of the transplantaible NPC cell line C15 was also easily detected (data not shown). When analysed using these same conditions, all six HL specimens showed a much more intense EBV-specific signal over the nuclei of epithelial cells in the upper layers of the epithelium, indicative of EBV-specific hybridization was observed in any area of these epithelia.

In situ RNA–RNA hybridization

Before analysing the HL specimens, the specificity of the RNA probes used in in situ RNA–RNA hybridization was validated using tissues and cell lines with patterns of EBV gene transcription characterized previously. In situ hybridization analysis of sections from the transplantable NPC C15 revealed strong nuclear labelling upon hybridization to the EBER1 and EBER2 antisense
Fig. 1. In situ hybridization detection of EBV DNA. (a) AW Ramos cells (1 EBV genome copy/cell) display small clusters of grains over the nuclei (3 days exposure, H & E counterstaining); (b) nuclear labelling of epithelial cells in the upper layers but not in the basal/suprabasal layers of HL (3 days exposure, H & E counterstaining).
Fig. 2. In situ hybridization of EBV RNA expression using reference cell populations. Nuclear labelling of C15 NPC cells upon hybridization with EBER-specific probes (a; 5 days exposure, H & E counterstaining), but not with control sense probes from the same plasmids (b; 5 days exposure, H & E counterstaining). Hybridization with an LMP mRNA-specific probe reveals weak to moderate labelling of C15 cells (c; 12 days exposure, H & E counterstaining), whereas hybridization with a control sense probe shows only background distribution of grains (d; 12 days exposure, H & E counterstaining). Hybridization with a BamHI W fragment antisense probe shows an intense cytoplasmic mRNA signal in X50-7 LCL cells (e, f; 6 days exposure, H & E counterstaining), whereas no corresponding signal is seen with the control sense probe (g; 6 days exposure, H & E counterstaining); the BamHI W fragment antisense probe did not detect such a signal in C15 NPC cells (data not shown).
Fig. 3. *In situ* RNA–RNA hybridization reveals strong nuclear labelling of the upper epithelial cell layers of an HL lesion with the EBER-specific antisense probes (a; 12 days exposure, H & E counterstaining) and with the control sense probes (b; 12 days exposure, H & E counterstaining). The same nuclear labelling is also seen with the LMP mRNA-specific probe (c; 10 days exposure, H & E counterstaining) and the *BamHI* W fragment RNA probe (d; 6 days exposure, H & E counterstaining). In the same sections no labelling of basal-suprabasal epithelial cells was seen upon hybridization with the EBER-specific (a), LMP mRNA-specific (c) and *BamHI* W fragment RNA (d) antisense probes.

However, no EBV RNA-specific signals were obtained using either the EBER-, LMP- or *BamHI* W fragment-specific probes (Fig. 3a, c, d), even after deliberate over-exposure of the slides. Likewise we did not see labelling in any area of control (non-HL) epithelial tissues. We considered the possibility that the presence of large quantities of replicative EBV DNA in some cells might interfere with RNA–RNA hybridization in adjacent latently infected
cells. However, the present methods clearly detected the expression of EBERs, LMP mRNA and *BamH1* W fragment exon-containing transcripts in the B95.8 LCL, in which up to 10% of the population was clearly in the productive cycle (data not shown).

**Immunohistology**

The HL lesions were stained first with MAb BZ1, which is specific for the EBV-encoded immediate early protein BZLF1 and clearly identifies the productively infected subpopulation of cells in the B95.8 cell line. As previously reported (Young et al., 1991), this reagent showed strong nuclear staining of epithelial cells in the upper layers of HL lesions (Fig. 4a); this staining was localized to the same foci of EBV replication as previously reported (Young et al., 1991). Immunohistology sections and control slides of normal stratified epithelium. The Ki67-positive cells were strictly confined to the basal layer (Fig. 4b), exactly as in control epithelial material.

Finally we probed for LMP 1 expression by immunoblotting with the CS1-4 MAb. In the majority of cases the results were completely negative; however, in six of 20 cases very weak bands in the 60K region of the gel (i.e. that expected for LMP) were observed after long exposure of the autoradiographs.

**Discussion**

There are at least three routes by which EBV could infect the epithelial cells of HL: (i) persistence of the viral genome in latently infected basal cells with activation of virus replication in the upper cell layers in a differentiation-dependent mode, (ii) continual infection of maturing epithelial cells from EBV-carrying B lymphocytes infiltrating the epithelium and (iii) continual direct

**Immunoblotting**

The presence of replicating EBV in HL lesions was analysed by immunoblotting using a human serum (EE) with unusually high titre antibodies against productive cycle antigens. This reagent detected the 45K to 60K EA-D complex and certain other productive cycle antigens both above and below this *M* range (i.e. VCA components and BZLF1 protein) in all 20 biopsies (Fig. 5a), exactly as in the productively infected B95.8 cell line. No such reactivity was seen in an EBV-negative BL cell line (BL41) or in six normal tongue biopsies.

Probing HL extracts with the RA39 serum (a selected human serum with high titre antibodies against the various EBNA species seen in LCLs) revealed a strong band at approximately 66K in 14 of the 20 HL specimens (Fig. 5b). No such reactivity was observed in EBV-negative BL31 cells and the six normal tongue biopsies. This 66K band was identified as EBNA 1 by virtue of its reactivity with an EBNA 1 monospecific antibody preparation derived from a selected human serum, Mo, by affinity purification of EBNA 1-reactive antibodies on a Gly-Ala peptide column (Rowe et al., 1991). Two features of the EBNA 1 immunoblotting data are worthy of note. First, the intensity of the EBNA 1 band was strongest in those biopsies expressing high levels of viral replicative antigens. Second, the EBNA 1 proteins in these HL specimens all appeared to be of the same size; this was surprising because individual virus isolates often encode EBNA 1 proteins of different *M*, (Ernberg et al., 1989). The polyspecific human serum RA39 did react with additional species on the immunoblot, but in none of these cases could the additional bands be confirmed as latent cycle antigens. For instance, the two distinct bands around 45K and 50K detected in HLs using the RA39 serum (Fig. 5b) lie in the same *M*, range as EBNA LP, but using an EBNA LP-monospecific antibody preparation affinity-purified from a human serum using a β-galactosidase–LP fusion protein, we could not detect EBNA LP reactivity in this region in any of the biopsies. Similarly the diffuse band identified using RA39 in HL biopsies and lying above EBNA 1 (Fig. 5b) is in the correct *M*, range for EBNA 2, but was not detected by the EBNA 2-specific MAb PE2; in fact, PE2 did not show any specific reactivity in immunoblots on HL material.

Finally we probed for LMP 1 expression by immunoblotting with the CS1-4 MAbs. In the majority of cases the results were completely negative; however, in six of 20 cases very weak bands in the 60K region of the gel (i.e. that expected for LMP) were observed after long exposure of the autoradiographs.
Fig. 4. Immunohistological staining of a HL biopsy reveals strong expression of the BZLF1 immediate early protein in the upper epithelial cell layers (a; MAb BZ1, APAAP, haematoxylin counterstaining). Staining with MAb Ki-67, indicative of proliferating cells, reveals labelled nuclei only in the basal layer of HL epithelium (b; APAAP, haematoxylin counterstaining). Staining with the EBNA 2-specific MAb PE-2 shows weak labelling of epithelial cell nuclei in the upper layers (c; APAAP, no counterstaining). Staining of the same biopsy with the LMP-specific MAb CS1-4 gave no detectable membrane/cytoplasmic staining (d; APAAP, haematoxylin counterstaining); note that the nuclear staining apparent in the more differentiated cell layers in this black and white print is entirely due to the haematoxylin counterstaining. Arrows indicate the basal cell layer.

infection of the maturing epithelium by infectious virus present in the saliva. In this study, we have demonstrated that although high levels of EBV DNA and of EBV-encoded productive cycle antigens are present in the upper epithelial layers of HL, viral DNA and viral gene products are not detectable in the underlying basal/suprabasal layers.

In situ hybridization is as yet the only technique for demonstrating viral nucleic acids at the single cell level. Previous in situ hybridization studies have demonstrated the presence of replicative EBV DNA in the upper layers of HL in the absence of detectable levels of viral DNA in the lower layers (Löning et al., 1987; De Souza et al., 1989). However, in these studies in situ hybridization was
performed with biotinylated probes of limited sensitivity and it was therefore assumed that latent EBV infection in basal epithelial cells was below the threshold of detection. In this study we have employed the most sensitive in situ hybridization technique available (Niedobitek & Herbst, 1991) and in control experiments have confirmed its ability to detect the single copy of the viral genome present in AW Ramos cells (Fig. 1a). Using this technique, we found no evidence of EBV DNA in the basal and suprabasal epithelial cells of HL. It is still formally possible that, for technical reasons, latent EBV genomes are more difficult to detect in this cellular environment than in the reference AW Ramos cell line; however, the strong inference from these in situ DNA hybridization studies is that HL is a purely productive lesion with no underlying latent infection.

We next attempted to address the same question by in situ detection of specific EBV transcripts. This phase of the work brought to light a technical problem which can easily lead to false positive results if assays are not adequately controlled. Thus, in the upper, productively infected epithelial cell layers, analysis of EBV gene expression is hampered by what appears to be hybridization of RNA probes to replicative EBV DNA without prior denaturation of double-stranded viral DNA. This same phenomenon has been described previously in work on cytomegalovirus (McCarrey et al., 1989). Several attempts were made to overcome the problem. U.v. irradiation of tissue sections with doses sufficient to inactivate virus, although not interfering with the detection of RNAs, did not render viral DNA inaccessible to the probes. Pretreatment of tissue sections with RNase-free DNase reduced hybridization of both antisense and sense probes to viral DNA but could not abolish it under conditions consistent with preservation of tissue morphology. Thus, analysis of EBV gene expression at the RNA level was not possible in productively infected epithelial cells. However, the autoradiographic signal generated by this cross-hybridization was confined to the upper epithelial layers and did not interfere with the subsequent evaluation of basal/suprabasal cells.

The probes used for in situ RNA–RNA hybridization were capable of detecting three well documented sets of transcripts that are constitutively expressed in virus-transformed LCLs. Thus, the small non-polyadenylated EBERs are the most abundant viral RNAs in LCLs (Sample & Kieff, 1990) and are also strongly expressed in three EBV-associated tumours, BL (Rowe et al., 1987a), Hodgkin’s disease (Pallesen et al., 1991; Wu et al., 1990) and NPC (Gilligan et al., 1990; G. Niedobitek et al., unpublished results). LMP mRNA represents the most abundant virus-encoded mRNA species in LCLs and is also detectable in EBV genome-positive cases of Hodgkin’s disease (G. Niedobitek et al., unpublished observations), as well as in a number of NPCs (e.g. C 15; Fig. 2c). Similarly, the BamHI W fragment exon-containing mRNAs are easily detectable in LCLs, in

Fig. 5. EBV gene expression in HL. (a) Immunoblot showing predominant expression of the 45K to 60K EA-D complex in HL biopsies, along with BZLF1 and some higher Mr VCA components. The immunoblot was probed with the human serum EE, which reacts predominantly with these productive cycle antigens. Lane 1, B95.8; lane 2, BL41; lane 3, normal tongue; lanes 4 to 5, HL biopsies. (b) Immunoblot probed with the human serum RA39, which reacts predominantly with EBNA 1, EBNA 2a, EBNA 3a, EBNA 3c and EBNA LP expressed in the X50-7 LCL. Lane 1, X50-7; lane 2, BL31; lanes 3 to 5, HL specimens. The bands seen in HL biopsies are at 66K, 50K and 45K. Identification of the 66K band as EBNA 1 was further supported by immunoblotting with an EBNA 1-monspecific antibody preparation; the lower Mr bands did not correspond to known EBV latent proteins (see text) and are thought to be productive cycle antigens.
which they are associated with transcription from the BamHI C or W fragment promoters (Kieff & Liebowitz, 1990). Therefore it is highly significant that none of these three sets of latent viral RNAs was ever detected in the basal/suprabasal layers of HL lesions (Fig. 3). The lack of detectable EBER RNAs is consistent with recent results from Northern blotting analysis of HL biopsies (Gilligan et al., 1990), whereas the lack of detectable LMP mRNA is consistent with the absence of anti-LMP MAb staining in the same cell layers (see Fig. 4d). Indeed, it was only after prolonged exposure of immunoblots that any hint of LMP was obtained in HL biopsies and the cellular origin of this very weak signal, observed at the whole tissue level, remains to be determined. Thus, although LMP does have the potential to alter human epithelial cell behaviour in vitro and in vivo (Dawson et al., 1990; Wilson et al., 1990), the morphological alterations in epithelium associated with HL do not appear to be consequences of LMP expression.

The EBNA 1 protein is conventionally taken to be a marker of latent EBV infection, but our findings raise the possibility that (unlike the other EBNA s) EBNA 1 is also expressed during a purely productive infection. Thus, a characteristically sized EBNA 1 species was detectable by immunoblotting in the majority of HL biopsies (Fig. 5). However, the constant size of the band was surprising in that different virus isolates would be expected to encode EBNA 1 proteins of slightly different M, s (Ernberg et al., 1989). One possibility is that the lesions are infected with a common virus isolate; alternatively, tissue-specific processing of EBNA 1 might generate this uniformity. On the other hand we cannot totally discount the possibility that what is being detected is a cross-reactive cellular protein, which is present in HL lesions but not in normal epithelial biopsies, or even a cross-reactive viral antigen which is expressed when EBV replicates in epithelium, but which has never been detected in productively infected lymphoid cells. Further work will be needed to discriminate between these alternatives.

Histological localization of EBNA 1 expression in HL could not be achieved; no EBNA 1-specific MAb is yet available for immunohistological work and attempts to stain HL sections with anti-EBNA 1-positive human sera were always complicated by the very strong accompanying reactivity against productive cycle antigens. The correlation between the level of EBNA 1 expression and that of productive cycle antigens is nevertheless consistent with the protein being expressed as EBV replicates.

Although there was no indication that any of the other EBNA s were detectable in HL by immunoblotting, immunostaining with MAb PE2 revealed a distinct but weak nuclear staining in the upper layers of epithelium in three of five HL specimens tested. The specificity of this signal remains uncertain, but it is interesting that in a recently developed in vitro system for analysing productive EBV infections in human epithelial cells, a small subpopulation of cells develop the same weak nuclear staining with PE2 (Q. X. Li et al., unpublished results). Further studies will be needed to identify the source of this reactivity.

HL is a lesion supporting EBV replication in an immunocompromised host and as such it may serve as an exaggerated model of the situation in immunocompetent individuals. Our results strongly suggest that EBV does not infect basal epithelial cells in HL. Accordingly, the work calls into question earlier models of EBV persistence, which envisaged latent infection being maintained within the basal epithelial compartment (Rickinson et al., 1985; Allday & Crawford, 1988), and refocuses attention on the B lymphoid system as a reservoir of latent EBV. Similarly, results from two other recent studies of the EBV carrier state stress the importance of B cells as a site of virus persistence (Gratama et al., 1988; Yao et al., 1989a, b). If HL does indeed represent a focus of self-sustained virus replication, how might this be achieved in the absence of latent infection in the underlying basal layer? Continual delivery of infectious virus to maturing epithelium by infiltrating EBV-carrying B lymphocytes seems an unlikely route, given the general sparsity of inflammatory cells in HL lesions. Hence, this leaves the possibility of a continual direct infection of the maturing epithelium in HL with locally produced virus.

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References


EBV gene expression in oral hairy leukoplakia


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