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Epstein–Barr Virus Gene Expression and Epithelial Cell Differentiation in Oral Hairy Leukoplakia

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Hairy leukoplakia (HL) is an Epstein–Barr (EB) virus related lesion of oral mucosa that is principally associated with human immunodeficiency virus–induced immunosuppression. To understand the nature of EB virus involvement in these lesions, this study compares the distribution of EB virus DNA and EB viral gene products with the pattern of keratinocyte differentiation in 12 lateral tongue biopsies of HL. Evidence of replicating EB viral infection and abundant virus production was demonstrated in the superficial epithelium of most (92%) samples by means of* in situ *hybridization and immunocytochemical techniques. Epstein–Barr virus latent membrane protein also was identified in 45% of samples, suggesting that this viral gene product, which is usually associated with EB virus latent infection, may be transiently expressed during viral replication in HL epithelium. The absence of detectable EB virus involvement in basal keratinocytes, however, fails to support the theory that latent infection occurs in basal epithelium. From this study, EB viral gene expression in HL appears to be linked with epithelial maturation. Conversely, the normal patterns of keratinocyte differentiation in these lesions do not appear to be appreciably altered by association with EB virus. (Am J Pathol 1991, 139:1369–1380)

Hairy leukoplakia (HL) is a recently recognized white patch lesion of the oral mucosa associated with immunosuppressed states. The lesion principally arises on lateral tongue margin in individuals with human immunodeficiency virus (HIV) infection,[1] but also has been described, less frequently, in HIV-seronegative patients receiving immunosuppressive therapy for acute leukemia[2] and after bone marrow[3] or solid organ transplantation.[4] Demonstration of replicating Epstein–Barr (EB) virus in HL epithelium by DNA hybridization,[1,5,6] immunocytochemical,[1,7,8] and ultrastructural methods[1,7,9] is now regarded as pathognomic for this condition and prerequisite to confirm the clinical diagnosis. The presence of EB virus replication in HL represents a unique clinical manifestation of EB virus infection in nonmalignant squamous epithelium and, as such, has important implications on the pathobiology of EB virus in normal individuals as well as its role in the development of human neoplasia.

Natural infection with EB virus occurs in more than 90% of the world population either subclinically during childhood or as acute self-limiting infectious mononucleosis (IM) in teenage years. After primary infection, the virus establishes lifelong persistence in healthy seropositive individuals, who remain asymptomatic but intermittently shed low levels of infectious viral particles in oropharyngeal secretions. The precise mechanisms involved in this long-term carrier state are not known, although the main *in vivo* reservoirs of persistently infected cells are thought to be in epithelial cells and circulating B cells. The B lymphotropic nature of EB virus is well established by its capacity to produce permanent B lymphoblastoid cell lines (BLCLs) *in vitro*. These are latently infected and express latent gene products, EB virus nuclear antigen (EBNA) types 1–6, and latent membrane protein (LMP), which are integral to continued cell proliferation. Whereas limited viral replication occurs spontaneously in latently infected cells, or can be induced by chemical agents *in vitro*, the failure to consistently demonstrate virus production in B cells suggests an alternative host cell target to complete the full viral cycle.

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The oncogenic properties of EB virus in two histogenetically diverse human tumors, Burkitt's lymphoma and nasopharyngeal carcinoma (NPC), attest to the dual target cell specificity of the virus. Support for EB virus replication in normal squamous epithelia has been derived from clinical and experimental studies. Linear EB viral DNA and RNA transcripts, EBNA and lytic cycle antigens, early antigen (EA), and viral capsid antigen (VCA) indicative of active viral replication have variously been demonstrated in desquamated epithelial cells of throat washings from patients with acute IM[10] and in healthy seropositive individuals,[11] in parotid tissue,[12] and in normal ectocervical[13] and adenoidal[14] epithelial cells after exposure to EB virus *in vitro*. Direct viral entry into these cells may involve EB virus-binding receptor molecules (CD 21 antigen),[15] which are expressed on stratified squamous epithelium in a differentiation-dependent manner.

Studies on the interactions between EB virus and normal squamous epithelium have generally been limited by the resistance of long-term cultured epithelial cells to EB virus infection *in vitro*. The phenotypic interrelationships of infected HL cells may be significant to the development of this lesion and also may reflect the mode of viral persistence in normal squamous epithelium. With these objectives in mind, this study examines the pattern of EB virus gene expression and epithelial cell differentiation in lateral tongue biopsies of HL from HIV-seropositive individuals using immunocytochemical and *in situ* hybridization techniques.

Materials and Methods

Fresh biopsies of white patch lesions from lateral margins of tongue were obtained from 12 HIV-seropositive individuals (11 men, 1 woman, aged 25 to 55 years). Criteria for the diagnosis of HL was based on the clinical appearances of the lesion, HIV status of the patient, and failure of the lesion to respond to antifungal therapy. At the time of biopsy, six patients were receiving antiviral therapy with zidovudine either as a single agent or combined with acyclovir and antifungal agents (fluconazole, nystatin, miconazole), or antifungal agents alone. Control lateral tongue biopsies were obtained from non–HIV-infected individuals with frictional keratosis (2 cases) and two HIV-negative, EB-virus–seropositive healthy male volunteers.

Portions of each sample were processed by snap-freezing in isopentane precooled in liquid nitrogen and by routine histologic methods of formalin fixation and paraffin wax embedding. Phenotyping studies were performed on 5-μ cryostat sections using peroxidase anti-peroxidase immunocytochemistry[16] with the panel of monoclonal and polyclonal reagents to EB virus gene products and epithelial differentiation markers shown in Table 1. Additional antisera were used to define EB-virus–associated B cell antigens (CD21, CD23) markers expressed on infiltrating mononuclear cells, including leukocyte common antigen (CD45), pan-B cells, pan-T cells (CD 2) and T cell subsets (CD4 and CD8), antigen-presenting cells (CD1a), and immunoregulatory molecules, including class I and II major histocompatibility (MHC) antigens and the cell adhesion molecules (CAMs), leukocyte function antigen (LFA)-3 (CD58), and intercellular adhesion molecule (ICAM)-1 (CD54). Immunoenzymatic detection of EBNA was performed by the anticomplement immunoperoxidase method.[27] Indirect immunofluorescence (IF) methods were additionally used to detect EA, VCA, and membrane antigen (MA) complex associated with EB virus lytic infection, EBNA species, and LMP. Simultaneous expression of EBNA and EB virus lytic antigens was investigated in combined labeling studies with ACIF-FITC (fluorescein isothiocyanate) and indirect IF-TRITC (tetramethylrhodamine isothiocyanate) techniques.

In situ hybridization studies for EB virus DNA were performed by two methods: 1) PBR322 cloned *Bam* HI W restriction fragment probe biotinylated by nick translation was applied to 10-μ cryostat sections and hybridized to target DNA as described by Brigati et al.[28] 2) A synthetically produced oligonucleotide probe (supplied by Dr N. J. Maitland, Pathology Department, Bristol University, UK) corresponding to 40 nucleotides from the *Bam* HI W fragment of the EB virus genome and labeled with digoxigenin at the 3′ end using terminal transferase (Boehringer Mannheim, Germany) was applied to paraffin-embedded tissue sections as described by Cubie and Norvall.[29] Targeted probe in method 1) was identified by a three-stage system using monoclonal antibody (MAb) to biotin (Dakopatts, Copenhagen, Denmark), biotinylated rabbit anti-mouse immunoglobulin antibody (Dakopatts), and avidin-biotin peroxidase complex (ABC Px; Dakopatts). Peroxidase signals were developed with 3′3′ diaminobenzidine and intensified by gold-silver precipitation (Amersham, Copenhagen, Denmark). In method 2), targeted probe was identified with anti-digoxigenin antibody conjugated to alkaline phosphatase and developed with nitroblue tetrazolium/bromochloroindolyl phosphate substrate (Gibco-BRL Ltd, Grand Island, NY).

Results

Histology

Histologic appearances of the biopsies from HIV-seropositive cases were consistent with HL and featured thickened superficial parakeratosis containing candida hyphae, swollen and vacuolated prickle cells, and acan-

Table 1. *Antibody Panel*

Specificity*	Designation†	Source‡	References
EB virus-associated antigens			
EBNAs 1–4, 6	RT; AT	Human serum	
EBNAs 2B + 3	WC	Human serum[a]	17
EBNA 2A + 2B	PE2	[b]	18
EA	EA-d	[c]	19
VCA; MA	F3.23; 813	Seralab; Biogenesis	
LMP	S12	[a]	20
CD23	MHM6	Dakopatts	
CD21	HB5; B7; B2	Seralab; Orthomune; Becton Dickinson	
Epithelial differentiation antigens			
Involucrin	DH1B4	Rabbit antibody[d]	21
Cytokeratins:			
1, 10 (st/corn epith)	LH1	[e]	22
10 (st/sup-basal epith)	RKSE60; LH2	ICN Immunochemicals; [e]	22
4, 13 (st/non-corn epith)	6B10, 1C7	[e]	22
14 (st/sup-basal epith)	LL001	[e]	22
7 (simple epith)	RCK105; LP1K	Eurodiagnostics; [e]	22
18 (simple epith)	Le61	[e]	22
8, 5 (simple epith)	RCK102	Eurodiagnostics	
8, 7, 18 (simple epith)	Cam 5.2	[f]	23
19 (simple & st epith)	LP2K	[e]	22
8, 17, 18, 19 (simple & st epith)	PKK1	Dakopatts	
Cell proliferation ag	Ki67	Dakopatts	
Immune-associated antigens			
CD45	PD7	Dakopatts	
Pan-B cell ag	L26	Dakopatts	
CD2; CD4; CD8; CD1a	T11; T4; T8; T6	Dakopatts; Coulterclone	
CD58 (LFA-3)	TS2/9	[g]	24
CD54 (ICAM-1)	RR1/11	[g]	24
Class I MHC	W6/32	[f]	25
Class II MHC	1B5	[f]	26

* St/corn epith = stratified cornifying epithelium; st/sup-basal epith = stratified suprabasal epithelium; st/non-corn epith = stratified non-cornifying epithelium.

† Murine monoclonal antibodies unless otherwise stated.

‡ Reagents supplied as gifts from (a) Dr. D. Thorley-Lawson, Department of Pathology, Tufts University, Boston, MA; (b) Dr. M. Rowe and Dr. L. Young, Cancer Research Campaign Laboratories, Department of Cancer Studies, University of Birmingham Medical School, UK; (c) Dr. G. Pearson, Department of Microbiology, School of Medicine, Georgetown University, Washington DC; (d) Keratinocyte Laboratory, ICRF, UK; (e) Cell Structure Laboratory, ICRF, UK; (f) Director's Laboratory, ICRF, UK; (g) Professor T. Springer, Laboratory of Membrane Immunochemistry, Dana-Farber Institute, Boston, MA.

thosis (Table 2). Inflammatory cell infiltration of the lamina propria was not usual except in four samples that contained low to moderate numbers of mononuclear cells. Frictional keratosis identified in white patch lesions from HIV-seronegative individuals showed moderate superficial parakeratosis without prickle cell vacuolation or candida hyphae. Normal lateral tongue mucosa in control biopsies from healthy HIV-seronegative, EB-virus–seropositive men showed nonkeratinizing squamous epithelium with minimal superficial parakeratosis in one biopsy.

In Situ *Hybridization and Identification of EB Virus Gene Products*

All HL samples showed evidence of EB virus infection either by *in situ* hybridization or by immunocytochemistry (Table 2). Epstein–Barr virus DNA was detected in 10 of 11 samples tested. Intense hybridization signals were usually concentrated over the superficial layers of hyperparakeratotic epithelium (Figure 1a). In most samples, a gradual diminution in signal intensity was noted on vacuolated cells of underlying spinous epthelium, which varied from moderate amounts to a few grains of precipitated probe label (Figure 1b). No hybridization signals were detected in basal epithelium of HL or in control biopsies of normal and non-HL lateral tongue.

By contrast, nuclear staining for EBNA species, detected with high-titer human sera and anti-complement immunocytochemistry in 8 of 12 samples, was predominantly localized to cells in the middle and upper spinous cell layers (Figure 2). These cells were below the site of maximal EB viral DNA hybridization, and corresponded to areas of marked prickle cell vacuolation. In some samples, EBNA-positive nuclear staining also extended to the surface at the edges of the lesion and was present in the lower spinous cell layer, but was not detected in basal epithelium in any sample. Expression of EBNA 2 species, defined by MAb PE2 and high-titer human serum, WC, was detected in four of eight and three of six samples,

Table 2. *Clinicopathologic Data and Demonstration of EB Virus DNA, Viral Gene Expression and CD21 Antigens in HL*

Case no.	Histology*			Rx†	Identification‡ of					
	HPK	VPC	CH		EB virus	EBNA		VCA, EA, MA	LMP	CD21
					DNA	1	2			
1	+	+	+	−	+	+	nt	+	−	−
2	+	+	+	−	+	+	(+)	+	(+)	−
3	+	+	+	−	+	+	nt	+	−	+
4	+	+	+	−	+	−	nr	nr	nt	−
5	+	+	+	−	+	−	−	−	−	−
6	+	+	+	−	+	+	−	−	−	−
7	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	(+)	+
9	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	−	nt	−	−
11	+	+	−	+	nr	−	nt	+	−	+
12	+	+	−	+	−	−	−	−	(+)	+

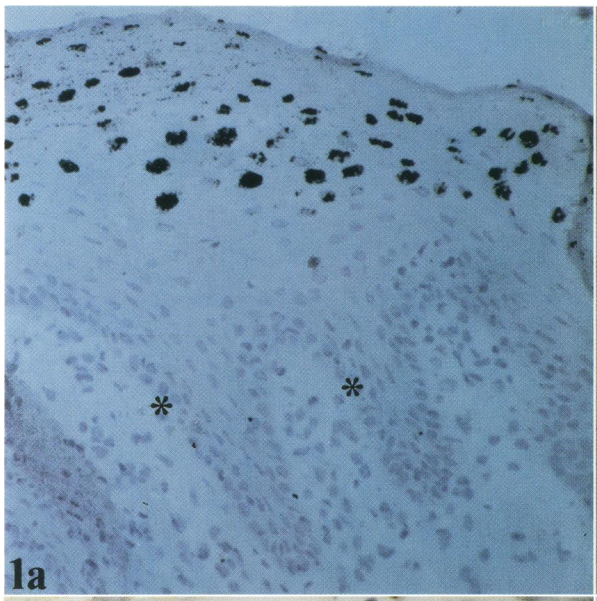
* HPK = hyperparakeratosis, VPC = vacuolated prickle cells, CH = candida hyphae.
† + = patients receiving treatment at the time of biopsy consisting of antifungal agents (Nystatin or Miconazole) only in Cases 8 and 9, anti-viral agent (Zidovudine) only in Cases 7 and 11 and combined anti-fungal + viral agents (Zidovudine + Fluconazole or Zidovudine + Acyclovir + Fluconazole) in Cases 10 and 12, respectively.
‡ Staining reaction: + = positive; (+) = diffuse positive/equivocal; − = negative; nt = not tested; nr = no result due to lack of epithelium in sample.

respectively. Concordant results (two positive, one negative) were obtained in three samples, which were tested with both WC and PE2 antisera. The distribution of EBNA 2 (PE2) and EBNA 2B+3 (WC) was similar to the patterns seen with human sera containing high activity to most EBNA types. Evidence of EB virus replication was demonstrated in 7 of 10 samples by the presence of nuclear and cytoplasmic staining for VCA or EA lytic cycle antigens (Figure 3a, b) and MA complex. The well-circumscribed nature of the lesion was clearly illustrated by the pattern of lytic cycle antigen staining in some samples. This formed a band of intense staining in the vacuolated spinous cell layer and at the edges of the lesion (Figure 3a). Superficial cells of lesional epithelium were usually unreactive or showed markedly reduced staining (Figure 3b), and no staining was evident in suprabasal cells or basal epithelium. Similarities between the topographic distribution of lytic cycle antigen expression and EBNA-containing cells were confirmed by double IF analysis with combinations of human antisera to EBNA and MAbs to VCA or EA. Cells with simultaneous labeling for EBNA and EA/VCA were demonstrated in the upper and middle layers of spinous epithelium (Figure 4). Cytoplasmic staining also was present in spinous cells as well as in flattened parakeratotic cells of superficial epithelium. In the absence of nuclear staining for EBNA in superficial epithelium, this cytoplasmic staining was attributed to anti-VCA antibodies known to be present in the human sera used for EBNA demonstration. Of 11 biopsies tested, LMP-positive cells were demonstrated in two samples (Figure 5a), with a further three samples showing equivocal diffuse positive staining. Small foci of LMP-positive cells appeared to be randomly distributed in the middle or upper epithelium. No LMP-positive cells were detected in the basal cell layers or in nonlesional epithelium. No immunocytochemical staining for LMP (Figure 5b) or other EB virus latent or lytic cycle antigens was evident in control lateral tongue biopsies.

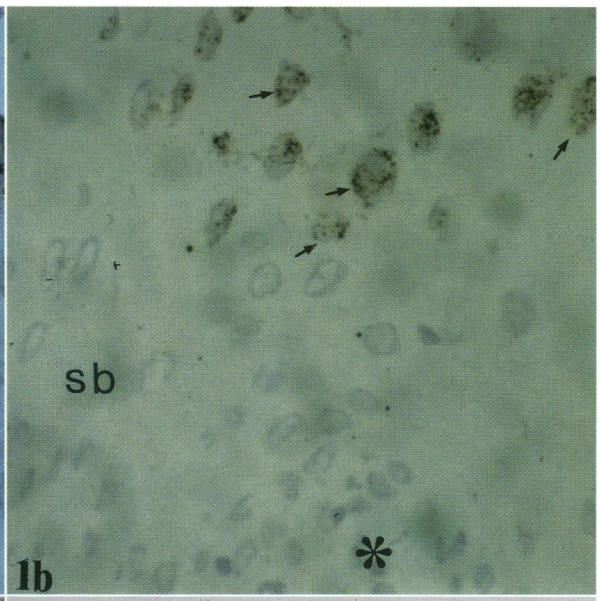
Immunophenotypic Analysis of HL

The hyperparakeratotic epithelial layers in HL were conspicuously unreactive with most antisera of the pheno-

Figure 1. **a:** *Intense EB virus DNA hybridization signals in middle/upper spinous layer and superficial epithelium of HL biopsy (Case 9). No hybridization signals are detectable in basal cells (*). (Cryostat section; hybridization signal detected with ABC Px and gold-silver precipitation, ×250).* **b:** *EB virus DNA hybridization in HL biopsy (Case 6) shows varying signal intensity (→) in lower spinous layer keratinocytes adjacent to suprabasal cells. Basal epithelial cells (*) show no detectable signals. (Cryostat section; hybridization signal detected with ABC Px and gold-silver precipitation, ×630).*
Figure 2. *Nuclear staining for EBNA species 1–4,6 expression in vacuolated prickle cells (vpc) of HL biopsy (Case 2). Hyperparakeratotic superficial epithelium (sp) and basal epithelial cells (*) are EBNA negative. (Cryostat section stained by ACIPx with human serum, AT, ×400).*
Figure 3. **a:** *Strong staining for VCA (mAb F323) in spinous layer keratinocytes of HL biopsy (Case 8). VCA$^+$ cells extend to the mucosal surface at the margin of the lesion (◀) whereas the hyperparakeratotic superficial epithelium (sp) is mainly unreactive. (Cryostat section; PAP method, ×160).* **b:** *Detail of lesional epithelium at (◀) in HL biopsy shown in* **(a)** *showing the clearly defined band of VCA$^+$ spinous cells, weak positive staining on sparse superficial cells and marked absence of staining on suprabasal and basal (*) cells. (Cryostat section; PAP method, ×400).*
Figure 4. *Simultaneous labelling of HL biopsy (Case 8) with FITC conjugated human antiserum (WC) to EBNA 2B + 3 and TRITC labelled EA-D mAb shows differences in the distribution of EB virus infected keratinocytes. EBNA+, EA-D+ cells (→) are located in mid and upper spinous epithelium (*), whereas superficial cells (sp) show no nuclear staining for EBNA. IF staining is poorly detectable in suprabasal (sb) cells. (Basal epithelium which is not illustrated is also EBNA−). (Cryostat section, ×630).*



1a



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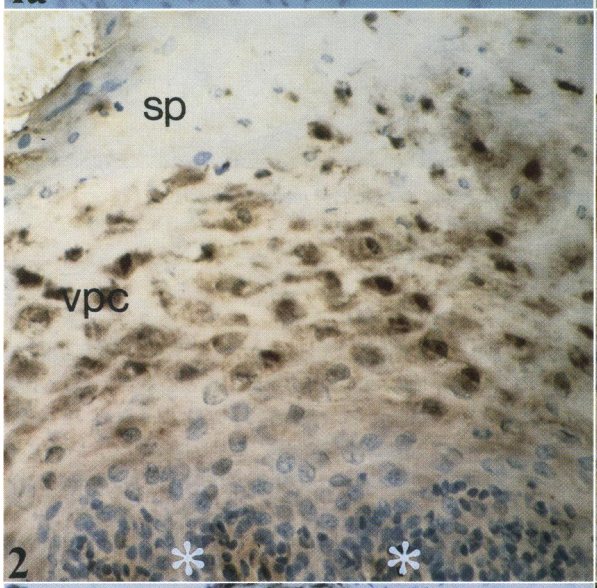

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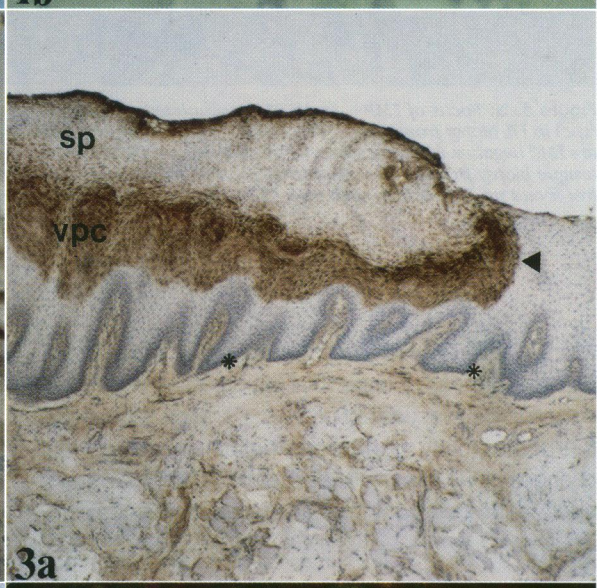
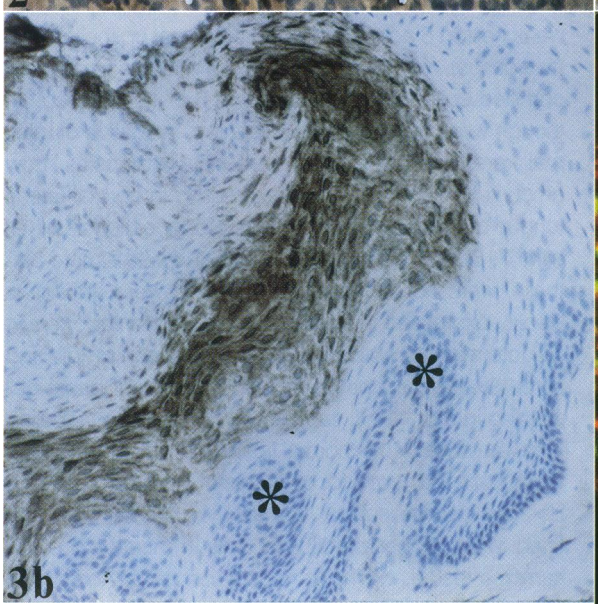


3a



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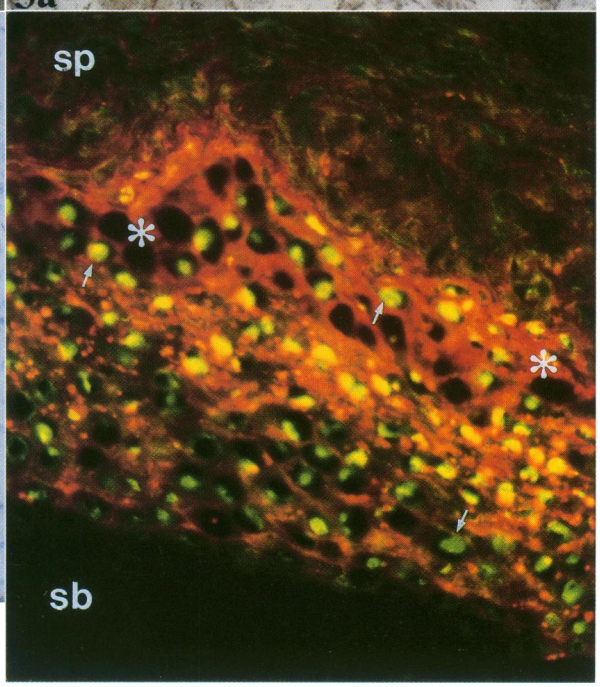

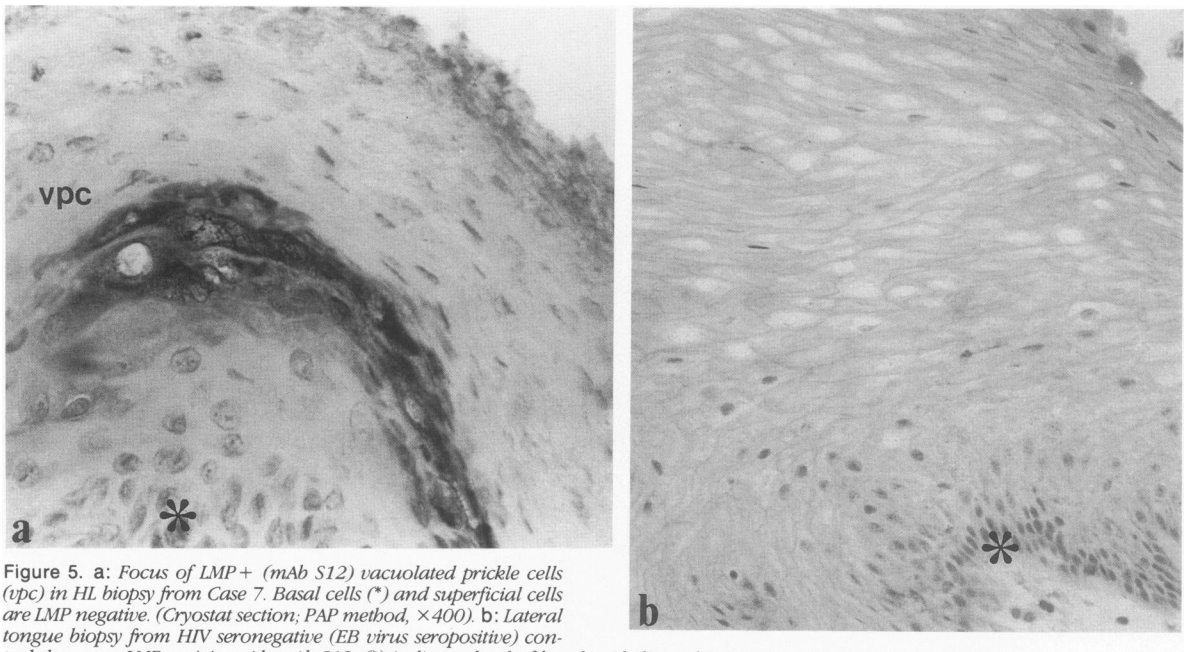

Figure 5. a: *Focus of LMP+ (mAb S12) vacuolated prickle cells (vpc) in HL biopsy from Case 7. Basal cells (*) and superficial cells are LMP negative. (Cryostat section; PAP method, ×400).* b: *Lateral tongue biopsy from HIV seronegative (EB virus seropositive) control shows no LMP activity with mAb S12. (*) indicates level of basal epithelium. (Cryostat section; PAP method, ×250).*

typing panel. This lack of immunoreactivity, which clearly delineated areas of superficial hyperparakeratosis, was not evident on vacuolated prickle cells, or on surrounding morphologically normal epithelium. Parakeratotic changes in the superficial epithelium of control biopsies also was associated with weak or absent antibody staining.

Epithelial Cell Differentiation in HL

No major differences were observed between the phenotypic profiles of epithelial differentiation markers in most HL biopsies and control tissues (Table 3). Qualitative variations in the normal patterns of expression were observed in some HL samples, however. Positive staining was demonstrated in HL epithelial cells adjacent to hyperparakeratotic areas and in vacuolated prickle cells of the lesions.

Involucrin, a precursor cornified envelope protein, was clearly expressed in suprabasal cells of all but two samples, providing evidence of keratinocyte terminal differentiation in HL (Figure 6a). Positive staining also was observed on basal cells as well as on hyperparakeratotic epithelium in several samples. Cytokeratin groups that characterize stratified squamous (keratins 5, 14, and 19), noncornified (keratins 4 and 13) epithelia were demonstrated in the majority of HL samples. *Keratins 5* and *14* were strongly expressed at all cell layers, including basal epithelium (Figure 6b), whereas *keratins 4* and *13* were expressed in suprabasal cells only (Figure 6c). Variable staining patterns observed in a minority of samples consisted of focal expression of keratins 4, 13, and 14 in areas of prickle cell vacuolation (Figure 6d) and minimal staining elsewhere in the epithelium (three cases), or weak keratin 5 activity limited to the basal epithelial layer (two cases). *Keratin 10,* which is classified as a marker of cornified epithelium, was absent in 8 of 11 samples, although weak suprabasal cell staining, indicative of minimal keratinization, was present in two biopsies. Low amounts of keratin 10 were expressed in normal lateral tongue but not in frictional keratosis tongue biopsies. *Keratins 7, 8,* and *18,* normally associated with simple epithelia, were not expressed in HL and were absent or weakly expressed in control tissues. Conversely, *keratin 19* determinants, which may be expressed in simple and some stratified epithelia, showed intermittent basal cell staining in 5 of 11 HL samples. This linear-type labeling also was observed in normal lateral tongue. Ki67 antigen expression was used for evidence of increased cell proliferation in these lesions. Nuclear Ki67 staining was confined to foci of basal epithelial cells in most (5/7) of HL samples tested, similar to control tissues, although two samples contained foci of nuclear stained vacuolated prickle cells.

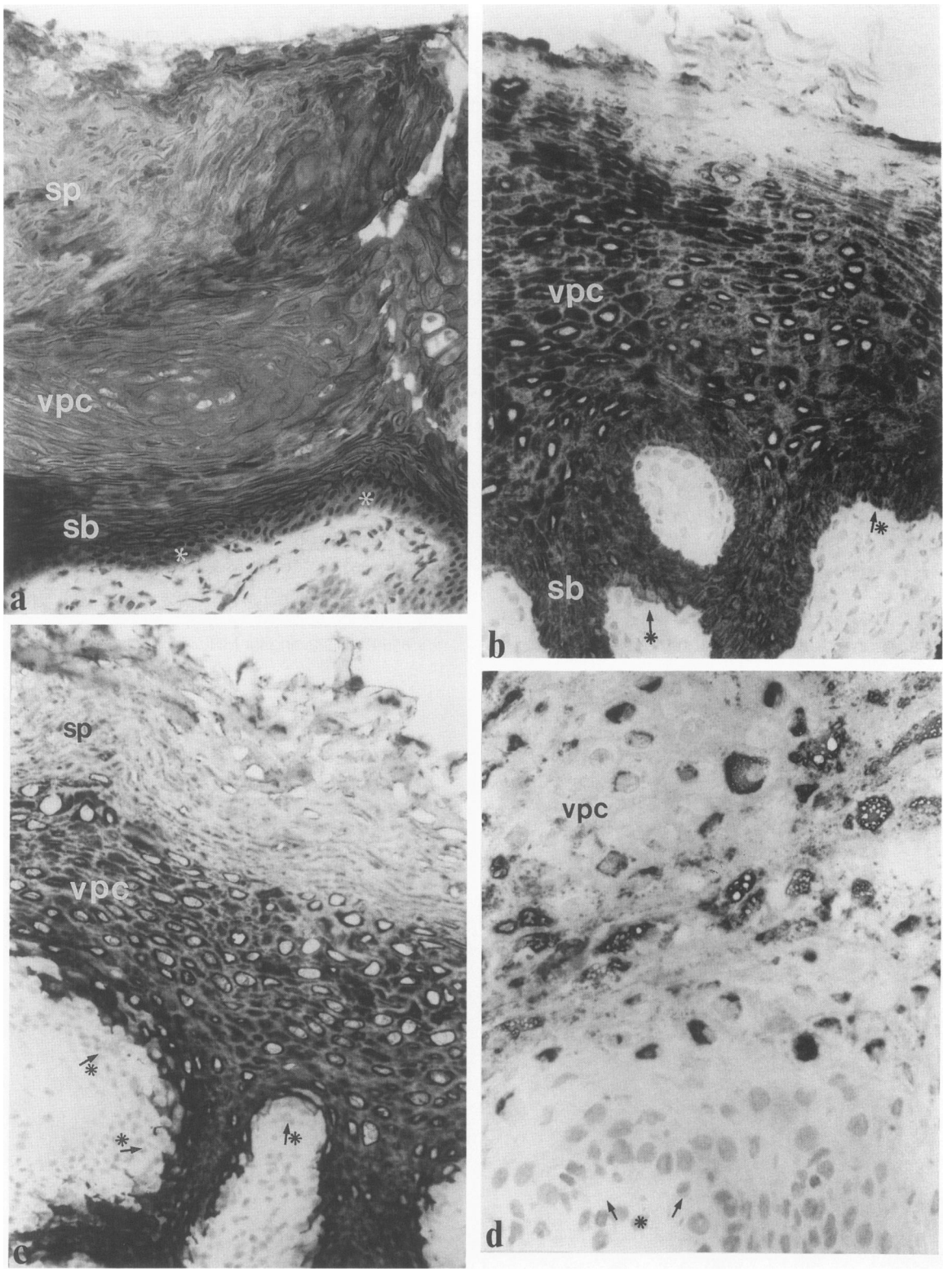

Figure 6. *Expression of* **(a)** *involucrin (polyclonal antiserum DH1B4),* **(b)** *keratin 14 (mAb LLOO1) and* **(c)** *keratin 4 (mAb 6B10) in HL biopsy from Case 8. The presence of involucrin and marked absence of keratin expression in hyperparakeratotic superficial epithelium (sp) is typical of most cases.* **d**: *HL biopsy from Case 7 shows focal positive staining for keratin 4 in vacuolated prickle cells (vpc). (*) indicates level of basal epithelium. (Cryostat sections; PAP method, ×250 and 400).*

Table 3. *Epithelial Differentiation, Immune Response, and Leukocyte-function Antigens in HL and Normal Lateral Tongue Biopsies*

Antigen (mAb)	HL phenotype*†				% (+/no tested)	Normal tongue			
	B	SB	S	SP		B	SB	S	SP
Involucrin	−	+	+	(+)	83 (10/12)	−	+	+	+
Cytokeratins									
8, 5 (RCK 102)	++	++	+	−	75 (9/12)	++	+	+	−
14 (LL001)	++	++	++	−	60 (6/10)	++	++	++	++
4 (6B10)	−	+	+	−	73 (8/11)	−	+	+	+
13 (1C7)	−	+	+	−	70 (7/10)	−	+	+	+
10 (RKSE60)	−	−	−	−	73 (8/11)	−	±	±	−
19 (LP2K, PKK1)	+	−	−	−	45 (5/11)	+	−	+	−
7, 8, 18 (Cam 5.2)	−	−	−	−	100	−	−/±	−/±	−
Class I MHC	+/++	+	+	−	67 (6/9)	++	+	+	+
Class II MHC	−	−	−	−	67 (6/9)	+	−	−	−
CD54	±	±	(+)	−	42 (5/12)	−	−	−	−
CD58	+	+	+	−	50 (6/12)	+	+	+	+
Ki67	+	−	−	−	71 (5/7)	+	−	−	−

* B = basal; SB = suprabasal; S = spinous layer including vacuolated prickle cells; SP = superficial (including hyperparakeratinized) epithelium.

† Staining reaction: ++ = intense; + = strong; (+) = focal positive; ± = weak; − = negative.

Expression of EB Virus Associated Cell Activation Antigens and Immunoregulatory Molecules in HL

Pooled monoclonal antisera to EB virus receptor molecules (CD 21 antigen) showed the same patterns of activity as normal biopsies in six HL samples with staining in middle and superficial spinous layer keratinocytes (see Table 2). Areas of hyperparakeratotic epithelium were CD 21 antigen-negative, and all cell layers were unreactive in six samples. Expression of EB-virus–associated B cell activation antigen CD 23 was uniformly negative throughout the epithelium in all HL and control samples.

Patterns of staining for MHC antigens and CAMs (Ta-

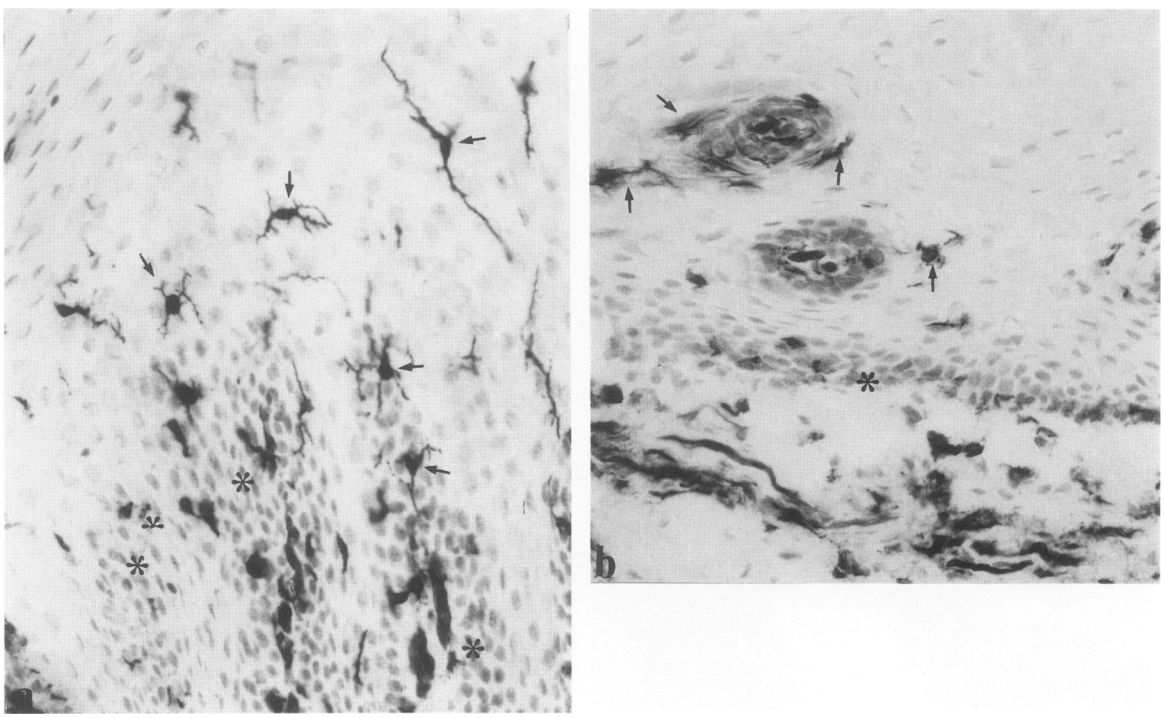

Figure 7. **a:** *HL biopsy (Case 11) stained for Class II MHC antigens (mAb 1B5) shows prominent numbers of dendritic LC type cells (→) in suprabasal sites adjacent to basal epithelium (*→). **b:** Class II MHC antigen staining in normal lateral tongue biopsy (HIV seronegative, EB virus seropositive control) demonstrates fewer numbers of less dendritic LC type cells (→) in suprabasal epithelium. Class II MHC antigens are also expressed on endothelial cells in both the HL and non-HL sample, and on basal epithelium in the normal control (*→). (Cryostat sections; PAP method, ×250).*

ble 3) were variable in individual HL and control samples. Whereas several HL tissues expressed class I antigens on all keratinocytes except in hyperparakeratotic epithelium, class II antigens were less commonly detected and were almost exclusively expressed on basal epithelium in three of nine samples. Small foci of suprabasal keratinocytes expressing class II antigens were present in one sample. In the control tissues, basal epithelium showed stronger class I antigen expression than suprabasal layers and class II antigen staining on basal cells only in normal tongue (Figure 7b), and no MHC antigen activity was demonstrable in the frictional keratosis samples. Dendritic cells consistent with epidermal Langerhans' cells (LCs) were identified in three HL samples by their morphophenotypic appearances with antibodies to class II (Figure 7a) and CD1a antigens. Langerhans cell aggregates appeared to be located either suprabasally at lymphoepidermotropic sites, or within areas of ballooned prickle cells in the upper epithelium. Langerhans cells appeared to be more numerous in these HL samples than in either of the normal tongue control biopsies (Figure 7b). The limited amount of tissue available, however, precluded extensive serial sectioning for quantitative studies to verify this observation.

Cell adhesion molecules phenotyping demonstrated that HL keratinocytes in 6 of 12 samples expressed LFA—3 (CD58) at all cell levels (except hyperparakeratotic areas), and seven samples lacked ICAM-1 (CD 54) activity, similar to normal tongue. Positive staining for ICAM-1 in five samples was either localized to foci of prickle cells or diffusely distributed throughout the epithelium. One of these latter samples contained a prominent inflammatory cell infiltrate in the lamina propria. Hemopoietic cell populations identified in 4 HL biopsies consisted of low to moderate numbers of activated (Class II antigen-positive) T lymphocytes, mainly of CD8 type, which showed varying degrees of epidermotropism.

Discussion

Identification of EB virus DNA and phenotypic expression of EB virus lytic infection in most HL samples of this study confirms the findings of others and supports the assumption that replicating EB virus has a causative role in HL. By using cytohybridization and immunocytochemical techniques, this study shows different patterns in the cellular distribution of DNA hybridization signals and viral gene products. In most samples, lytically infected cells, containing EB virus DNA, and co-expressing EBNA species, EBNA2, and lytic cycle antigens, EA, VCA and MA, occupied the middle and upper prickle cell layers of epithelium, whereas superficial keratinocytes with the most intense EB virus DNA hybridization signals were almost exclusively associated with cytoplasmic expression of viral structural antigens. Nuclear staining for EBNA was not detected in the hyperparakeratotic cells of superficial epithelium. Clearly identifiable but less intense signals for EB virus DNA were present in low numbers of suprabasal cells that failed to exhibit phenotypic evidence of viral infection. This topographic distribution of EB-virus–infected cells in HL tends to support the view that active viral replication is linked to epithelial cell differentiation. These patterns would be consistent with viral replication progressing in parallel with keratinocyte maturation, resulting in an accumulation of free virus particles in the upper epithelium before their release from the mucosal surface. The absence of detectable EBNA in superficial epithelial cells is consistent with the loss of EBNA expression associated with lytic conversion of latent infection in BLCLs *in vitro*.[30]

In normal squamous epithelium, active EB viral replication has been linked to keratinocyte differentiation by the patterns of viral gene expression and presence of EB virus DNA and EB virus RNA in exfoliated oropharyngeal cells from 80% of patients with IM, and in a small proportion of healthy seropositive individuals.[10] Concurrent salivary shedding of free virus indicated productive infection in these subjects, which in asymptomatic individuals attests to the long-term carrier state of EB virus infection *in vivo*. Abundant EB virus DNA and cellular expressions of lytic infection also have been identified in terminally differentiating cells of human cervical biopsies and primary cultures of ectocervical epithelium exposed to EB virus *in vitro*.[13,31] In B cells, active viral replication and release of free virus has not been confirmed *in vivo*. Productive infection occurs, however, albeit rarely, in BLCLs *in vitro*, and is thought to involve a minority population undergoing terminal differentiation.[30] The implication of normal squamous epithelium as a reservoir of EB-virus–infected cells in normal seropositive individuals has been thought to need a latently infected epithelial component for persistent infection to be maintained. Epstein–Barr virus latent gene expression is predominantly associated with B cells and is central to immortalization of BLCLs *in vitro*. Normal basal epithelial cells also may be capable of supporting EB virus latency,[10,31] but this has yet to be fully substantiated *in vivo*. Latent EB virus infection in epithelium was initially inferred by exclusive EBNA staining in ectocervical cell cultures exposed to EB virus *in vitro*.[13] Isolates of EB-virus–infected cultured keratinocytes corresponding to different maturational stages also have been shown to differentially express latent and lytic viral gene products. This has been taken as further evidence that different events of the EB virus cycle are influenced by the state of keratinocyte differentiation.[31] Latent membrane protein expression in isolated cells of some HL biopsies of this study has not been previously reported

and suggests that LMP may be transiently expressed at some stage of the lytic cycle in epithelial cells. The absence of detectable EB virus DNA and EB virus gene expression in the basal epithelium of these lesions, however, does not support the theory that latent infection occurs in undifferentiated cells at this level. Conversely, it is possible that low copies of EB viral genome are present in basal cells but below the level of detection by the nonisotopic DNA labeling methods used in this study.

The present findings show no evidence that EB virus infection appreciably alters normal phenotypic patterns of epithelial differentiation in HL. Involucrin and cytokeratin profiles in morphologically normal epithelium and areas of prickle cell vacuolation in most samples were essentially the same as normal control biopsies and consistent with nonkeratinizing squamous epithelium of oral mucosa,[32] which forms the lateral margins of the tongue. The profound absence of cytokeratin expression in superficial areas of HL probably reflects changes due to hyperparakeratinization and increased cell senescence rather than specific effects of EB virus infection. Similar losses in activity were noted with the relatively minor parakeratotic changes in superficial layers of control biopsies. Epstein–Barr virus infection has been suggested to confer increased growth capacity to HL epithelium.[31] This was not supported by the pattern of staining with the cell proliferation marker Ki 67, which in most HL samples showed the normal distribution confined to basal epithelium. Other immunohistologic studies have also failed to demonstrate increased keratinocyte proliferation in HL with recently generated MAbs to cytokeratins 6 and 16,[33] which are markers of high epithelial turnover.[22] Similarly CD 23 antigen, which acts as an autocrine growth factor in BLCLs and is released in soluble form by NPC tumor cells *in vitro,*[34] is not expressed in HL.

Epstein–Barr virus binding to epithelium involves CD 21/C3d receptor molecules that are constitutively expressed on surface and spinous layer keratinocytes of nonkeratinized squamous epithelium.[35,36] The pattern of CD 21 antigen expression in normal oral mucosa, including lateral tongue, closely corresponds to areas of EB virus DNA localization in HL. Epitopes of CD 21 molecules were not identified in 50% of HL samples in the present study, which is reminiscent of the loss of CD 21 antigen expression on B cells after EB virus infection. It may be significant, however, that HL samples containing CD 21 antigen-positive keratinocytes were all obtained from patients receiving antiviral or antifungal treatment at the time of biopsy. 'Regeneration' of CD 21 antigens may reflect an initial response to therapy, or up-regulation by EBNA2,[37] but does not appear to correlate with the overall clinical behavior of the lesions.

The intriguing site-specific nature of HL raises several questions about the mode of viral entry and pathogenesis of HL. Hairy leukoplakia characteristically develops on the lateral margins of tongue, but also may affect the ventral surface or buccal mucosa. From the pattern of keratinocyte differentiation and EB virus receptor expression, normal oral mucosa is a potential target for EB virus infection *in vivo.* In support of this, clinically normal mucosa from HIV-infected individuals has been found to contain EB virus DNA by standard *in situ* hybridization methods[6] and by DNA amplification using the polymerase chain reaction.[38] Apart from oropharyngeal epithelium, however, there is no evidence that EB virus replication is a regular occurrence in oral mucosa of healthy non–HIV-infected, EB-virus–seropositive individuals. The high amounts of free virus shedding from the oropharynx of HIV-infected individuals, and immunosuppressed patients in general, increases the potential for EB virus infection in oral mucosal epithelium. The susceptibility of lateral tongue margin to repeated minor trauma may explain the site predilection of HL. Subsequent parakeratotic changes in the surface epithelium, and possibly concurrent fungal infection, might provide a suitable local environment for EB virus entry, and might facilitate access to specific virus-binding receptor sites in the upper epithelium. The pattern of keratinocyte-dependent viral gene expression reported in this study indicates that, in addition to superficial lytic infection with active viral production, evidence of EB virus latent gene expression occurs in epithelial cells of a proportion of cases. These cells may be derived from a putative pool of cells with persistent latent infection in the basal epithelium, or, alternatively, may represent keratinocytes that become secondarily infected after replicative infection in HL is established. The source of these latently infected cells and their contribution to permissive viral infection in HL remains to be investigated.

The causative role of other viruses in HL is unresolved. Human immunodeficiency virus has not been demonstrated in these lesions and initial studies, indicating coinfection with human papilloma virus (HPV)[1] was not found subsequently to be a consistent feature of HL.[5] Uncommon HPV genotypes have been identified in non-HL oral mucosal lesions in HIV seropositive patients,[38] however, and recently hybridization for herpes simplex virus RNA has been reported in an HL-like lesion of an HIV-seronegative bone marrow graft recipient.[3] Hairy leukoplakia currently is thought to be an opportunistic infection by EB virus in an immunocompromised host in which several other infective agents may have a contributory role. The lack of an inflammatory response in HL, either as subepithelial lymphocytic infiltration or induction of class II antigens or ICAM-1 determinants on keratinocytes, may reflect impairment of cell-mediated immunity associated with HIV infection. The presence of inflammatory cell infiltrates in some samples of this study

also has been observed by others.[8] T cells probably control permissive EB virus infection in healthy immunocompetent individuals, because viral replication and production is increased with defective T cell functions in immunosuppressed states.[39] The extent to which the absence of a local T cell response contributes to persistent viral replication in HL is not known. Furthermore LC depletion, which has been suggested to be of pathogenic significance in HL,[40] was not observed in all biopsies examined. Quantitative studies with a larger series of normal control samples are required, however, to substantiate the observation from this study. Quantitative variations in LC occur at different squamous epithelial sites,[41] and their paucity in areas of thickened epithelium may explain the low numbers seen in normal tongue biopsies and absence in most HL samples.

Given the diagnostic importance of EB virus in HL, reliable methods for laboratory investigation are essential. The rate of EB virus detection in the present study was higher by *in situ* hybridization than by immunocytochemistry, although concordant results for EB virus DNA and tissue expression of viral gene products was obtained in eight cases. Sampling errors introduced by the limited amount of biopsy tissue available and differences in tissue section thickness required for each technique could account for the discrepancy between positive *in situ* hybridization results and absent immunocytochemical staining in two samples and failure to identify EB virus DNA or full viral gene expression in two cases. Nevertheless both technologies are within the scope of most routine laboratories. Because conventional methods of histological tissue preparation are not appropriate for EBNA detection, or many immunohistologic investigations, cryopreservation of surgical biopsies is recommended.

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