Absence of Langerhans Cells in Oral Hairy Leukoplakia, an AIDS-Associated Lesion

Troy E. Daniels, D.D.S., M.S., Deborah Greenspan, B.D.S., John S. Greenspan, B.D.S., Ph.D., Evelyne Lennette, Ph.D., Morten Schiødt, D.D.S., Dr. Odont., Vibeke Petersen, B.S., and Yvonne de Souza, M.S.

Department of Stomatology, School of Dentistry, University of California (TED, DG, JSG, MS, VP, YdeS), San Francisco, California; and Virolab Inc. (EL), Berkeley, California, U.S.A.

Oral hairy leukoplakia (HL) is a recently described manifestation of human immunodeficiency virus (HIV) infection in which Epstein-Barr virus (EBV) has been shown to replicate. To seek evidence for a local defect in mucosal immunity, we assessed the presence of epithelial Langerhans cells (LC) in these lesions and in autologous nonlesional mucosa. We used monoclonal antibodies against HLA-DR, HLA-DQ, and T6 antigens to identify LC in biopsy specimens of HL from 23 homosexual men. In all lesion specimens, LC either were not detected or were present only in greatly reduced numbers with at least 1 of the antibodies. In nonlesional oral mucosa from the same patients, LC were detected with all 3 antibodies in 11/12 specimens (92%) and were found in approximately normal numbers with at least 1 antibody. There was close correlation between the absence of LC and positive staining for EBV, human papillomavirus antigens, and candidal hyphae in the epithelium. We conclude that LC are absent or greatly reduced in the lesions of HL. Absence of normal LC function may be important in the pathogenesis of HL and may reflect an event in the pathogenesis of other features of the acquired immune deficiency syndrome. *J Invest Dermatol 89:178–182, 1987*

Oral hairy leukoplakia (HL), a newly described lesion, is associated with the ultimate development of acquired immune deficiency syndrome (AIDS) and clearly contains Epstein-Barr virus (EBV) and perhaps other intraepithelial viruses [1,2]. Found predominantly in immunosuppressed homosexual men and occasionally in others at risk for developing AIDS [3], the lesion appears clinically as white, irregularly surfaced patches on the lateral margins of the tongue. The lesion is usually bilateral and has a histologic appearance similar to that of the flat wart of skin or the uterine flat condyloma. The Centers for Disease Control have now placed HL in the new classification of infections associated with the human immunodeficiency virus (HIV) (group IVc—secondary infectious diseases) [4].

Human epithelial Langerhans cells (LC) participate in antigen processing in vitro and in vivo and, together with recirculating T lymphocytes and regional lymph nodes, appear to constitute a skin-associated lymphoid tissue [5]. They are derived from bone marrow precursors and can be recognized by electron microscopic observation of Birbeck granules and by several antigenic markers, including the histocompatibility antigens HLA-DR and HLA-DQ and the thymocyte antigen T6 (reviewed in [6]). Human LC also express the helper-inducer T-lymphocyte antigen (T4), weakly in normal skin [7] but strongly in various inflammatory conditions [8].

Langerhans cells are present, although with different densities, in essentially all areas of normal human skin and mucosa [9–11]. In patients with AIDS and opportunistic infections, the numbers of LC in clinically normal skin were reduced more than 60% compared with appropriate controls [12]. We have examined the numbers of LC expressing the antigenic markers HLA-DR, -DQ, and T6 in lesions of oral HL and in clinically normal oral mucosa from these patients. We then examined the relationship between the number of LC and the presence of viral antigens and candidal hyphae within the epithelium to find out if changes in LC might be involved in the pathogenesis of HL.

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Reprint requests to: Troy E. Daniels, D.D.S., School of Dentistry, University of California, San Francisco, California 94143-0424.

Abbreviations:
AIDS: acquired immune deficiency syndrome
EBV: Epstein-Barr virus
HIV: human immunodeficiency virus
HL: hairy leukoplakia
HPV: human papillomavirus
LC: Langerhans cell(s)
PAS: periodic acid-Schiff

MATERIALS AND METHODS

Subjects and Biopsy Patients for this study were drawn from a population previously described [1] and consisted of 23 homosexual men who had oral HL. All had lesions on the lateral margin of the tongue, and one also had lesions on the buccal mucosa. The patients showed evidence of immunodeficiency, in that T-lymphocyte helper/suppressor ratios were reduced in all of 13 patients tested (mean = 0.6, range = 0.1–1.1) and all of 22 patients tested showed absent or reduced delayed hypersensitivity skin response to 4 antigen preparations (purified protein derivative, *Candida*, mumps, and trichophyton). None had AIDS at the time of diagnosis, but 48% of the patients have since developed AIDS [13].

Incisional biopsy specimens were obtained from each lesion at the time HL was diagnosed. As control tissue, clinically normal mucosa from the lateral tongue posterior to the lesions (3 specimens) or from the cheek (9 specimens) were obtained from 12 of the patients at the same time as the lesion specimen. Lesion and control specimens were immediately frozen and stored in liquid nitrogen until immunohistochemical examination. Sections stained with hematoxylin and eosin were prepared from each specimen. Additional specimens from 9 lesions were prepared for electron microscopic examination by fixing in 3% glutaraldehyde, postfixing in 1% buffered osmic acid, and embedding in Epon.

Culture specimens were obtained from the oral mucosa of each patient before biopsy and 15/23 (65%) revealed the presence of *Candida albicans*. Six of the patients were receiving antifungal drugs at the time of biopsy or in the weeks preceding biopsy; 2 of these had positive culture specimens.

Detection of Langerhans Cell Antigens Langerhans cell antigens were detected by immunohistochemistry on sets of 10-μm thick cryosections cut from each lesion and control specimen, which ranged from 3–5 mm in width. Sections were placed on formal gelatin–coated slides, fixed in cold acetone for 10 min, and allowed to air dry. After washing (Tris hydrochloride buffer was used for this and all subsequent washes), sections were incubated in 3% normal horse serum for 20 min at room temperature, washed again, then incubated in primary antibody for 30 min. We used dilutions of commercially available mouse monoclonal antibodies: anti-HLA-DR and anti-Leu-10 (HLA-DQ) from Becton-Dickinson Inc., Mountain View, California and anti-OKT6 from Ortho Diagnostics, Raritan, New Jersey. In control reactions, we substituted mouse ascites fluid for monoclonal antibody. We labeled antibody reactions on serial sections of each specimen by 2 methods: avidin-biotin-peroxidase complex [14] and peroxidase/antiperoxidase [15]. Sections were counterstained with 2% methyl green and read by 2 independent investigators. An average of 3 serial sections were stained with each antibody and examined at 400× magnification along the entire length of each specimen.

For identification purposes, LC were defined as a peroxidase-stained cell body with at least 1 dendrite, located within the epithelium. We used the following semiquantitative grading scheme to assess the LC in each specimen: 3 = approximately normal [11], 2 = reduced (having approximately half the number of LC observed in oral mucosal specimens from healthy individuals or in normal-appearing mucosa from postmortem specimens), 1 = few (less than 5 LC per section), and 0 = no LC observed.

Detection of Virus Antigens The presence of human papillomavirus (HPV) antigen was detected in situ by peroxidase/antiperoxidase immunohistochemistry. Epstein-Barr virus antigens were detected by anticomplement indirect immunofluorescence using characterized human antisera, and by indirect immunofluorescence with 3 mouse monoclonal antibodies specific for EBV viral capsid antigen, early antigen-restricted component, and early antigen-diffuse component, respectively. These methods and their controls have been described in detail [1,2].

Detection of Fungal Hyphae An average of 5 sections of 21 lesion and 12 control specimens were stained with periodic acid-Schiff (PAS) to detect the presence of fungal hyphae within the epithelium.

Statistical Analysis To determine the statistical strength of association between the absence of LC and the presence of HPV or EBV antigens or fungal hyphae, we used the Kendall rank correlation coefficient, a nonparametric significance test for multiple independent variables.

RESULTS

Examination of hematoxylin and eosin–stained sections of the lesions revealed the characteristic histopathologic features of HL, which include acanthosis, irregular hyperparakeratosis, areas of koilocytic cells in the epithelium, and little or no inflammatory cell infiltration in the subepithelial connective tissue [1,2]. The control sections appeared within normal limits, showing a very mild subepithelial lymphocytic infiltrate.

In 11/12 (92%) of the control sections, LC were graded as 2 or 3 with at least 2 monoclonal antibodies; in only 1 specimen were they graded as 1 or 2 with all antibodies (Table I). There were no significant differences in number of LC between control specimens from the buccal mucosa and those from the tongue. In lesion sections, LC either were absent, or were present in reduced numbers with all antibodies. LC were not detected with any of the 3 antibodies (grade 0) in 9/23 (39%) of the lesion specimens, and in 11/23 (48%) they were not detected with at least 1 antibody and were present only in greatly reduced numbers (grade 1) with the others. Langerhans cells in the remaining 3 specimens were graded as 1 or 2 with each antibody (Fig 1). Control reactions with ascites fluid were negative in all specimens. There were no differences in grading of LC identified by either of the 2 peroxidase-labeling methods. There were no significant differences in number of LC between patients who later developed AIDS and those who did not.

Further evidence for the absence of LC came from electron microscopic examination, which revealed 1 Birbeck granule–containing cell in only 1 of 9 lesion specimens (after an average of 113 cells in the epithelium of each specimen had been examined). Langerhans cells in that 1 positive specimen were graded as either 1 or 2 with each antibody.

Human papillomavirus antigen was identified in epithelial cell nuclei of 16/22 lesion specimens, and all 3 EBV antigens were

Table I. Relation of Presence of Langerhans Cells (LC), as Determined by Three Markers, to Presence of Human Papillomavirus (HPV) and Epstein-Barr Virus (EBV) Antigens in Hairy Leukoplakia and Control Tissue

	Hairy Leukoplakia					Controls (autologous)				
	LC Antigens			Viral Antigens		LC Antigens			Viral Antigens	
Grades for Presence of LC	OKT6	HLA-DR	HLA-DQ	HPV	EBV	OKT6	HLA-DR	HLA-DQ	HPV	EBV
Grade 0 Specimens without LC	12	15	14	11/14	13/14	0	1	0	0	0
Grade 1 Specimens with <5 LC per section	9	6	7	4/6	5/7	0	0	1	0	0
Grade 2 Specimens with reduced LC[a]	1	2	2	1/2	1/2	4	1	1	0/4	1/4
Grade 3 Specimens with normal numbers of LC	0	0	0	0	0	5	10	7	0/8	0/8
Total number of specimens	22	23	23	22	23	9	12	9	12	12

[a]Approximately half the number of LC observed in oral mucosal specimens from healthy individuals or in normal-appearing mucosa from postmortem specimens [10].

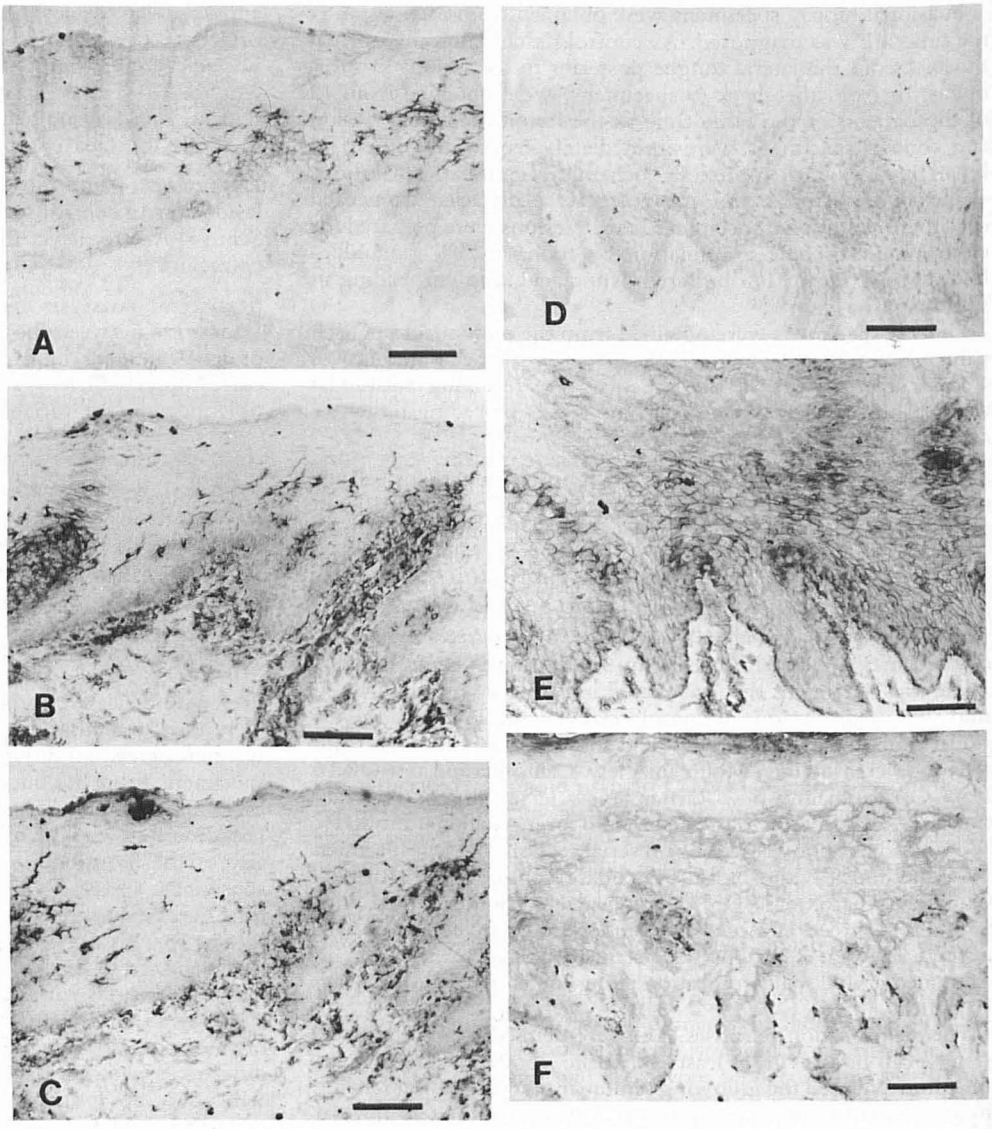

Figure 1. Absence of Langerhans cells (LC) in hairy leukoplakia. Semiserial sections of a clinically normal-appearing specimen of the buccal mucosa (*A–C*) and a specimen of hairy leukoplakia (*D–F*) from the same patient. Sections *A* and *D* are stained with anti-OKT6 and graded for LC as 3 and 1, respectively. Sections *B* and *E* are stained with anti-HLA-DR and graded for LC as 3 and 0, respectively. Note intense interepithelial cell staining in *E*. Sections *C* and *F* are stained with anti-Leu-10 (HLA-DQ) and graded for LC as 3 and 1, respectively. *Bars* = 100 μm.

identified in 19/23 lesion specimens. Human papillomavirus staining was absent from all 8 control specimens and EBV staining was absent in 7/8 controls. The 1 positive control was a specimen from the posterior lateral tongue overlying lingual tonsil tissue. There was close correlation between the absence of LC and the presence of HPV antigen ($p = 0.0006$) and EBV antigens ($p < 0.00001$).

Periodic acid-Schiff–positive fungal hyphae consistent with *C albicans* were present in the superficial epithelium of 10/21 (48%) of the lesion specimens but in none of 12 control specimens. In serial sections from each lesion, hyphae were either present in all or absent in all sections. Hyphae were present in 6/13 lesion specimens with grade 0 LC, 3/6 lesion specimens with grade 1 LC, and 1/2 lesion specimens with grade 2 LC. There was a significant correlation between the absence of LC and the presence of fungal hyphae ($p = 0.02$). In the 12 patients who provided control biopsy specimens (showing no fungal hyphae and grade 2 or 3 LC), the corresponding lesion specimens showed hyphae in 7/12 (58%) of cases.

Interepithelial cell staining was seen with anti-HLA-DR, but not with anti-Leu-10 or anti-OKT6, in 12/23 lesion and 6/12 control specimens. This staining was diffuse and intense in 3 lesion specimens (Fig 1*E*) and 2 control specimens, but patchy and less intense in the rest. In most cases, there was a similarity in the presence or absence of this staining between lesion and control specimens, but in others there was no association (Fig 1*B, E*).

DISCUSSION

Hairy leukoplakia is a specifically HIV-associated lesion found in several of the adult AIDS risk groups and is highly predictive of the development of AIDS [13]. It had never been described before the AIDS epidemic and has not been reported outside the AIDS risk groups. It is the first form of oral leukoplakia that shows consistent evidence for the presence of a virus, although HPV is found in some cases of the common forms of leukoplakia [16]. Hairy leukoplakia is the first lesion in which EBV is consistently found as a fully expressed virion, although EBV-DNA and some EBV antigens are found in Burkitt's lymphoma, nasopharyngeal carcinoma, and some other carcinomas [17–19]. Hairy leukoplakia also demonstrates that persistent, productive EBV infection can occur in oral epithelial cells [2].

Hairy leukoplakia is an epithelial hyperplasia of the oral mucosa and may contain papillomavirus in addition to EBV. No examples of malignant transformation of HL have been described. The absence of LC, which we report for the first time in this article, and coexistent systemic immunosuppression caused by HIV appear to be etiologically involved. It is not clear in what order and to what extent these factors contribute to the pathogenesis of HL. One possible schema is as follows: HIV initially infects circulating helper/inducer T lymphocytes. The T4 antigen molecule, common to both the circulating helper/inducer T lymphocytes and LC [7,8], is a receptor for HIV [20]. Since T4-bearing lympho-

cytes remain chronically infected or are destroyed by HIV [21], it is possible that epithelial LC or their precursors may be directly involved in the progression to HL. Loss of T4 lymphocyte function may also result in failure of lymphokine production upon which LC may depend for migration, antigen expression, or other functions. The lateral tongue has small (0.2–0.5 mm in diameter), recurring areas of epithelium that lack LC [11]. These small LC-free sites could conceivably have deficient antigen-processing ability, as noted previously in rodents [22]. These sites could then allow initial colonization by EBV or HPV (which would explain why the lesion occurs on the lateral margins of the tongue but rarely elsewhere). Loss of functioning T4 lymphocytes and LC (in areas adjacent to those naturally lacking LC) could then allow the virus to proliferate causing the epithelial hyperplasia of HL.

The characteristic absence of subepithelial inflammatory cell infiltration in HL lesions is especially interesting, given the presence of viral and fungal antigens within the epithelium. We have previously demonstrated that T6-positive LC and candidal hyphae coexist, but with marked subepithelial inflammation, in lesions of chronic hyperplastic candidiasis from otherwise healthy individuals [23]. Oral candidiasis is a frequent feature of patients at risk for developing AIDS [24] and of lesions of HL [1]. In the current study, the presence of candidal hyphae in lesion specimens and their absence in corresponding control specimens indicates a localized defect in some component of the mucosal immune response. This defect could be a result of loss of LC function, or of deficient T-helper lymphocyte function, or both. It is also possible that the passage of fungal or viral antigens through an epithelial surface deficient in LC could lead to the induction of antigen-specific tolerance, as was previously observed when chemical antigens were applied to LC-deficient epithelium [22]. Such tolerance could be a mechanism for disarming the host's immune system, contributing to the absence of inflammation in the HL lesions.

Epithelial cell expression of HLA-DR antigen has been observed in a variety of diseases in which there is prominent inflammatory cell infiltration [25–27]. In apparent contrast to those observations, many of our HL specimens showed mild to intense interepithelial-cell expression of HLA-DR, with no subepithelial inflammation. However, control specimens with HLA-DR expression had mild to moderately severe subepithelial lymphocytic infiltration. In general, the presence and intensity of epithelial HLA-DR expression were similar in lesion and control specimens from the same patient. There was no association in either lesion or control specimens between the presence or extent of this HLA-DR expression and the presence or absence of LC. There is no clear explanation for this epithelial expression of HLA-DR in HL, but several factors may be involved. Expression of HLA-DR by keratinocytes is modulated in vitro by gamma interferon [28], which is normally produced by T lymphocytes. In the presence of viruses and the absence of normal mucosal immune response in lesions of HL, it may be that the epithelial cells are able to express HLA-DR without the presence of lymphocytes, perhaps because gamma interferon is available from other sources, or because other substances can induce HLA-DR expression on human epithelial cells.

It remains to be seen whether the absence of LC, which we here report in the lesions of HL, is also a feature of other mucosal and skin lesions in individuals with HIV infection.

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