Oral Microbiology Immunology 2005: 20: 154–162
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

Lack of evidence for local immune activity in oral hairy leukoplakia and oral wart lesions

E. A. Lilly[1,4], J. E. Cameron[1,4], K. V. Shetty[2,4], J. E. Leigh[2,4], S. Hager[3], K. M. McNulty[1,4], C. Cheeks[3], M. E. Hagensee[1,4], P. L. Fidel Jr[1,4]

Departments of [1]Microbiology, Immunology, and Parasitology, [2]General Dentistry and [3]Medicine, Section of Infectious Disease, [4]Center of Excellence in Oral and Craniofacial Biology, Louisiana State University Health Sciences Center and School of Dentistry, New Orleans, LA, USA

Lilly EA, Cameron JE, Shetty KV, Leigh JE, Hager S, McNulty KM, Cheeks C, Hagensee ME, Fidel PL Jr. Lack of evidence for local immune activity in oral hairy leukoplakia and oral wart lesions.
Oral Microbiol Immunol 2005: 20: 154–162. © Blackwell Munksgaard, 2005.

Background: Oral warts, caused by human papillomavirus (HPV), and oral hairy leukoplakia (OHL) caused by Epstein-Barr virus (EBV), are common oral manifestations in HIV-infected persons. Although both conditions occur most often with reduced blood $CD4^+$ T-cell numbers, oral warts and OHL rarely occur simultaneously, suggesting that dysfunctions in other secondary local immune parameters are also involved. The present study evaluated tissue-associated proinflammatory and T-helper cytokine and chemokine mRNA expression and the presence of T cells in each lesion.
Methods: Biopsies were taken from lesion-positive and adjacent lesion-negative sites of HIV^+ persons with oral warts or OHL and lesion-negative sites from HIV^+ persons who were oral HPV or EBV DNA-positive (matched controls). Cytokine/chemokine mRNA expression was quantified by real-time polymerase chain reaction. CD3, CD4, and CD8 cells were identified by immunohistochemistry.
Results: No differences were detected in tissue-associated cytokine/chemokine mRNA expression in warts or OHL when compared to lesion-negative sites. Immunohistochemical analysis of T cells showed $CD8^+$ cells exclusively, but few cells were present in either lesion. No differences were detected between lesion-positive and -negative control sites of each pathologic condition.
Conclusion: Little evidence was found for local immune reactivity to either oral warts and OHL, suggesting that $CD4^+$ T cells are a primary host defense against both oral warts and OHL, but with nonimmune factors potentially responsible for the divergent prevalence of each.

Key words: cytokines; HIV; oral hairy leukoplakia; oral warts; T cells

Paul L. Fidel, Jr, Center of Excellence in Oral and Craniofacial Biology, Louisiana State University School of Dentistry, 1100 Florida Ave., New Orleans, LA 70119, USA
Tel.: +1 504 670 2734;
fax: +1 504 670 2736;
e-mail: pfidel@lsuhsc.edu
Accepted for publication October 23, 2004

Oral manifestations are common in HIV-infected persons. Oral candidiasis is the most common infection, followed by several viral lesions, including oral warts and oral hairy leukoplakia (OHL) (9, 10, 12). Oral warts, caused by human papillomavirus (HPV), can present in almost any location in the mouth as single, raised cauliflower-like lesions, termed squamous papillomas, or condylomas, which appear as multiple soft lesions. Approximately 2% of HIV^+ individuals will develop oral warts sometime during progression to AIDS. Oral HPV infection is more common in HIV^+ than in HIV^- persons; approximately 36% of HIV^+ persons are HPV DNA-positive, compared to 6% of HIV^- individuals (4). Hence, oral warts are extremely rare in HIV^- persons. OHL is characterized by abundant Epstein-Barr virus (EBV) replication and presents as white hair-like projections on the lateral border of the tongue (13, 14, 24). Like HPV, oral EBV is more common in HIV^+ persons; 42% of HIV^+ individuals are oral EBV DNA-positive, compared to 17% of HIV^- persons (1, 2). OHL therefore is equally rare in HIV^- persons. The introduction of highly active antiretroviral therapy (HAART) has been accompanied by a reduction in the number of OHL cases. However, the incidence of oral warts has increased, suggesting a

non-HPV-specific immune reconstitution or immune reconstitution syndrome (8, 16, 17).

The mechanisms by which HIV may cause an increase in the incidence of OHL and oral warts are poorly understood. The fact that these lesions are primarily and almost exclusively observed in HIV^+ individuals with low $CD4^+$ T cell counts (< 400 CD4 cells/ml) suggests that they are directly associated with T-cell immunosuppression. However, these lesions rarely occur simultaneously, suggesting that unique secondary local immune dysfunction(s) are also involved. Added to this are any effects of HIV alone may have on local immunity. In studies comparing HIV^- persons to HIV^+ persons without oral lesions, HIV has been shown to enhance oral tissue-associated chemokine mRNA (19, 26), with minor effects on cytokine mRNA (26).

This aspect of unique local immune dysfunction was recently suggested for oropharyngeal candidiasis. One observation showed a predominant Th2-type cytokine profile in the saliva of infected HIV^+ persons, a profile that is associated with susceptibility to mucosal candidiasis (18). Immunohistochemical analyses of tissue lymphocytes in the lesions of those with reduced $CD4^+$ T cells revealed an accumulation of $CD8^+$ T cells, but at a considerable distance from *Candida albicans* (22). The analyses were supported by increased CD8 cell-associated cytokine and chemokine mRNA expression in the lesion compared to HIV^+ persons without oropharyngeal candidiasis (19). From these data it was concluded that although $CD4^+$ T cells are the primary host defense against oropharyngeal candidiasis, $CD8^+$ T cells may serve as a secondary defense mechanism, but are defective or dysfunctional in their ability to interact with the organism.

The purpose of this study was to evaluate similar local immune parameters in oral warts and OHL through tissue-associated cytokine and chemokine mRNA and analysis of T cells in tissue, to gain a better understanding of the role of local immunity in the divergent prevalence of the two lesions.

Materials and methods

Subjects

Subjects were recruited and evaluated at the Louisiana State University Health Sciences Center (LSUHSC) HIV Outpatient Program (HOP) of the Medical Center of Louisiana at New Orleans and the Charity Hospital Dental Clinic. Informed consent was obtained from each participant, and all procedures were conducted in accordance with the Institutional Review Board at LSUHSC, New Orleans and the research committee of the HOP. Subjects were part of an established cohort (n=379) consisting of HIV-infected persons without lesions and those with oropharyngeal candidiasis, oral warts or OHL. A subset of this cohort was chosen for this analysis, including HIV^+ individuals who were lesion-negative with the appearance of healthy mucosa but who tested positive for oral HPV DNA (buccal mucosa) (n=14) or oral EBV DNA (tongue) (n=4), individuals who had oral warts [labial mucosa (n=4), buccal mucosa (n=3), lip (n=5)], and individuals with OHL (tongue) (n=11) persons. Sixty-one percent of HIV^+ persons in the subset were receiving highly active antiretroviral therapy (HAART). HAART was defined as three or more antiretroviral drugs. Other regimens were classified as non-HAART or ART. HIV^- healthy volunteers (n=5) were used in specified controls.

Diagnosis of oral warts and OHL

The diagnosis of oral warts was made on the clinical appearance of a solitary, raised cauliflower-like lesion or multiple soft lesions that can present on the tongue, lips, buccal mucosa or labial mucosa. The presence of white hair-like projections on the lateral border of the tongue was diagnostic for OHL. Biopsies were sent to the pathology department for examination to confirm the clinical diagnosis. Inclusion of patients was dependent on the positive pathologic diagnosis.

Sample collection and processing

Blood

Venous blood (10 ml) was collected from each subject. HIV status was verified in serum by enzyme-linked immunosorbent assay, followed by Western blot through the Clinical Immunology Laboratory at the LSU Health Sciences Center. CD4 lymphocyte counts were quantified by flow cytometry. Peripheral blood lymphocytes from healthy individuals were isolated by differential centrifugation using Ficoll Paque (Amersham Pharmacia Biotech, Piscataway, NJ), adjusted to 4×10^6 cells/ml, and incubated with phytohemagglutinin (PHA) (1000 μg/ml) for 24 h at 37°C in AIM-V Medium™ (Gibco, Invitrogen, Carlsbad, CA). The cells were harvested and RNA extracted using Ultraspec RNA Isolation Reagent (Biotecx Laboratories, Houston, TX) for use in a standard curve for real-time polymerase chain reaction (PCR).

Biopsy and tissue processing

Elliptical biopsies were taken from the lip, labial mucosa or buccal mucosa of HIV^+ $Wart^+$ individuals, the tongue of HIV^+ OHL^+ individuals, and from an adjacent uninfected site of the same tissue type, which served as a negative control. Biopsies were also taken from the buccal mucosa or tongue of HIV^+ lesion-negative individuals who tested positive for oral HPV or EBV DNA (matched controls). For real-time PCR analysis, tissue was placed in 4 ml of Hanks' Balanced Salt Solution (HBSS) (Gibco) for transport. Once in the laboratory, tissue was homogenized in 500 μl of Ultraspec RNA Isolation Reagent. RNA extracted from tissue and cultured peripheral blood lymphocytes was stored in frozen isopropanol until precipitated and resuspended in nuclease-free H_2O for cDNA synthesis. For immunohistochemical analysis, tissue was oriented for cross-sectional analysis in Tissue-Tek cyromolds (Miles Corp., Elkhart, IN), by using optimum cutting temperature (OCT) medium (Sakura Finetek USA, Inc., Torrance, CA). Tissue was snap-frozen and stored at − 80°C. Frozen tissue was sectioned (10 μm), collected on glass slides, fixed in acetone (5 min), and stored at − 20°C.

PCR detection of oral HPV and EBV DNA

HPV DNA detection

Saliva and tissue DNA extracts were tested for HPV DNA by a multiplex PCR reaction containing HPV L1-consensus primers (PGMY09/11) and β-globin primers (GH20/PC04) to test for sample adequacy. Primers and PCR reagents were kindly supplied by Roche Molecular Systems (Alameda, CA), and reactions were conducted according to Roche protocol (7). PCR contamination was avoided by utilizing separate areas for DNA extraction, PCR setup and gel electrophoresis. A master PCR mix was prepared for each PCR run, and water blanks were inserted after every fourth sample to detect contamination. Extracted DNA from cultured SiHa cells was used as an HPV^+ positive control. After 40 amplification cycles, PCR products which demonstrated the 254 base-pair β-globin band and the ~450 base-pair HPV band were considered HPV positive.

Herpesvirus DNA detection

Saliva and tissue DNA extracts were tested for herpesvirus DNA by a consensus PCR assay containing primers specific for the DNA polymerase gene of herpes simplex virus (HSV) types 1 and 2, Epstein-Barr virus, cytomegalovirus (HCV), and Kaposi's sarcoma herpesvirus. The assay was modified from Johnson et al. (15). The primer sequences were as follows: HSV-P1, 5-GTGGTGGACTTTGCCAGCCTG-TACCC-3 and HSV-P2 5-TAAACATG-GAGTCCGTGTCGCCGTAGATGA-3. A master mix containing Amplitaq Gold polymerase buffer, 1.25 mM $MgCl_2$, 250 μM deoxynucleoside triphosphates, 1 μM HSV-P1 and HSV-P2 primers, and 2.5 U Amplitaq Gold was used for each reaction (45 μl each), to which 5 μl of template DNA was added. Water blanks were inserted after every fifth sample as controls for indicating contamination. Positive controls included cell lines infected with HSV-1 strains McKrae and syn, and HSV-2 strain 333 kindly provided by Dr. J. Hill, LSU Lions Eye Center. EBV-positive Burkitt lymphoma B cell line (EB-1) was purchased from the American Type Culture Collection. CMV-positive patient culture was obtained from Dr. G. Sloop, Medical Center of Louisiana, New Orleans. KSHV-positive cell line (BCBL-1) was generously donated by the National Institute of Health AIDS Research and Reference Reagent Program. Each PCR run was subjected to 40 rounds of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and elongation at 72°C for 1 min. PCR products which demonstrated the herpesvirus amplicon (532–604 base-pairs) were considered positive. Herpesvirus-positive PCR products were subsequently typed by restriction fragment length polymorphism using *Bam*H1 or *Bst*U1 enzymes.

Real-time PCR

Predeveloped primers (for amplification) and probes (for detection of product) (Applied Biosystems, Foster City, CA) for each cytokine/chemokine (interferon (IFN)-α, interleukin (IL)-12, IL-4, IL-10, IL-1α, IL-6, tumor necrosis factor (TNF)-α, IL-2, IL-15, IFN-γ-inducible protein (IP)-10, RANTES, MCP-1, IL-8) were combined with TaqMan Universal PCR Master Mix (Applied Biosystems) for each reaction. cDNA was synthesized from total RNA using the TaqMan reverse transcription reagents (Applied Biosystems). Real-time PCR was performed in the iCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA). Two fluorescent probes, FAM-490 (target gene) and VIC-530 (housekeeping gene), were detected for each cDNA sample. PCR product was measured in consecutive cycles (real time) through a total of 40 cycles during each reaction using the iCycler iQ Multi-Color Real-Time PCR Detection System Software. Human cyclophilin was used as the housekeeping gene. For each cytokine/chemokine, an initial control experiment was performed whereby nuclease-free water, cDNA from PHA-stimulated peripheral blood lymphocytes and unstimulated peripheral blood lymphocytes (both from HIV^- healthy volunteers), and *C. albicans* cDNA were run in both singleplex and multiplex reactions with the respective primers to check for reagent compatibility. In each case, no amplification of nuclease-free water or *Candida* cDNA was detected, and amplification of peripheral blood lymphocytes cDNA was similar in both singleplex and multiplex reactions. cDNA from PHA-stimulated peripheral blood lymphocytes was used to generate a relative standard curve. Nuclease-free water was used as a negative control in each reaction. Unknown cDNA samples were quantified using the standard curve and normalized to cyclophilin cDNA. cDNA from unstimulated peripheral blood lymphocytes of HIV^- healthy volunteers were also quantified according to the standard curve and used as a calibrator to control for interassay variability. Finally, a ratio of the unknown sample to the calibrator was computed and data for each cytokine/chemokine was expressed as a relative amount.

Immunohistochemistry

Hematoxylin and eosin

Tissue sections were stained with hematoxylin and eosin (Biochemical Sciences, Inc., Swedesboro, NJ) according to the manufacturer's instructions in order to confirm tissue orientation.

Chromophore staining of cell surface antigen

Serial sections of lip and tongue biopsies were rehydrated in phosphate-buffered saline (PBS) for 5 min. The endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide (peroxidase blocking reagent; R & D Systems, Minneapolis, MN) for 5 min. After washing with PBS, nonspecific protein-binding sites in the tissue were blocked by incubating the tissue in normal mouse serum (R & D Systems) for 15 min. This was followed by the addition of two additional blocking reagents, avidin−0.1% sodium azide (avidin blocking reagent) (R & D Systems) and biotin−0.1% sodium azide (biotin blocking reagent) (R & D Systems) (each for 15 min) with a wash with PBS in between. Following blocking, the sections were washed with PBS and incubated overnight at 4°C with monoclonal mouse anti-human CD3, CD4, or CD8 antibodies (10 μg/ml; Dako Corp., Cambridge, MA). Negative controls consisted of tissue incubated with isotype-specific, purified mouse immunoglobulin (Dako Corp.). After overnight incubations, the slides were washed in PBS and incubated with a biotinylated goat anti-mouse immunoglobulin G (IgG; 10 μg/ml; R & D Systems) for 30 min. Thereafter, the sections were washed and incubated for 30 min with high-sensitivity streptavidin-horseradish peroxidase conjugate (R & D Systems). The substrate 3-amino-9-ethylcarbazole chromogen (AEC) (R & D Systems) was added to washed sections for 5–10 min. Mayer's hematoxylin (Sigma Diagnostics, St. Louis, MO) was used to counterstain. Slides were preserved using aqueous mounting medium (R & D Systems).

Tissue-associated lymphocyte analysis

Images of tissue-associated T cells in lip, buccal mucosa, and labial mucosa (wart) and tongue (OHL) tissue were acquired by a Nikon Eclipse E600 microscope. Defined areas (37,328 μm/each) representative of epithelium and lamina propria for each tissue (visualized at 10× magnification) were quartened off sequentially (five areas per slide), and the positively stained T cells in each area were painted using MetaView software (Universal Imaging Corp., Downingtown, PA). Three slides were examined from the lesion-positive and healthy tissue of each patient. A percent threshold of tissue-associated $CD8^+$ T cells was then determined per unit area (37,328 μm) for lesion-positive and adjacent healthy tissue from each patient. Samples from all available patients were included.

Statistics

Differences in the relative amounts of tissue-associated cytokine/chemokine in lesion-positive and healthy tissue were determined by the Mann–Whitney *U*-test. Differences in percentage threshold $CD8^+$ cells per unit area between lesion-positive and healthy tissue were determined by the

Student's *t*-test. Significant differences were defined as $P<0.05$ using a two-tailed test. All statistics were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

Results

Systemic T cell numbers and effect of HAART and HIV viral load on oral warts and OHL status

Information about the demographics, systemic immune status, HAART, and HIV viral load is provided in Table 1. The majority of subjects were male with a distribution of race. CD4, but not CD8, cell numbers were depressed, with the most significant decline being found in those with EBV or OHL. Roughly, at least half of the subjects in each group were on HAART. A minority of those with oral warts and/or oral HPV alone had HIV viral loads greater than 50,000 copies/ml, whereas approximately 50% of those with OHL and/or EBV alone had viral loads greater than 50,000 copies/ml. Of those without lesions, approximately 50% were coinfected orally (positive for both HPV and EBV DNA).

Tissue-associated cytokine mRNA expression in oral warts

To begin to evaluate differences in local immunity in oral warts, wart and lesion-negative healthy tissue from the same patient was evaluated for cytokine/chemokine mRNA expression. Results in Fig. 1 show no differences between the wart and adjacent lesion-negative, healthy-appearing tissue of the same patient for any of the cytokines or chemokines. Results were similar when wart tissue from the labial and buccal mucosa was compared to buccal mucosa from HPV^+ lesion-negative matched controls. The only exceptions were increased IL-1α and TNF-α in warts (data not shown).

T cells in oral warts

As another measure of local immune reactivity, we evaluated the presence of $CD3^+$, $CD4^+$, and $CD8^+$ cells in oral warts. Representative images from a lip wart biopsy and adjacent lesion-negative healthy site are shown in Fig. 2A and B. Both the wart and adjacent healthy sites showed small numbers of $CD3^+$ cells distributed between the epithelium and lamina propria. Most of the $CD3^+$ cells matched the presence of $CD8^+$ cells. The majority of $CD4^+$ cells did not appear to be $CD3^+$. Similar results were observed for warts on the buccal mucosa, and on labial mucosa and buccal mucosa from lesion-negative matched control patients (data not shown). These results were confirmed by quantitative analysis of positively stained cells, which showed no significant differences in the numbers of $CD8^+$ T cells between the lesions and adjacent lesion-negative healthy sites (Fig. 2C).

Tissue-associated cytokine mRNA expression in OHL

To assess differences in local immunity during OHL, lesion and adjacent lesion-negative, healthy-appearing tongue tissue from the same patient was evaluated for cytokine/chemokine mRNA expression. There were no differences between the lesion and healthy sites for any of the cytokines or chemokines (Fig. 3). Similar results were observed when OHL^+ tongue tissue was compared to tongue tissue from EBV^+ lesion-negative matched controls. The exception was decreased IL-1α (data not shown) in OHL lesions.

T cells in OHL lesions

$CD3^+$, $CD4^+$, and $CD8^+$ cells were also evaluated in OHL tongue lesions compared to healthy lesion-negative adjacent sites. Representative tongue images in Fig. 4A (OHL lesion) and Fig. 4B (adjacent lesion-negative healthy site) show that $CD3^+$ cells again matched $CD8^+$ cells rather than the $CD4^+$ cells. $CD8^+$ cells were sparsely present at the lamina propria interface and in the epithelium of both the lesion and adjacent healthy tissue. $CD4^+$ cells were present only in the lamina propria in both lesion and healthy sites. Similar results were found for lesion-negative tongue tissue from matched control patients (data not shown). These results were confirmed by quantitative analysis of positively stained cells, which showed no significant differences in the numbers of $CD8^+$ T cells between lesions and adjacent lesion-negative healthy sites (Fig. 4C).

Discussion

Oral warts and OHL continue to be common manifestations in HIV^+ persons, second only to oropharyngeal candidiasis. While the incidence of OHL has decreased due to HAART, cases of oral warts have increased, according to some reports (8, 17). There is a paucity of information on the local immune reactivity in oral warts and OHL. Although both are most common under conditions of reduced CD4 cell numbers, the infrequent simultaneous presence of both lesions suggests that local mechanisms are also involved. This emphasizes the need to understand the local immune response(s) in each.

We recently reported on the effects of HIV and systemic $CD4^+$ T-cell numbers on tissue-associated immune mediators in this cohort (19, 22). The studies showed that the presence of HIV in the absence of any oral lesions resulted in enhanced tissue-associated chemokine (IP-10, RANTES, IL-8), but not cytokine, mRNA expression, and none was further influenced by blood CD4 cell numbers ($n<200$ vs $n\geq200$ cells/ml) (19). Finally, immunohistochemical analysis found no differences in T-cell distribution in buccal mucosa tissue from HIV^- and HIV^+ individuals irrespective of blood CD4 cell number (22). In fact, $CD4^+$ T cells were virtually absent in the tissue, leaving only $CD8^+$ T cells. With these data already established for this cohort, this study began by examining immune reactivity in oral warts and OHL using adjacent lesion-negative healthy tissue from

Table 1. Demographics, immune status, and HIV viral load in the cohort

	Group			
	HPV^+ $lesion^-$ (n = 14)	$Wart^+$ (n = 12)	EBV^+ $lesion^-$ (n = 4)	OHL^+ (n = 11)
Gender	71% male	92% male	100% male	91% male
Race	36% C[a]	58% C	25% C	64% C
	57% AA[b]	33% AA	75% AA	36% AA
	7% H[c]	8% H		
% with CD4 < 500 cells/ml	64%	92%	75%	91%
Mean CD4 (cells/ml)	329	310	186	210
Mean CD8 (cells/ml)	900	1005	988	745
% on HAART	42%	58%	75%	55%
% Viral load > 50,000 copies/ml	17%	17%	50%	55%
Mean viral load (copies/ml)	55,960	16,145	180,462	84,968

[a]Caucasian. [b]African American. [c]Hispanic.

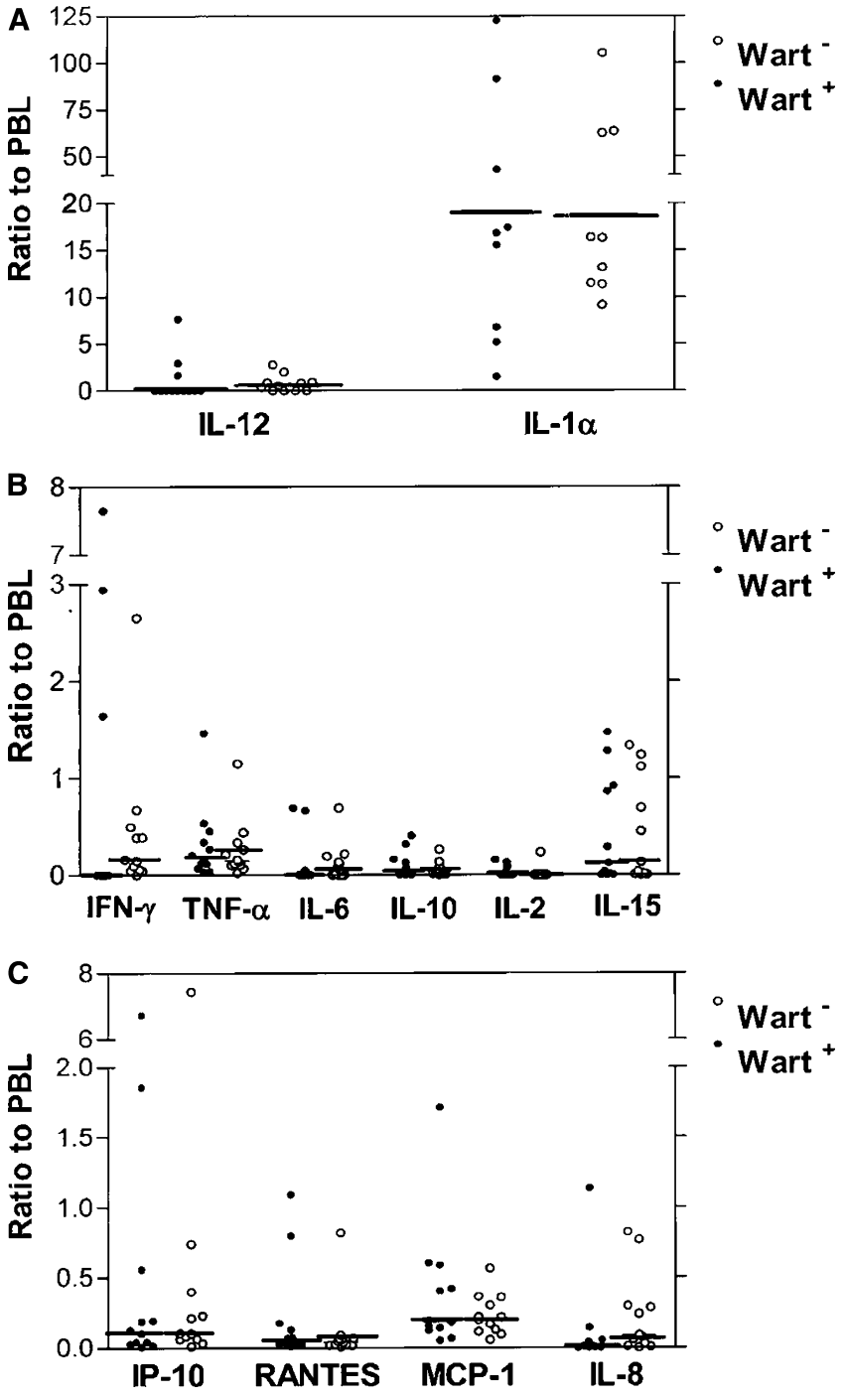

Fig. 1. Tissue-associated cytokine/chemokine mRNA expression in oral warts. Cytokine/chemokine mRNA expression was evaluated in the warts and adjacent lesion-negative buccal mucosa, labial mucosa, and lip biopsy tissue of HIV^+ patients (n=12). Results are expressed as a ratio to unstimulated peripheral blood lymphocytes (PBL) from healthy HIV^- persons with medians illustrated. A) Cytokine mRNA concentrations generally higher than unstimulated PBL. B) Cytokine mRNA concentrations generally below unstimulated PBL. C) Chemokines.

the same patient as well as lesion-negative persons with confirmed oral HPV or EBV DNA (infected) as matched controls.

No differences were found in tissue-associated cytokine/chemokine mRNA expression between warts or OHL and adjacent lesion-negative sites. Although adjacent positive and negative sites from the same patient may present some problems as controls due to the close proximity of the infected and clinically healthy tissue biopsies, similar results with few exceptions were observed when compared to tissue from lesion-negative matched control patients. Thus, it appeared that lesion-negative sites from each patient served as an effective control. However, in the case of HPV we recognize that, since buccal mucosa was examined exclusively from HPV^+ lesion-negative patients [due to issues of consent for other more sensitive biopsy sites (i.e. lip, labial mucosa)], results may differ with the inclusion of additional sites. In the case of EBV, for similar reasons of consent, the number of lesion-negative tongue biopsies was small and results may have been different with higher patient numbers. We feel therefore that it is premature to suggest that the increased IL-1α and TNF-α in warts compared to HPV^+ lesion-negative persons, and the decreased IL-1α in OHL compared to EBV^+ lesion-negative persons, represent true differences, when this is not further supported by evidence of cellular activity or inflammation from immunohistochemical analyses.

Indeed, the finding of a lack of differences in tissue-associated cytokines was supported by low numbers of T cells in warts and OHL. In all cases, $CD8^+$ T cells were matched more closely to $CD3^+$ T cells than to $CD4^+$ T cells, similar to the results found for HIV^- or HIV^+ persons with and without oropharyngeal candidiasis (22). In the case of OHL, like oropharyngeal candidiasis, $CD4^+$ T cells were absent, presumably due to their lack of availability under immunocompromised conditions, whereas $CD4^+$ $CD3^-$ cells are most likely macrophages or dendritic cells (22). In warts, one might have expected some level of tissue $CD4^+$ T cells in HPV^+ persons based on generally higher numbers of blood $CD4^+$ T cells, but this was not the case. At any rate, the overall lack of immune reactivity in warts and OHL is supported further by the small differences detected in Th1, Th2, and proinflammatory cytokines in saliva between patients with warts or OHL and matched controls (Fidel, unpublished).

Although HAART has been associated with an increased incidence of oral warts, HAART had no demonstrable influence on the local immune responsiveness, or lack thereof, in this cohort. This was readily evaluable since 61% of the cohort was defined as being on HAART. Alternatively, protease inhibitors and antiviral drugs on HPV replication may play more of a role. Irrespective of this, there are few data to support that an increase in oral warts under HAART is due to immune reconstitution and thus oral warts

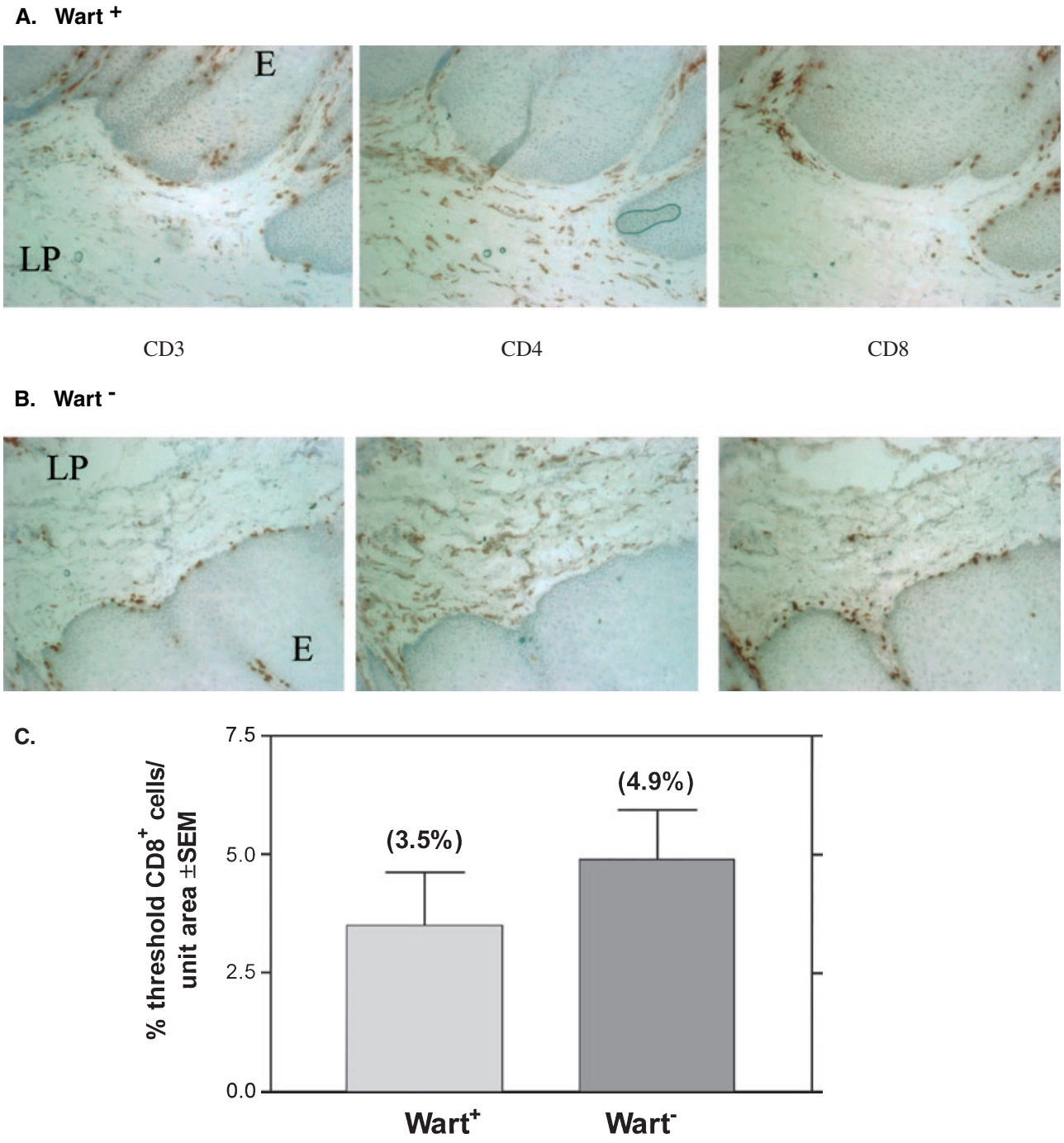

Fig. 2. Distribution of T cells in (A) warts and (B) adjacent lesion-negative lip biopsies of HIV^+ individuals. Representative images of a wart and adjacent wart-negative site from 10 HIV^+ patients examined. Magnification 10×. LP, lamina propria. E, epithelium. C) Threshold analysis of $CD8^+$ cells in lip, buccal mucosa, and labial mucosal warts and adjacent healthy tissue of the same patient (n=5). A percentage threshold of $CD8^+$ cells in warts and lesion-negative sites was quantified per unit area (37,328 μm) for all patients tested. Mean threshold provided in parentheses.

would not be classified as an immune reconstitution disease.

In contrast to warts and OHL, oral candidiasis lesions had increases in RNA for several cytokines and chemokines as well as an accumulation of $CD8^+$ T cells (19, 22), suggesting the potential for secondary host defenses acting against infection when $CD4^+$ T cells are below a protective threshold and are not available. It is interesting that two viral-induced lesions failed to show any evidence of $CD8^+$ T-cell responsiveness when cytotoxic T lymphocytes are active against both HPV and EBV (6, 20, 23), and blood $CD8^+$ T-cell numbers were at immunocompetent levels in the patients. A previous study had shown increased EBV-specific cytotoxic T-lymphocyte responses in HIV^+ individuals with OHL using peripheral blood mononuclear cells (6). Moreover, acute EBV infection, such as infectious mononucleosis, is characterized by increases in $CD8^+$ cytotoxic

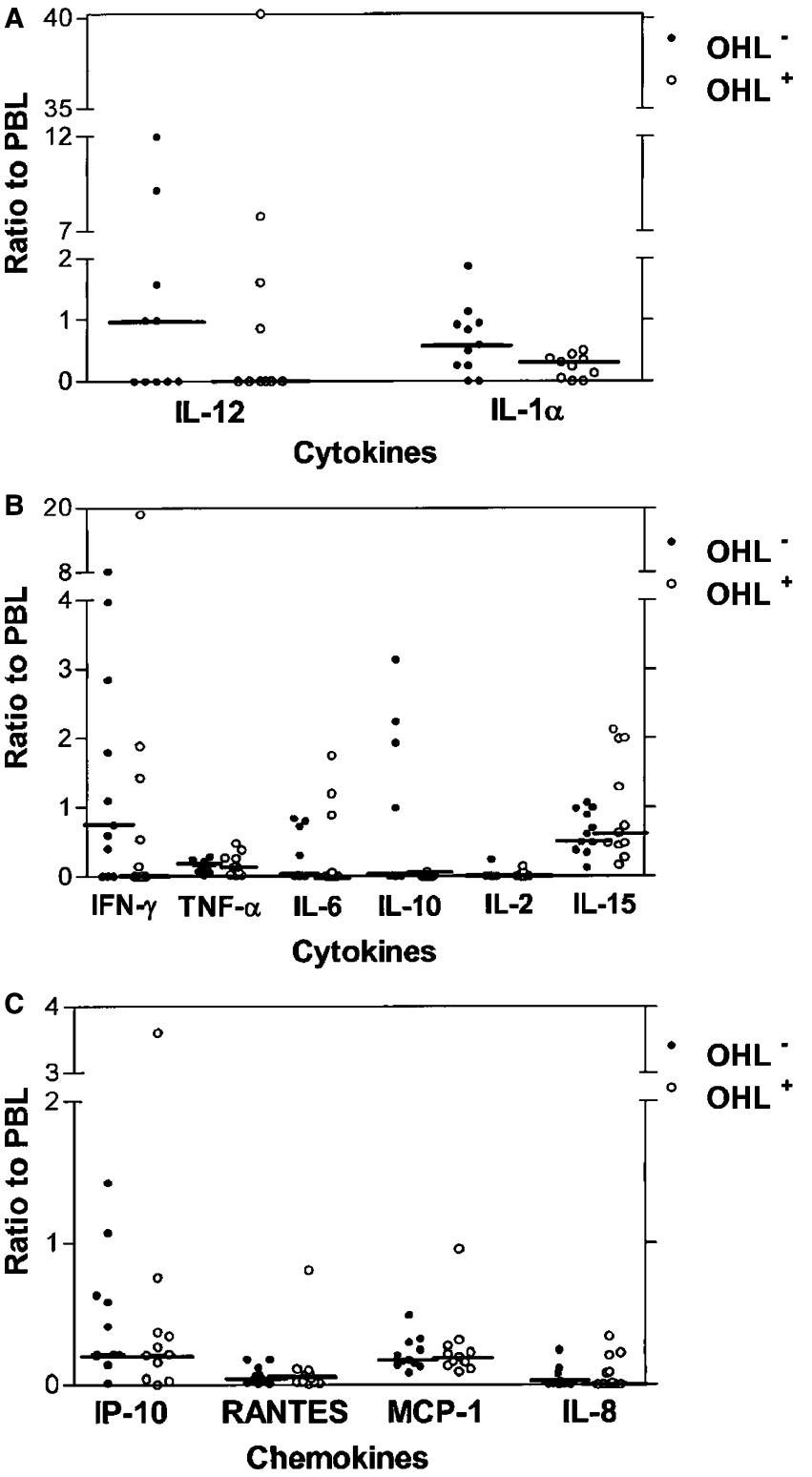

Fig. 3. Tissue-associated cytokine/chemokine mRNA expression in OHL. Cytokine/chemokine mRNA expression was evaluated in the lesions and adjacent lesion-negative tongue tissue of HIV^+ patients with OHL (n=11). Results are expressed as a ratio to unstimulated peripheral blood lymphocytes (PBL) from healthy HIV^- persons with medians illustrated. A) Cytokine mRNA concentrations generally higher than unstimulated PBL. B) Cytokine mRNA concentrations generally below unstimulated PBL. C) Chemokines.

T lymphocytes in response to EBV-infected B cells (3). In cervical carcinomas with HPV present, the virus triggers specific cytotoxic T-lymphocyte responses (21). In the case of oral candidiasis, the $CD8^+$ T cells are not considered to be formal cytotoxic T lymphocytes, but a putative non-major histocompatibility complex restricted activity. Therefore, it is possible that cytotoxic T lymphocytes are not active in the oral cavity and are not formally recruited other than those induced by HIV itself.

In light of the results showing little local immune reactivity, it would have been interesting to compare the results to HIV^- persons with identical lesions to get a better understanding of the role of immunosuppression. However, both OHL and oral warts are extremely rare in HIV^- persons, and thus such an analysis is not possible. We recognize, too, that negative results are difficult to interpret and require significantly more scrutiny. As such, it is possible that the lack of evidence of immune reactivity was technical or an issue of assay sensitivity. However, the same general cohort was used for studies evaluating HIV^- persons and HIV^+ persons with and without oropharyngeal candidiasis where differences were clearly demonstrated (19). Thus, it is unlikely that the negative results were artificial in any way.

The general lack of activity in both oral warts and OHL lesions raises intriguing questions about the role of host defenses against these lesions. Although there appears to be immune-based susceptibility in oral warts and OHL, suggesting a strong primary and possibly exclusive role for $CD4^+$ T cells, there does not appear to be evidence for any type of secondary cellular (innate or adaptive) immune response against either virus in the oral mucosa. This is supported by a recent study showing that persistent oral epithelial Epstein-Barr virus replication decreases local Langerhans cell numbers, which suggests immune evasion (25). It is also intriguing that oral warts, while occurring with higher CD4 cell numbers than those with oral candidiasis, together with little or no evidence of secondary local immune reactivity, are not more common than oral candidiasis lesions. This is especially true based on the relatively high rates of oral infection by HPV in the HIV^+ population. A similar argument could be made for OHL based on the high rates of oral EBV infection. Perhaps cell-mediated immunity plays a relatively limited role in protection against either oral HPV or EBV.

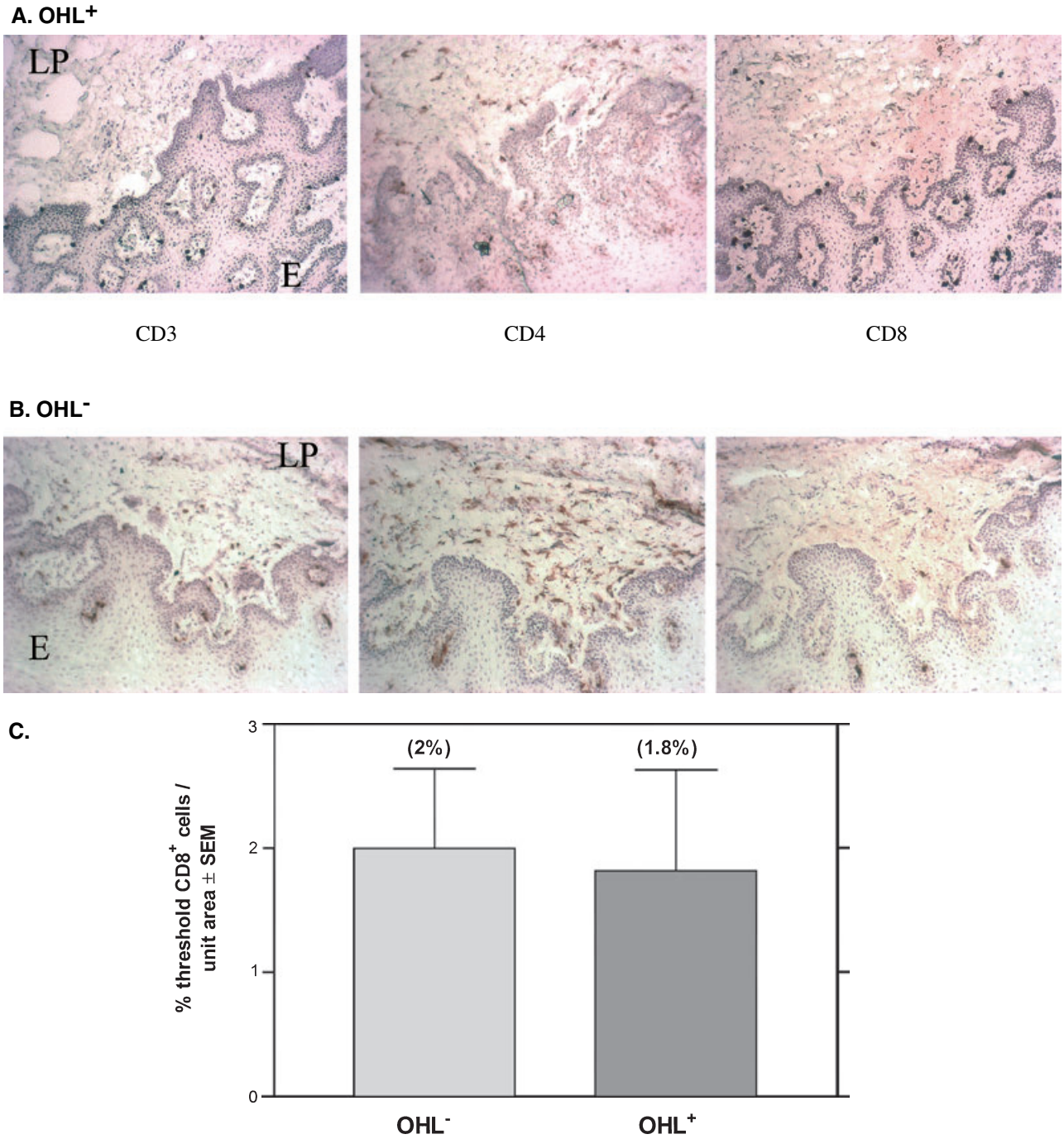

Fig. 4. Distribution of T cells in (A) OHL lesions and (B) adjacent lesion-negative tongue tissue of HIV^+ individuals. Representative images of an OHL lesion and adjacent lesion-negative site from seven HIV^+ patients examined. Magnification 10×. LP, lamina propria. E, epithelium. C) Threshold analysis of $CD8^+$ cells in OHL^+ tongue and adjacent healthy tissue of the same patient (n=5). A percentage threshold of $CD8^+$ cells in OHL lesions and healthy adjacent tissue was quantified per unit area (37,328 μm) for all patients tested. Mean threshold provided in parentheses.

The question then becomes what responses are protecting HPV or EBV-infected immunocompromised persons from developing oral lesions? Perhaps humoral immunity is important. This study did not address antibodies, and the question will require evaluation of both HPV and EBV-specific antibodies in saliva. Alternatively, nonimmune factors may be more important. As such, is there a role for virologic factors in susceptibility to oral warts and OHL? For example, is there an effect of HIV on HPV or EBV that is more critical than immunosuppression on the development of oral warts and OHL? In oral candidiasis there is a definite association of high HIV viral load on the incidence of symptomatic infection (5, 11), and this may be more important than CD4 cell numbers (Fidel, unpublished). According to our cohort, high HIV viral load is not a strong risk factor for either oral warts or OHL. But that does not necessarily preclude a role for HIV in these

lesions. Future studies should be directed toward the influences of virologic factors on both lesions and, through those associations, a better understanding of the host response and susceptibility to each lesion in HIV disease may be revealed.

Acknowledgments

This work was supported by Public Health Service grant DE 12178 from the National Institute of Dental and Craniofacial Research (NIDCR) at the NIH, and the Louisiana Board of Regents through the Millennium Trust Health Excellence Fund, HEF (2000–05)-04.

References

1. Ammatuna P, Campisi G, Giovannelli L, Giambelluca D, Alaimo C, Mancuso S, et al. Presence of Epstein-Barr virus, cytomegalovirus and human papillomavirus in normal oral mucosa of HIV-infected and renal transplant patients. Oral Dis 2001: **7**: 34–40.
2. Ammatuna P, Capone F, Gaimbelluca D, et al. Detection of Epstein-Barr virus (EBV) DNA and antigens in oral mucosa of renal transplant patients without clinical evidence of oral hairy leukoplakia (OHL). J Oral Pathol Med 1998: **27**: 420–427.
3. Callan MF. The evolution of antigen-specific $CD8^+$ T cell responses after natural primary infection of humans with Epstein-Barr virus. Viral Immunol 2003: **16**: 3–16.
4. Cameron JE, Mercante DE, O'Brien M, Gaffga AM, Leigh JE, Fidel P Jr, et al.The impact of highly active antiretroviral therapy (HAART) immunodeficiency human papillomavirus (HPV) on infection in the oral cavity of HIV seropositive adults. (in press).
5. Campo J, Del Romero J, Castilla J, Garcia S, Rodriguez C, Bascones A. Oral candidiasis as a clinical marker related to viral load, CD4 lymphocyte count and CD4 lymphocyte percentage in HIV-infected patients. J Oral Pathol Med 2002: **31**: 5–10.
6. de Jong A, Palefsky J, Stites D, Nakagawa M. Human immunodeficiency virus-positive individuals with oral hairy leukoplakia are able to mount cytotoxic T lymphocyte responses to Epstein-Barr virus. Oral Dis 2000: **6**: 40–47.
7. Gravitt PE, Peyton CL, Alessi TQ, Wheeler CM, Coutlee F, Hildesheim A, et al. Improved amplification of genital human papillomaviruses. J Clin Microbiol 2000: **38**: 357–361.
8. Greenspan D, Canchola. AJ, MacPhail LM, Cheikh B, Greenspan JS. Effect of highly active antiretroviral therapy on frequency of oral warts. Lancet 2001: **357**: 1411–1412.
9. Greenspan D, Greenspan JS. Oral 'hairy' leukoplakia in male homosexuals: evidence of association with both papillomavirus and a herpes-group virus. Lancet 1984: **ii**: 831–834.
10. Greenspan D, Greenspan JS, Hearst NG, Pan LZ, Conant MA, Abrams DI, et al. Relation of oral hairy leukoplakia to infection with the human immunodeficiency virus and the risk of developing AIDS. J Infect Dis 1987: **155**: 475–481.
11. Greenspan D, Komaroff E, Redford M, Phelan JA, Navazesh M, Alves ME, et al. Oral mucosal lesions and HIV viral load in the Women's Interagency HIV Study (WIHS). J Acquir Immune Defic Syndr 2000: **25**: 44–50.
12. Greenspan JS, Barr CE, Sciubba JJ, Winkler JR. Oral manifestations of HIV infection: definitions, diagnostic criteria and principles of therapy. Oral Surg Oral Med Oral Pathol 1992: **73**: 142–144.
13. Greenspan JS, Greenspan D, Lennette E, et al. Replication of Epstein-Barr virus within the epithelial cells of oral 'hairy' leukoplakia, an AIDS-associated lesion. N Engl J Med 1985: **313**: 1564–1571.
14. Itin P, Lautenschlager S. Viral lesions of the mouth in HIV-infected patients. Dermatology 1997: **194**: 1–7.
15. Johnson G, Nelson S, Petric M, Tellier R. Comprehensive PCR-based assay for detection and species identification of human herpesviruses. J Clin Microbiol 2000: **38**: 3274–3279.
16. King MD, Reznik DA, O'Daniels CM, Larsen NM, Osterholt D, Blumberg HM. Human papillomavirus-associated oral warts among human immunodeficiency virus-seropositive patients in the era of highly active antiretroviral therapy: an emerging infection. Clin Infect Dis 2002: **34**: 641–648.
17. Leigh JE. Oral warts rise dramatically with use of new agents in HIV. HIV Clin 2000: **1**: 7–8.
18. Leigh JE, Steele C, Wormley FL JrLuo W, Clark RA, Gallaher WR, et al. Th1/Th2 cytokine expression in saliva of HIV-positive and HIV-negative individuals: a pilot study in HIV-positive individuals with oropharyngeal candidiasis. J Acquir Immune Deficiency Syndr Hum Retrovirol 1998: **19**: 373–380.
19. Lilly E, Hart DJ, Leigh JE, Hager S, McNulty KM, Mercante DE, et al. Tissue-associated cytokine expression in HIV-positive persons with oropharyngeal candidiasis. J Infect Dis 2004: **190**: 604–612.
20. Luxton J, Shepherd P. Human papillomavirus antigens and T-cell recognition. Curr Opin Infect Dis 2001: **14**: 139–143.
21. Mota F, Rayment N, Chong S, Singer A, Chain B. The antigen-presenting environment in normal and human papillomavirus (HPV)-related premalignant cervical epithelium. Clin Exp Immunol 1999: **116**: 33–40.
22. Myers TA, Leigh JE, Arribas A, Hager S, Clark RA, Lilly E, et al. Immunohistochemical evaluation of T cells in oral lesions from human immunodeficiency virus-positive persons with oropharyngeal candidiasis. Infect Immun 2003: **71**: 956–963.
23. Rickinson AB, Moss DJ. Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. Annu Rev Immunol 1997: **15**: 405–431.
24. Scully C, Porter S, Di Alberti L, Jalal M, Maitland N. Detection of Epstein-Barr virus in oral scrapes in HIV infection, in hairy leukoplakia and in healthy non-infected people. J Oral Pathol Med 1998: **27**: 480–482.
25. Walling DM, Flaitz CM, Hosein FG, Montes-Walters M, Nichols CM. Effect of Epstein-Barr virus replication on Langerhans cells in pathogenesis of oral hairy leukoplakia. J Infect Dis 2004: **2004**: 1656–1663.
26. Zehnder M, Greenspan JS, Greenspan D, Bickel M. Chemokine gene expression in human oral mucosa. Eur J Oral Sci 1999: **107**: 231–235.