IMMUNOPHENOTYPING OF THE INFLAMMATORY RESPONSE OF POTENTIALLY
PRECANCEROUS ORAL MUCOSAL LESIONS

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ABSTRACT

Andres David Flores Hidalgo: Immunophenotyping the inflammatory
response of potentially precancerous oral mucosal lesions
(Under the direction of Ricardo Padilla)

Objective: Identify the type and distribution of CD4+ and CD8+ T-lymphocytes in oral mucosa specimens to potentially distinguish underlying alterations or patterns between oral epithelial dysplasia and oral lichen planus.

Methods: For this pilot study, 15 archival tissue samples received at UNC Oral and Maxillofacial Pathology Laboratory, diagnosed as oral lichen planus, moderate to severe epithelial dysplasia and overlapping equivocal cases between dysplasia and lichen planus were selected. Dual staining with CD4 and CD8 antibodies was carried out on each case. Slides were scanned in the Aperio ScanScope FL (Leica Biosystems, Wetzlar, Germany) and archived. Histomorphometric analysis of the cells that expressed biomarkers of in the epithelium and connective tissue regions was performed.

Results: No differences were found in amount and ratio of CD4+/CD8+ lymphocytes between the three groups analyzed. The intraepithelial CD8+ lymphocyte distribution was strikingly different between lichen planus and moderate to severe epithelial dysplasia.

Conclusions: The localization of CD8+ cells can be potentially as an adjunctive diagnostic procedure to distinguish oral epithelial dysplasia from other inflammatory entities such as lichen planus.

"My brother is one of my true heroes. Steady and sober where I am impulsive and emotional."
Mark McKinnon
I dedicate this thesis to my brother, Lino.

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LIST OF ABBREVIATIONS

LP – Lichen planus

OED – Oral epithelial dysplasia

MHC – Major histocompatibility complex

NK cells – Natural killer cells

DNA - Deoxyribonucleic acid

SCC – Squamous cell carcinoma

PCNA – Proliferating cell nuclear antigen

IL – Interleukin

IFN – Interferon

TNF – Tumor necrosis factor

WHO – World Health Organization

LM – Lichenoid mucositis

IF – Immunofluorescence

IHC – Immunohistochemistry

CD4 – Cluster of differentiation 4

CD8 – Cluster of differentiation

LITERATURE REVIEW

The oral cavity is lined by mucosa comprised of stratified squamous epithelium and supporting connective tissues. Clinical lesions in the oral mucosa often present as pathognomonic changes, such as geographic tongue or Fordyce granules. Frequently, however, and sometimes as non-specific areas of red, white, ulcerated mucosa, or combination of these that require pathologic examination for diagnosis. The majority of the time, lesions examined histologically by pathologists will exhibit enough features to reach a specific diagnosis. Occasionally, some lesions have similar histologic architectural alterations in the presence of inflammation of different etiologies such as trauma, allergic reactions, autoimmune diseases, and infectious diseases. Some lesions can also show a similar inflammatory response when undergoing malignant transformation. Lichen planus (LP) and oral epithelial dysplasia (OED) are entities with different etiology, treatment and prognosis, but sometimes they can share similar or overlapping histopathologic features as described above (1).

Oral Leukoplakia

Oral leukoplakia is a clinical term used to describe a mucosal lesion that exhibits a white surface that does not rub off and is not diagnosable clinically as a specific disease. Upon histological evaluation, these are usually diagnosed as either hyperkeratosis, epithelial dysplasia, squamous cell carcinoma (SCC), lichen planus, oral hairy leukoplakia, etc. Oral leukoplakia has a worldwide prevalence of 2%, which represents the most common "risk" lesions for the oral mucosa, with annual overall malignant transformation rates ranging from 0.7% to 2% in various populations and geographical areas. Therefore, surveillance of precursor lesions is an ongoing challenge in many healthcare systems(1). Despite progress

in the understanding of basic mechanisms involved in malignant transformation and substantial improvements in molecular diagnostics, there is no single marker or panel of markers available yet, that allow for reliable prediction of malignant transformation of leukoplakia for the individual patient. Therefore, it is recommended that all leukoplakias undergo histologic examination for final and precise diagnosis (1).

Biology of Oral Lichen Planus

The initial clinical description of lichen planus is generally attributed to Ferdinand Ritter von Hebra, who in 1860 termed the condition "lichen ruber planus"(2). He is credited with the first scientific description of the disease on the skin. Lichen planus obtained its name because of the lacy white lines that bear a close resemblance to the symbiont, lichen, a composite organism consisting of a fungus and a photosynthetic partner living together in a symbiotic relationship, seen growing on rocks and trees. However, Ferdinand Ritter von Hebra used the term lichen to denote skin lesions which are characterized by a macular-papular skin eruption. Louis Wickham is acknowledged as the first to describe the characteristic, fine, white or grey lines known as Wickham striae or dots seen on the top of the pruritic papular rash of lichen planus of the skin. Wickham striae are also seen in oral LP. (3,4)

The oral mucosa appears to have a limited immunological repertoire, predominantly a lichenoid-type reaction(5). This is characterized by delayed type IV hypersensitivity reaction, dominated by cytotoxic cluster of differentiation 8 positive (CD8+) T-lymphocytes inducing apoptosis of the basal keratinocytes. It represents the final common immunopathological pathway secondary to a variety of insults, such as the development of autoantibodies against self-antigens, interaction with allergens such as various drugs or dental materials, viruses such as Hepatitis C, mechanical and chemical trauma, and even emotional stress(6). However, the specific pathophysiology, including the precise triggering factors, remain unknown and elusive.

The antigen that serves as the trigger and/or driver of the immune responses seen in idiopathic oral lichen planus is unknown. It is likely to be an endogenous peptide or a protein sequence innate to the basal keratinocytes; therefore, LP can be characterized as an autoinflammatory condition(1). It is also likely that the supposed exogenous triggers for oral LP, such as dental materials, flavoring agents, certain drugs, viruses and even trauma serve to expose such self-antigens, or, alter the normal innate peptide sequences so that they are perceived by the immune-surveillance cells as being "foreign" or as antigenic targets(5,7). The immune responses to this, as yet, unidentified antigen develops in three stages: T-cell migration to the epithelium, T-cell activation, and induction of apoptosis of the basal keratinocytes.

T-Cell Migration into the Epithelium

Two hypotheses have been proposed to explain the migration of T-cells into the epithelium. The "chance encounter" hypothesis suggests that normally circulating, antigen-specific CD8+ cytotoxic T-cells enter the epithelium for routine surveillance and by chance encounter the putative antigen when it is present in the epithelium (8,9). Alternatively, the keratinocytes direct the CD8+ cytotoxic cells to migrate into the epithelium by the release of cytokines that allow the lymphocytes to "home-in" on the antigen-bearing basal keratinocyte, the so called "direct migration" hypothesis (10).

T-Cell Activation

The lymphocytic infiltrate that histologically characterizes the oral LP lesions is comprised predominantly of T-cells (5,10). The majority of the T-cells in proximity to the damaged and dying basal keratinocytes and within the epithelial layers are predominantly activated cytotoxic CD8+ T-cells(6). Cytotoxic CD8+ T-cells bind to the antigen on the major histocompatibility complex Class I (MHC) site of keratinocytes, which releases cytokines that attract other lymphocytes and immune cells into the site of the developing LP lesion. The CD8 +T-cells are also activated by the CD4+ helper cell found in the lamina propria. In oral LP

lesions, helper CD4+ T-cells are also activated by antigens associated with Class II MHC presented by the antigen-presenting cells or Langerhans cells, or by the keratinocytes themselves, which are induced to present antigens on their Class II MHC sites (1,11,12). Langerhans cells are increased in number in oral LP lesions and also have up-regulated Class II MHC expressing Langerhans cells and keratinocytes which in turn promote CD4+ T-cell secretion of cytokines interleukin-2 (IL-2) and interferon-Ý (IFN- Ý) (9,13). These cytokines and probably others, together with the presentation of an antigen associated with MHC class I on basal keratinocytes, promote cytotoxic CD8+ T-cell induction of apoptosis in keratinocytes (14,15). Hence, it is likely that in oral LP there is a cycle of self-inducting and self-perpetuating T-lymphocyte activation.

Basal Keratinocyte Apoptosis

The apoptosis of the basal keratinocytes that characterizes all forms of LP, is mediated predominantly by hyperactive, cytotoxic CD8+ T-cells(16). It has been previously reported that in patients with cutaneous LP, the T-cells are usually more cytotoxic and active than T-cells present in non-lesional areas of the skin(17,18). Also, it has been shown that the most cytotoxic T-cell clones were CD8+ and the least cytotoxic were CD4+. This finding also indicates that the apoptosis of the basal keratinocytes so characteristic of cutaneous and oral LP is induced by the cytotoxic CD8+ T-lymphocytes activated by a putative basal keratinocyte antigen associated with the MHC class I(5,13,19).

The induction of keratinocyte apoptosis by CD8+ T-cells in LP and oral LP can occur by three established pathways:

1. T-cell secretion of tumor necrosis factor-alpha (TNF-α) which binds TNF-α receptor 1 on the keratinocyte surface.

2. T-cell surface expression of CD95L (Fas ligand), which binds CD95(Fas) on the keratinocyte surface. CD95L is a type-II transmembrane protein that belongs to the TNF family that in binding with its receptor induces apoptosis in

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the target cells. Fas-induced apoptosis by the perforin pathway are the two main mechanisms by which cytotoxic T-lymphocytes induce cell death in cells expressing foreign antigens.

3. Infusion of granzyme B by T cells into the keratinocytes. Granzymes are serine proteases that are released from cytoplasmic granules within cytotoxic T-cells and natural killer cells (NK cells). Granzyme B induces apoptosis within virus-infected cells. Cytotoxic T-cells release X perforin, a protein that attacks the target cells by forming a multimeric complex that enters cells through the mannose 6-phosphate receptor. Granzyme B is released to cause apoptosis by various pathways, including the cleaving of caspases (especially caspase-3), which in turn activates caspase-activated DNase and this enzyme degrades DNA, therefore inducing the apoptotic cascade culminating in cell death(6,10,13).

Malignant transformation of Oral Lichen Planus

There is a small chance of malignant transformation in patients with lichen planus, particularly in patients with either the erosive or so-called plaque-type form(20,21). Ever since the first clinical report about malignant transformation in LP was published in 1924, there has been an unresolved controversy regarding whether LP should be considered a premalignant condition (22-24). To this date, the reported malignant transformation varies from 0.4% to 12.5% with an overall average rate of 1.09% cited in a recent meta-analysis and systematic review of 7,806 patients in 16 studies, making the World Health Organization (WHO) categorize these lesions as premalignant (25,26). However, the majority of the studies have not shown evidence of premalignant potential as convincing, consistent, or conclusive as that characterizing OED (27,28). Besides OED, many other disorders clinically and histopathologically can resemble lichen planus in the oral cavity.

A currently favored explanation for the transformation of oral LP into SCC is that the inflammatory infiltrate underlying the surface epithelium is affected by the release of cytokines associated with oxidative stress, which, in turn, activate transcription factors in the epithelial cells with premalignant potential (6,29). A variety of scenarios may lead to a clinical and histologic presentation of lichenoid mucositis (LM), including local or systemic allergy, immune-related disease including LP, chronic ulcerative stomatitis and dysplastic oral lesions (30). One point not yet determined is whether the proposed mechanism for transformation could be applied to all etiologies of lichenoid lesions or whether it applies only to classic/idiopathic oral LP (31). In the case of dysplastic oral lesions eliciting a lichenoid response, if this model is applicable, it would seem that the lichenoid response in an already premalignant lesion could exacerbate and accelerate the progression to SCC. To date, it is unclear whether oral LP is an independent risk factor for malignant transformation.

Numerous studies have also investigated various mechanisms involved in carcinogenesis including P53 mutation, PCNA mutation, loss of heterozygosity at the tumor suppressor gene loci, and cytogenetic abnormalities (32-35). None of the data shows convincing or consistent findings about the premalignant potential of oral lichen planus. The inflammatory infiltrate associated with oral lichen planus has been proposed to be a mechanism for malignant transformation (36). This proposal has some merits as other chronic inflammatory diseases have also been linked to cancerous transformation such as colon cancer in long-standing inflammatory bowel disease precipitated by intestinal microflora, and Barret's esophagitis (37). Conversely, other data suggests that inflammatory and immune systems may inhibit tumorigenesis (38).

The histopathologic features of lichen planus fall on a spectrum, potentially influenced by the stage of the disease at the time of the biopsy, recent therapy of the condition, the clinical types and/or the anatomic sites of involvement. Oftentimes, biopsies of OED can mimic oral LP on low magnification, exhibiting a prominent band-like chronic inflammatory infiltrate

subjacent to the basal keratinocytes (30,39,40). This inflammatory infiltrate may be there in an attempt to inhibit tumorigenesis progression.

Oral Lichen Planus vs Epithelial Dysplasia

Various disorders may present with clinical and histopathologic features similar to oral LP (41,42). Although oral epithelial dysplasia usually presents as a solitary lesion with variable proportions of white and red color change and ulceration, multifocal presentation is well recognized as seen, for example, in patients with advanced tobacco-related mucosal injury and proliferative verrucous leukoplakia (43).

Oral epithelial dysplasia is usually associated with a band-like chronic inflammatory cell infiltrate in the superficial lamina propria which, when viewed with low-magnification microscopy, may offer substantial histopathological mimicry of LP(29). In 1985, the term "lichenoid dysplasia" was introduced to differentiate cases of epithelial dysplasia exhibiting lichenoid inflammation from oral lichen planus(44). This term was eventually discontinued and due to the ambiguity generated when rendering a straightforward diagnosis. Considerable clinical and histologic overlap has been found between LP and OED of the oral cavity, especially with the entity called proliferative verrucous leukoplakia, raising the question of whether the cases of oral LP that purportedly transform to SCC in fact represent undetected premalignant lesions with a nonspecific inflammatory response that mimics oral LP (37,45). The incidence of lichenoid features in OED and OSCC has been investigated by Fitzpatrick et al. (2014)(25), in which lichenoid features were found, at least focally, in 29% of 352 dysplasia or SCC cases. In the cases that showed lichenoid features, band-like inflammatory cell infiltration, of mostly lymphocytes, and basal keratinocyte degeneration were the most frequent features encountered, accounting for 74% and 30% of the lichenoid features, respectively(29).

There are still no widely accepted diagnostic criteria for LP. Microscopic differentiation of OED from LP is based on recognition of cytologic atypia in squamous epithelial cells and

identification of disturbance in the maturation pattern in cases of dysplasia, and their absence in LP(25). However, discrimination of mild epithelial dysplasia, with chronic interface mucositis, from LP, with reactive cellular atypia, can be challenging, requiring subjective assessment of ostensibly objective morphologic features(39,46). The WHO 2017 criteria do not address a way to distinguish or exclude OED from oral LP diagnosis(47).

Little has been written concerning the frequency of individual lichenoid characteristic in dysplastic lesions, although Krutchkoff and Eisenberg(44) argued that the presence of liquefactive degeneration of the basal membrane of the epithelium was rarely noted in dysplastic lesions and may serve to differentiate such lesions from non-dysplastic LM. Nonetheless, they also warned that this was not always the case and that the presence of atypical features should overrule any lichenoid feature that might be coincident (29).

Considerations about the Microscopic Diagnosis of Oral Epithelial Dysplasia

The efficacy of microscopic identification and grading of precursor lesions as an indicator of potential malignant transformation has long been contested in the literature. Current evidence recognizes carcinogenesis of the epithelium as a multistep, progressive, cumulative process of genetic mutations that culminate in tumor formation and ultimately invasion and metastasis(48). Although transformation into oral cancer is not linear in its development, there is general agreement that it begins as epithelial hyperplasia and progresses through dysplasia, with more severe dysplastic changes signifying more extensive genetic aberrations(43,49). The timeframe for this process is not known, but it is thought to be a relatively slow process, with malignant transformation occurring within 10 years(49,50). Although this model may suggest that oral SCC is an inevitable conclusion to OED, this is not the case, even in the absence of definitive surgical intervention(51). Conversely, malignant transformation may occur despite active treatment and follow-up of mucosal lesions exhibiting dysplastic changes(51).

The diagnosis of epithelial dysplasia is often predicated on a combination of architectural and cellular abnormalities. Cellular abnormalities include proliferation of immature or atypical-appearing keratinocytes in the basal and parabasal areas of the oral epithelium as well as nuclear pleomorphism, high nuclear-to-cytoplasm ratio, increased mitotic activity, and hyperchromatism in individual cells (52). Architectural abnormalities include irregular epithelial stratification, elongated rete ridges extending into the submucosa (43), and loss of normal maturation, characterized by increased cellular density in the superficial epithelium, crowding of cells, and loss of polarity especially in the basal zone(30).

The diagnostic dilemma between lichen planus and epithelial dysplasia appears to have more implications than the overlapping histopathologic features that can make the diagnosis difficult for the pathologist. Absence of broad consensus regarding the diagnostic criteria has been identified as the major obstacle to assuring the validity of studies investigating the potential of oral LP to undergo malignant transformation. This lack of diagnostic criteria becomes a problem for pathologists when evaluating cases with overlapping features between LP and OED, especially because the management of these condition is significantly different. A patient diagnosed with symptomatic LP will often be prescribed steroids or immunomodulators that will inhibit the inflammatory reaction targeting the superficial oral mucosa. In contrast, when a diagnosis of OED is made, a surgical resection of the lesion and close clinical follow-up is recommended as well as avoidance of topical steroids or other medications that may suppress the immunosurveillance of the area.

New diagnostic techniques in pathology are focusing on the inflammatory response induced by the neoplasm, instead the tumor itself, to distinguish similar neoplastic entities, for early diagnosis of cancer in premalignant lesions, prognostic value, etc. This can be accomplished by evaluating and targeting lymphocytes specialized in orchestrating and regulating the immune cells and the immunosurveillance against malignant transformation.

In this study, we utilize immunohistochemical antibody phenotyping of the CD4+ and CD8+ T-lymphocytes within the inflammatory response in oral mucosal samples in order to differentiate between the patterns of LP and OED in cases with overlapping histologic features.

AIMS AND OBJECTIVES

Objective

Identify the type and distribution of CD4+ and CD8+ T-cells in oral mucosa specimens to potentially distinguish underlying alterations or patterns between oral epithelial dysplasia and oral lichen planus.

Null Hypothesis: there are no statistical significant differences between the presence and distribution of subtypes of T-lymphocytes in the inflammatory response in oral lichen planus and epithelial dysplasia.

Alternate hypothesis: there are statistical significant differences between the presence and distribution of different subtypes of T-cells in the inflammatory response in oral lichen planus and epithelial dysplasia.

MATERIALS AND METHODS

This study protocol was registered, reviewed and approved by the University of North Carolina at Chapel Hill Institutional Review Board under the number 16-5988. All study procedures were performed in accordance with the Declaration of Helsinki and Research Committee Regulations. The study was designed as a pilot retrospective study. The population or sample consisted of archival tissue samples from the UNC Oral and Maxillofacial Pathology Laboratory. For this pilot study, 5 cases of each group were selected for testing.

Specimen Selection

Oral tissue samples received between July 1, 2005 to January 31, 2016, at the UNC at Chapel Hill Oral and Maxillofacial Pathology Laboratory that were diagnosed with Oral Lichen Planus or Epithelial Dysplasia were collected. A third group of specimens was selected composed of equivocal cases those with overlapping features between LP and OED (Figure 1). Biopsy accession files and medical records from samples were reviewed for case selection.

Figure 1. Examples of cases selected per group (Stain: Hematoxylin and Eosin)

Inclusion and Exclusion Criteria

Inclusion Criteria:

For inclusion in the study, the specimens selected met the following criteria:

1.   Cases had sufficient tissue for additional analysis.

2.   Lichen planus samples were from the buccal mucosa only.

3.   Epithelial dysplasia samples were from tongue, buccal mucosa or floor of mouth:

      i.   Moderate to severe epithelial dysplasia: cytological and architectural premalignant changes limited to the lower two thirds of the total thickness of the epithelium.

      ii. A lichenoid inflammatory reaction in the upper connective tissue.

4.   Equivocal cases with overlapping features, were from buccal mucosa and lateral tongue.

5.   Samples were from subjects over 21 years of age.

Exclusion Criteria:

Any of the following was regarded as a criterion for exclusion from the study:

1. Tissue samples from subjects with previous history of cancer of any type.

2. Patients with documented prior history of treatment with steroids prior to the biopsy date

3. History of underlying immunosuppression from any cause.

4. Subjects under 21 years of age

5. Subjects with prior history of radiation treatment to the head or neck.

Data Collection and Processing

The hematoxylin and eosin slides were reviewed and the diagnosis confirmed by two board-certified oral and maxillofacial pathologists (RP, VM). After the final case selection, the paraffin blocks were selected and two unstained slides sectioned for immunofluorescence (IF) and immunohistochemistry (IHC) studies.

Study Procedures

Dual IF was carried out on the Bond fully-automated slide staining system (Leica Microsystems Inc., Norwell MA). CD4 and CD8 antibodies (catalog numbers NCL-L-CD4-368 and NCL-L-CD8-4B11, respectively) were purchased from Leica Biosystems. Slides were deparaffinized in Bond dewax solution (AR9222), hydrated in Bond wash solution (AR9590) and sequentially stained for CD4 and CD8. Specifically, antigen retrieval for CD4 was performed for 20 min at 100^0C in Bond-epitope retrieval solution 2 pH 9.0 (AR9640). After pretreatment, slides were incubated for 30 min with CD4 antibody (1:200) followed with Bond polymer (DS9390) and TSA Cy5 (PerkinElmer, Boston, MA). After completion of CD4 staining, a second round of antigen retrieval was performed for 10 min at 100^0C in Bond-epitope retrieval solution 2 pH 9.0. Slides were then incubated with the CD8 antibody (1:200, 30 min) which was detected with Alexa488 labeled goat anti-mouse secondary antibody (Invitrogen, Carlsbad, CA). Nuclei were stained with Hoechst 33258 (Invitrogen, Carlsbad, CA). The stained slides were mounted with ProLong Gold antifade reagent (Molecular Probes, Inc. Eugene, OR 97402).

Analysis of IF Images:

Slides were scanned in the Aperio ScanScope FL (Leica Biosystems, Wetzlar, Germany) using a 20X objective and images were archived in TPL's eSlide Manger database (Leica Biosystems, Wetzlar, Germany).

Figure 2. Example of annotations made on IF/IHC slides.

For all eSlides, Aperio ImageScope (Aperio ImageScope Version 12.3.2.8013; Leica Biosystems, Wetzlar, Germany) software was used to highlight two regions named Epithelium or Zone 1 and Connective Tissue or Layer 2. Images were annotated by the investigators based on H&E staining (Figure 2). The criteria utilized for the annotation regions were as follows:

1. The epithelial section was outlined following the contour of the basement membrane, involving all the layers of the epithelium and including inflammatory cells infiltrating through the basement membrane, extending to the surface of the stratum corneum.

2. The connective tissue section involved included encircling the area immediately subjacent to the basement membrane.

3. Any cells with double expression for CD4 and CD8 were excluded from analysis.

Automated digital analysis of images was run separately in these two regions. Tissue Studio software (Tissue Studio version 2.5 with Tissue Studio Library version 4.2; Definiens Inc., Carlsbad CA), specifically, the Nuclei and Simulated Cells algorithm in the IF Portal, was used to detect and enumerate cells that co-expressed biomarkers of interest in the annotated

regions. Briefly, nuclei were digitally detected by the presence of Hoechst stain (nuclear counterstain). From these nuclei, a cell simulation was performed – cell margins were grown out from nuclear boundaries (Figure 3). For this data set, positivity thresholds for CD4 and CD8 were determined by measuring the average staining intensities both inside and outside simulated cells. Measurements were made from a total of six regions from an algorithm training set that contained three of the sixteen images (15 cases of all three groups and control). The training set images were chosen to encompass the full range of staining intensities present in the entire analysis data set (images with high, medium, low or negative staining for CD4 and CD8). Once thresholds were set, the algorithm evaluated each cell individually for the presence of CD4 and CD8. Cells that were negative for both markers or positive for CD4, CD8 and both CD4 and CD8 were enumerated by the algorithm. Microsoft Excel 2013 was used to determine the percentage of cells that co-express each marker.

Figure 3. Graphic representation of the region analysis and cells classification made by the software (Zone 1: Epithelium; Zone 2: Connective Tissue)

Formula's for Co-Expression Percentage Determinations:

% of CD4+ cells = (# CD4+ cells/Total cells) *100

% of CD8+ cells = (# CD8+ cells/Total cells) *100

% CD4+ cells that co-express CD8 = (# of cells that co-express CD4 and CD8/#CD4+ cells) *100

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% CD8+ cells that co-express CD4 = (# of cells that co-express CD4 and CD8/#CD8+ cells)*100

Data Analysis

A statistical software package (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) was used to calculate confidence intervals (CI) in order to stablish an estimated difference between cell populations by layer analyzed.

Statistical Analysis

Confidence intervals and power statistical analysis were conducted on the preliminary data to establish significant different and the necessary sample to stablish a statistical significant difference. All data was entered into SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp). Mean, standard deviation and 95% confidence intervals were computed for all continuous variables.

Analysis of the Distribution of CD8+ T-cells in Lichen Planus vs Epithelial Dysplasia

The second part of the study consisted of the analysis of the distribution of infiltrating CD8+ T-cells in the epithelial portions of all selected specimens. Aperio ImageScope (Aperio ImageScope Version 12.3.2.8013; Leica Biosystems, Wetzlar, Germany) software was used to divide Zone 1, encircling the epithelium, into two different areas named upper half and lower half or basal area. The upper half was defined as the area between the stratum corneum and half the thickness of the epithelial layer. The lower half or basal area, was defined as the segment between the basal layer of the epithelium and a line drawn through the midpoint of the thickness of the epithelium (Figure 4). All focal areas with tangential sections were not measured and analyzed, to avoid false positives.

Figure 4. Example of analysis of distribution of CD8+ lymphocytes within epithelium or Zone 1. The yellow lines mark the epithelial thickness and the red line the approximate half of the region analyzed.

The investigator then separated all the specimens with the following criteria:

1.   All the tissue sections with the majority of CD8+ cells concentrated in the basal layer of the epithelium were classified as Group A.

2.   The specimens with the majority of CD8+ cells scattered through the thickness of the epithelium, and mostly found in the upper half the Zone 1 were classified as Group B.

All results were compared with the original diagnoses and hematoxylin and eosin stained tissue sections.

RESULTS

Baseline Characteristics

The total number of cells and ratio of CD4+ and CD8+ T-cells by category in all tissue sections are summarized in Table 1. All the cells that the software identified on IHC and IF as double positive for CD4 and CD8 antibodies were excluded from the analysis to prevent false positive due to overlapping positivity of adjacent cells. Although the presence of double positive CD4/CD8 T-cells in internal organs and peripheral tissue has been reported, flow cytometry seems to be the best way to identify them (53). Also, the published data is still controversial and currently little is known about their function and development.

	Number of CD4+ Cells		Number of CD8+ Cells		Total of lymphocytes		% of CD4+ Cells		% of CD8+ cells	
	Epithelium	Connective tissue	Epithelium	Connective Tissue	Epithelium	Connective Tissue	Epithelium	Connective Tissue	Epithelium	Connective Tissue
1	29	36	2075	5594	2104	5630	1.4	0.6	98.6	99.4
2	48	269	860	7928	908	8197	5.3	3.3	94.7	96.7
3	424	4329	118	416	542	4745	78.2	91.2	21.8	8.8
4	716	2652	1127	2085	1843	4737	38.8	56.0	61.2	44.0
5	1511	12459	40	448	1551	12907	97.4	96.5	2.6	3.5
6	573	212	2345	1848	2918	2060	19.6	10.3	80.4	89.7
7	1826	11572	860	2125	2686	13697	68.0	84.5	32.0	15.5
8	13	130	1105	3422	1118	3552	1.2	3.7	98.8	96.3
9	1828	4487	905	1001	2733	5488	66.9	81.8	33.1	18.2
10	167	5661	477	4108	644	9769	25.9	57.9	74.1	42.1
11	957	1953	1474	1761	2431	3714	39.4	52.6	60.6	47.4
12	0	3	2942	3672	2942	3675	0.0	0.1	100.0	99.9
13	510	4513	253	1069	763	5582	66.8	80.8	33.2	19.2
14	3060	3919	7095	1498	10155	5417	30.1	72.3	69.9	27.7
15	69	469	2594	14606	2663	15075	2.6	3.1	97.4	96.9

Table 1. Total number and ratio of CD4+ and CD8+ lymphocytes by zone analyzed.

A graphic representation of the percentiles of quantified cells by zone and group are displayed in Figure 5.

Figure 5. Graphic representation of averages of percentages of cells counted by zone in all the specimens selected.

CD4+/CD8+ Ratio in Oral Lichen Planus

Zone 1:

The average number of CD4+ cells infiltrating the epithelium in all cases of LP was 545.6 cells, representing 44.2% of all CD4+ and CD8+ cells quantified in Zone 1. The average number of CD8+ cells was 844, accounting for 55.8%. The average ratio of CD4+/CD8+ cells infiltrating the epithelium in all cases of confirmed LP was 2:3 respectively.

Zone 2:

Regarding the cells present in the lichenoid infiltrate in Zone 2, the average amount of CD4+ cells in the connective tissue was 3949, accounting for 49.5% of all purely positive CD4+/CD8+ cells quantified. The average amount of CD8+ cells was 3294.2 representing 50.5% of cells quantified. The ratio of CD4+/CD8+ cells in the Zone 2 was 1:2 respectively.

CD4+/CD8+ Ratio in Epithelial Dysplasia

Zone 1:

The average amount of CD4+ T-lymphocytes encountered in all cases with confirmed moderate to severe epithelial dysplasia was 881.4 which represents 36.3% of all cells. The average of CD8+ cells was 1138.4, accounting for 62.4%. This constitutes a CD4+/CD8+ ratio of 1:2 respectively.

Zone 2:

The average of CD4+ T-lymphocytes present in the underlying lichenoid infiltrate in all confirmed OED was 4412.4 which represents 62.4% of all immunoreactive T-cells. The CD8+ cell average in Zone 2 was 2500.8 which represents a 52.1% of all CD4+/CD8+ cells, resulting in a 2:1 ratio.

Statistical Analysis

For the most part, the CD4+/CD8+ T-cell ratio was very similar for both oral lichen planus and epithelial dysplasia. However, a slight increase of cytotoxic CD8+ T-cells was encountered in Zone 1 and 2 in the OED group.

Power Analysis:

With the use of a statistical software package (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp), statistical power was calculated to detect the significance that could be achieved for the given sample size, and plausible population parameters for the statistical model judged appropriate for the research questions. The standard deviation of CD4+ cells found infiltrating Zone 1 for the combination of LP and OED groups, was 739.4; for CD8+ cells was 764.3. It was calculated that a sample size of 76 specimens would be necessary to significantly demonstrate a difference in the increase of CD8+ cells infiltrating and underlying epithelial dysplasia when compared to lichen planus confirmed specimens.

Power was estimated as the proportion of replication of results in which the null hypothesis is correctly rejected for alpha = .05 (type I error). Power was calculated greater or equal to .80.

Confidence Intervals:

Confidence intervals (CI) were performed to identify a plausible range of the true population value by accounting for distributional assumptions. Confidence level was set at 95%. Medians about percentiles per zone analyzed can be seen in Table II.

Quantiles			
CD4+ T-cells			
Lichen Planus - Zone 1 (Epithelium)			
Level	Quantile	95% Confidence Limits Distribution Free	
50% Median	424	29	1511
Lichen Planus - Zone 2 (Connective Tissue)			
50% Median	2652	36	12459
Epithelial Dysplasia - Zone 1 (Epithelium)			
50% Median	573	13	1828
Epithelial Dysplasia - Zone 2 (Connective Tissue)			
50% Median	4487	130	11572
CD8+ T-cells			
Lichen Planus - Zone 1 (Epithelium)			
Level	Quantile	95% Confidence Limits Distribution Free	
50% Median	860	40	2075
Lichen Planus - Zone 2 (Connective Tissue)			
50% Median	2085	416	7928
Epithelial Dysplasia - Zone 1 (Epithelium)			
50% Median	905	477	2345
Epithelial Dysplasia - Zone 2 (Connective Tissue)			
50% Median	2125	1001	4108

Table 2. Confidence Intervals (median about percentiles) calculated per zone analyzed.

Confidence Intervals in Lichen Planus vs Epithelial Dysplasia in Zone 1

Although power analysis estimated statistical significance between groups, confidence intervals were calculated to identify possible difference between the CD4+/CD8+ ratio. The overlapping of confidence intervals can be observed graphically in Figures 6 and 7.
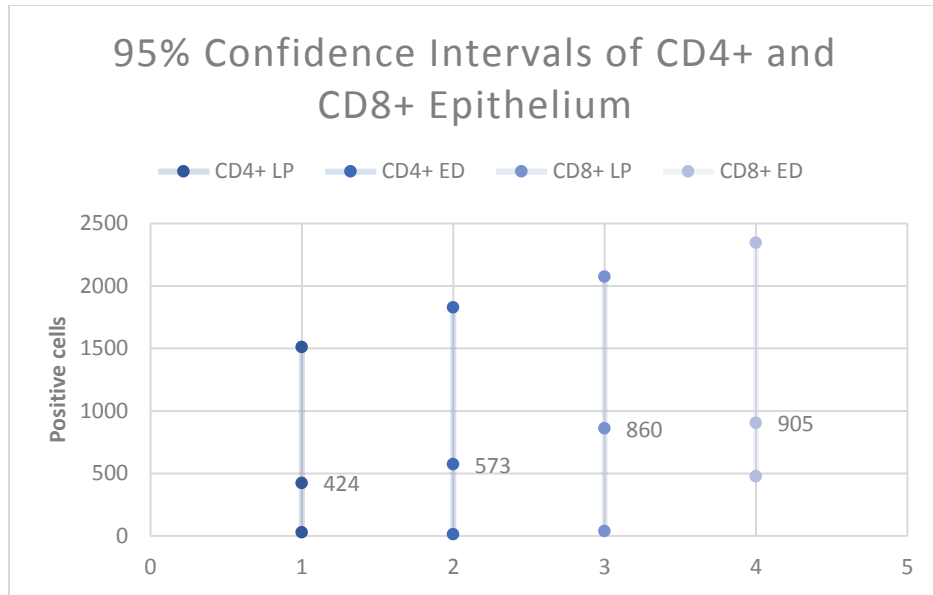
Figure 6. Overlapping confidence intervals (95%) - CD4+ and CD8+ cells infiltrating the epithelium (Zone 1) by group.

Confidence Intervals in the Lichen Planus vs Epithelial Dysplasia in Zone 2
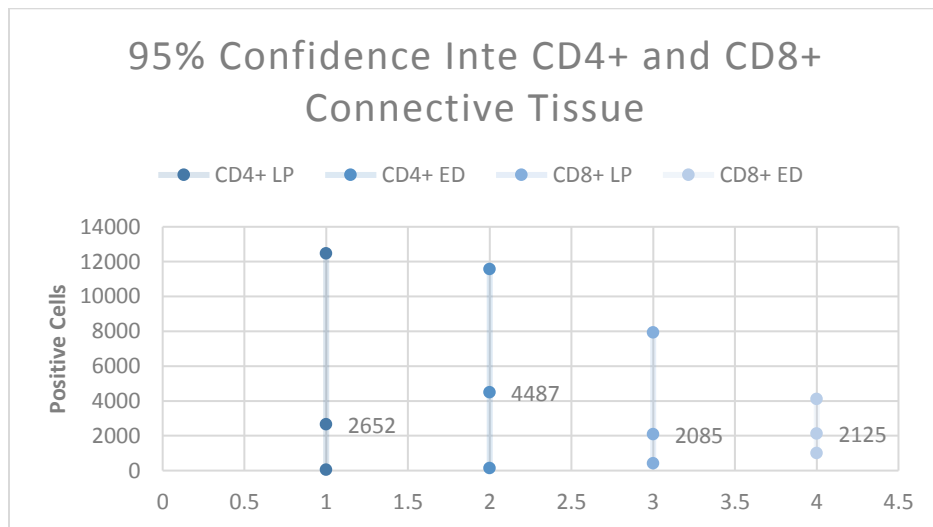

Figure 7. Overlapping confidence Intervals (95%) - CD4+ and CD8+ cells underlying the epithelium (connective tissue / Zone 2).

Analysis of the Distribution of CD8+ T-cells in Lichen Planus vs Epithelial Dysplasia

The full results of the morphological distribution of CD8+ cells are summarized in table 3. The biggest difference in the comparison of oral lichen planus with epithelial dysplasia was the pattern of infiltration of CD8+ cells.

Diagnosis	Case	Observations
LP	1	CD8+ cells in basal layer and below epithelium or Layer 1
LP	2	CD8+ cells below epithelium and infiltrating basal layer of epithelium
LP	3	CD8+ cells below epithelium
LP	4	CD8+ cells below epithelium and infiltrating basal layer of epithelium
LP	5	CD8+ cells below epithelium and infiltrating basal layer of epithelium
Dysplasia	6	Multiple CD8+ cells found in upper half of the epithelium
Dysplasia	7	Atrophic epithelium, minimal amount of CD8+ cells in upper half
Dysplasia	8	CD8+ cells found in upper half
Dysplasia	9	Upper half of epithelium, some infiltrating normal appearing epithelium
Dysplasia	10	CD8+ in upper half throughout later 1, and infiltrating salivary gland ducts

Table 3. Distribution of CD8+ cells in the Epithelium in Oral Lichen Planus vs Epithelial Dysplasia.

The greatest infiltration of CD8+ T-cells was observed in the OED group, where the cytotoxic cells appeared to infiltrate the dysplastic epithelium and follow the atypical features. Since all the samples selected for this group consisted of moderate to severe dysplasia, the CD8+ cells were localized to the area from the basal layer to the upper half of the epithelium or Zone 1. This because the CD8+ T-cells follow the malignant epithelial changes in the tissue.
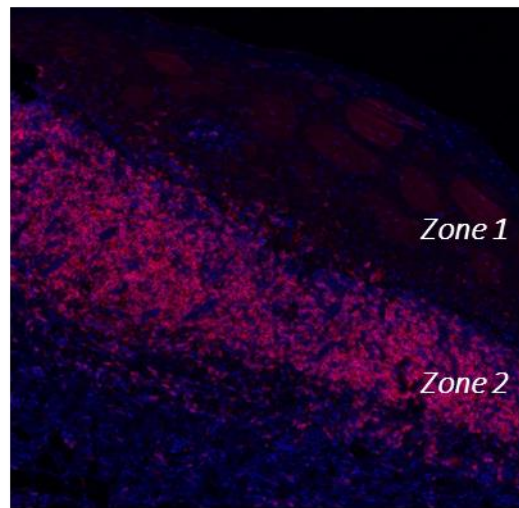
DISCUSSION

This study attempted to evaluate the population of T-cells comprising the inflammatory lichenoid infiltrate underlying the epithelium in oral lichen planus and epithelial dysplasia, specifically regarding the quantity and distribution of two major subtypes of lymphocytes.

Phenotype of Inflammatory Cells in Oral Lichen Planus

Established oral lichen planus lesions are typically found to contain T-cells with alpha-beta receptors, including CD4+ and CD8+, designated helper and cytotoxic respectively. Both subtypes can be found within the epithelium and lamina propria infiltrates, and are involved in a type 1 immune response, where CD4+ T-cells produce Th1 soluble factors. This response likely includes cell-mediated cytotoxicity, as CD8+ T-cells have been described to be localized to the epithelial-connective tissue interface and sometimes adjacent to apoptotic keratinocytes(6). In our study, the majority of CD4+ cells were found in the connective tissue underlying the involved epithelium, and CD8+ cytotoxic cells were mostly found infiltrating the keratinocytes of the basal layer (Figure 8). Khan et al. (2003) reported that CD4+ helper T-cells are prominent in early lesions, mainly in the superficial connective tissue with occasional cells in the epithelium (Figure 9), which is also consistent with the study of Firth et al (2015)(10) study, where regulatory T-cells expressing FoxP3 and IL-7, such as helper CD4+ lymphocytes were mostly found in the superficial connective tissue underlying the epithelium.

Figure 8. Distribution of CD4+ and CD8+ lymphocytes detected by IF/IHC. Blue - Nuclei stained with Hoechst. Red - CD4. Green - CD8. Note the scattered CD8+ lymphocytes infiltrating the lower half of the epithelium (400x magnification).

Figure 9. Presence of CD4+ lymphocytes mainly in the connective tissue and basal membrane some. Red - CD4+ cells (400x magnification).

Also, most of the intraepithelial lymphocytes were CD8+ cells and the proportion of these lymphocytes was higher in the superficial than the deeper lamina propria, in consonance with what has been previously established in the literature(54). CD8+ T-lymphocytes were usually found in close proximity to the epithelial basement membrane. This finding correlates with the studies of Khan et al. (2003), where the presence of CD8+ lymphocytes in oral lichen

planus, were associated with basal epithelial cells undergoing apoptosis(13,15). It has been also described, that the presence of activated CD8+ cells expressing human leukocytic antigen surface antigen, is detected not only close to damaged epithelial cells but also to the basement membrane and epithelial areas of erosion (55). No significant differences in the ratio of CD4+/CD8+ (2:3) in the infiltrating and underlying t-lymphocytes were encountered in the present study.

The trigger for keratinocyte apoptosis in oral lichen planus is unknown. However, the localization of CD8+ cells can be explained by the previously mentioned studies and by Sugerman et al. (2000), who reported that CD8+ lesional cells recognize antigen associated MHC class I on lesional keratinocytes. Following antigen recognition and activation, CD8+ cytotoxic T-cells trigger keratinocyte apoptosis, resulting in the clinical and histological appearance of LP (16).

Phenotype of the Inflammatory Infiltrate in Oral Epithelial Dysplasia with Lichenoid Features

In the oral cavity, the most common lesions clinically recognized as potentially malignant are leukoplakia and erythroplakia, but it is also apparent that as many as 50% of oral SCCs arise from apparently clinically normal mucosa (56). The prognostic significance of an individual lesion is difficult to determine. None of the currently available molecular markers have proved to be prognostically significant nor have yet been evaluated in large prospective studies. At present, therefore, the diagnosis and grading of OED is based on a combination of architectural and cytological changes, but these are subjective and there is considerable variability between pathologists in grading of the lesions. In a study by Fitzpatrick et al. (2014), 29% of 352 OED observed, have lichenoid features, which consisted of a band-like inflammatory cell infiltrate underlying and infiltrating the epithelium and basal cell degeneration(29). This finding is consistent with all the specimens encountered in our study, since the majority of these features were identified and used in case selection (Figure 10).

Figure 10. Hematoxylin and Eosin tissue section of a sample with moderate to severe epithelial dysplasia. (Magnification 400X)

As far as the CD4+/CD8+ ratio of cells underlying moderate to severe OED was concerned, no statistically significant differences were encountered in any of the layers. There was a moderate increase in the inflammatory cell infiltrate in the OED group compared to oral LP. The infiltrates of both of the CD8+ and CD4+ cells were significantly greater in number than in LP and deeper into the tissue Zone 1 as seen in Figure 11.

Figure 11. CD4+ and CD8+ cell infiltrate within Zone 1. Yellow line highlights the epithelium-connective tissue interphase.

CD4+/CD8+ cell ratio in Oral Lichen Planus vs Epithelial Dysplasia with Lichenoid Features

Regarding the CD4+/CD8+ ratio, the CD8+ lymphocytes were found to be slightly increased in comparison to the CD4+ cell population in OED, whereas the ratio was slightly tipped in favor of CD4+ lymphocytes in LP. This suggests an important change in the behavior of the inflammatory infiltrate from a cytotoxic activity that is aimed to eliminate the lesion in OED to a more regulatory and suppressive in LP. This change can be observed in Figure 12.

Figure 12. Graphic representation of averages of percentages of cells present in Layer 1 and 2 of oral lichen planus vs dysplasia.

An important change can be appreciated in the date for premalignant lesions where the CD4+/CD8+ cell ratio changes from the position of CD8+ lymphocytes. It is already known CD4+/CD8+ cell infiltrate is significantly higher in moderate to severe dysplasia in comparison with benign lesions and in SCC (9,40). This can be explained by the relationship between immunosurveillance and tumor progression, in which carcinogenesis results from crosstalk of cancer-cell-intrinsic factors and host immune system or "cell-intrinsic" effects(57). Hirota et al. (1990), also demonstrated an increased number of T-helper cells over T-cytotoxic cells within the T-cell subpopulation of oral SCC lesions. The above suggests an overall switching of the immune response in favor of pro-tumor immunity dominated by a CD4+ cell population that might help in tumor initiation and progression as suggested by Strauss et al. (2007). Further studies need to be conducted to identify the subpopulations of CD4+ cells that are directly responsible for such increase in the CD8+ population.

There was no statistically significant difference between the numbers of cells encountered in the different zones analyzed in our study. This indicates that the CD4+/CD8+ T-cell ratio is similar between both lesions and, therefore cannot be used to differentiate them.

However, the localization of CD8+ cells infiltrating the epithelium can be useful in differentiating these dysplastic lesions with lichenoid features and LP.

In our study, in all the cases with moderate to severe epithelial dysplasia, a striking population of CD8+ lymphocytes were found following the dysplastic changes in the epithelial zone. Since cytotoxic CD8+ cells will try to eliminate the cells undergoing malignant transformation within the epithelial layer, it makes sense that in moderate to severe OED, these types of inflammatory cells follow the malignant changes throughout the thickness of this morphological and cytological changes within the tissue. For this reason, the localization of CD8+ cells could be used to differentiate OED and LP in equivocal cases. Of course, more proven cases are necessary, along with clinical follow-up of the patient's progression to confirm this theory. Also, tangential sectioning of tissue can obscure the true position of these CD8+ lymphocytes, biasing the results.

CONCLUSIONS

The inflammatory component of the oral cavity is a complex system that, at this point in time, is still not well understood, especially with respect to potentially premalignant mucosal lesions. The diagnostic dilemma addressed in this study may be eventually solved by identifying cells with specific functions within the inflammatory population that can be used to track early malignant changes within the mucosal epithelium. However, there is no specific marker or panel of markers available yet that allow reliable prediction of malignant transformation of leukoplakia in the oral cavity. Further studies regarding specific subtypes of CD4+ and CD8+ T-cells are necessary to distinguish between OLP and OED and reliably predict the malignant transformation of OED and those equivocal cases previously mentioned.

Results of this study suggests that the distribution of CD8+ by immunohistochemistry can help in differentiating moderate/severe oral epithelial dysplasia, from LP and potentially other inflammatory conditions. Further research with more cases in comparison with diverse inflammatory models, is necessary to establish conclusive results, which will be part of the continuation of this pilot study.

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