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**A retrospective and prospective  
analysis of Oral Hairy Leukoplakia in a  
South Australian HIV-infected  
population**

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*This thesis is dedicated to Mum and Dad for their continual support and encouragement over the years.*

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## **ABSTRACT**

Oral hairy leukoplakia (OHL) is an oral lesion that, prior to the advent of AIDS in 1981, had not been reported in the literature.

In 1984, Greenspan *et al*, described a white, non-removable lesion, which they called oral “hairy” leukoplakia (OHL), which was confined to the lateral borders of the tongue. OHL was associated in this initial report with both papillomavirus and a herpes-type virus. It was also observed that the presence of this lesion was associated with progression of HIV disease in many of the patients in the study. The importance of OHL as an indicator of immunosuppression and prognosis of HIV disease was soon realised. It is included by the Centers for Disease Control in the 1993 Revised Classification System for HIV Infection and Expanded Case Surveillance Case Definition for AIDS Among Adolescents and Adults.

In the 13 years since the first description of OHL, many studies, primarily from the United States and Europe, have investigated the lesion with respect to aetiology and pathogenesis, histologic features and relevance to HIV disease progression. A close relationship has been observed between OHL and Epstein-Barr virus (EBV).

There has not been any Australian studies which have described, in detail, the behaviour and characteristics of OHL in Australian HIV-infected

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patients. Anecdotal evidence indicated that OHL may not be as strong an indicator of HIV progression or of immunosuppression in South Australian patients as had been reported by international studies

This research project was designed in two sections. The first part of the project comprised a retrospective analysis of OHL in 197 patients who had attended the Medically Compromised Patient Unit of the Adelaide Dental Hospital. The prevalence of OHL in these patients was examined with respect to factors such as length of time of HIV infection, CD4+ T-lymphocyte counts, AIDS-defining illness and concurrent medication. The second, prospective analysis, of OHL examined the lesion in 20 patients who had clinical evidence of the lesion. Exfoliative cytology smears from OHL lesions of these patients were examined using light microscopy and transmission electron microscopy.

The results from this project found that the prevalence of OHL in South Australian patients was comparable to that found in international studies. The presence of OHL was not related to CD4+ T-lymphocyte count or AIDS-defining illness, nor did the length of time a patient had been infected with HIV relate to the presence of OHL. An association was observed between a reduced prevalence of OHL in patients taking the antiviral medications AZT and aciclovir. The prevalence of oral *Candida* infection in relation to the presence of OHL was not statistically significant.

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The prospective analysis of OHL found that the clinical appearance of OHL in the group of 20 patients was varied. It could present as a smooth, flat white lesion, to one that was corrugated and “shaggy”. The size of the lesions was not related to medication or CD4+ T-lymphocyte count. OHL was observed to occur over a large range of CD4+ T-lymphocyte counts.

Examination of exfoliative cytology specimens revealed *Candida* infection in 25% of cases. The light microscopic appearance of squamous epithelial cells did not reveal any marked changes in the morphology of the cells. Ultrastructural examination of squamous epithelial cells demonstrated the presence of herpes-type viruses (consistent with EBV) within the cell nuclei and extracellularly. Nuclear degeneration, as described in other studies, was also observed, as was the presence of fungal organisms.

Generally, these results concur with those from other reports in the literature relating to OHL. These results do support the notion that OHL is not an indicator of immunosuppression in South Australian patients, of course longitudinal studies are required to ascertain the relationship of OHL to HIV disease progression. Although supporting the possible role for EBV in the aetiology of OHL, these results do not provide unequivocal support for EBV as the sole aetiologic agent for the pathogenesis of OHL. The role of co-factors in the pathogenesis of this lesion requires further investigation.

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## **DECLARATION**

This work contains no material which has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis being made available for loan and photocopying.

Richard M. Logan

November 1997

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## **LIST OF ABBREVIATIONS**

<b><i>Acic.</i></b>	Aciclovir
<b><i>AIDS</i></b>	Acquired Immune Deficiency Syndrome
<b><i>AZT</i></b>	Zidovudine
<b><i>BSA</i></b>	Bovine serum albumin
<b><i>Clarith.</i></b>	Clarithromycin
<b><i>Clofaz.</i></b>	Clofazamine
<b><i>CMV</i></b>	Cytomegalovirus
<b><i>ddC</i></b>	Zalcitabine
<b><i>ddl</i></b>	Didanosine
<b><i>d4T</i></b>	Stavudine
<b><i>EA-D</i></b>	Diffuse early antigen
<b><i>EBV</i></b>	Epstein-Barr virus
<b><i>Etham.</i></b>	Ethambutol
<b><i>Flucon.</i></b>	Fluconazole
<b><i>Ganc.</i></b>	Ganciclovir
<b><i>H and E</i></b>	Haematoxylin and eosin
<b><i>HIV</i></b>	Human immunodeficiency virus
<b><i>HPV</i></b>	Human papilloma virus
<b><i>HSV</i></b>	Herpes simplex virus
<b><i>HZV</i></b>	Herpes zoster virus
<b><i>Itracon.</i></b>	Itraconazole
<b><i>Ketocon.</i></b>	Ketoconazole

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<b>KS</b>	Kaposi's sarcoma
<b>LMP-1</b>	Latent membrane protein
<b>MAC</b>	<i>Mycobacterium avium</i> complex
<b>NHS</b>	Normal horse serum
<b>OHL</b>	Oral hairy leukoplakia
<b>PAS</b>	Periodic Acid - Schiff
<b>PBS</b>	Phosphate buffered saline
<b>PCP</b>	<i>Pneumocystis carinii</i> pneumonia
<b>Pent.</b>	Pentamidine
<b>Rifam.</b>	Rifampicin
<b>TB</b>	Tuberculosis
<b>TEM</b>	Transmission electron microscopy
<b>TMP/SMX</b>	Trimethoprim/sulfamethoxazole
<b>WHO</b>	World Health Organisation

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## **Chapter 1**

### **INTRODUCTION**



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## **1. INTRODUCTION**

The first cases of Acquired Immune Deficiency Syndrome (AIDS) were identified in the United States in 1981 (Gottlieb *et al*, 1981; *MMWR*, 1981). The first indication of this new disease was the diagnosis of *Pneumocystis carinii* pneumonia (PCP) and Kaposi's Sarcoma (KS) in previously healthy, homosexual men. The viral aetiologic agent for this syndrome was eventually isolated in 1983 by both French and American investigators. It was shown to be a retrovirus and, in 1986, was named the Human Immunodeficiency Virus (HIV) .

Oral manifestations as a result of immunosuppression due to HIV infection have been widely reported in the literature. The majority of people infected with HIV experience oral lesions during the course of their disease (Greenspan and Greenspan 1996).

Oral manifestations of HIV infection can be divided into four broad groups according to their aetiology. These groups are:

1. Bacterial infections (for example, periodontal diseases)
2. Fungal infections (for example, oral *Candida* infections)
3. Viral infections (for example, herpes simplex infection)
4. Neoplastic lesions (for example, non-Hodgkins lymphoma)

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Many of these lesions have been reported to have an atypical presentation in patients who are infected with HIV (Greenspan and Greenspan, 1996). Oral lesions may present with increased frequency and severity when occurring in the context of HIV infection. In addition some oral manifestations had not been described before the AIDS epidemic. An example of such a lesion was oral hairy leukoplakia.

In 1984, Greenspan *et al*, reported this previously undescribed oral lesion in HIV positive, homosexual men. The white, non-removable lesion, which they called oral "hairy" leukoplakia (OHL), was confined to the lateral borders of the tongue. A high prevalence of *Candida* was found in association with OHL. More interestingly however, papillomavirus and a herpes-type virus were found in association with OHL. It was also observed that a proportion of the patients in the study developed AIDS-defining illnesses within a relatively short time following the diagnosis of OHL.

The importance of OHL as an indicator of immunosuppression and prognosis of HIV disease was soon realised. It is included in the 1993 Revised Classification System for HIV Infection and Expanded Case Definition for AIDS Among Adolescents and Adults (MMWR, 1992) (Appendix I). The European Clearinghouse on oral problems related to HIV infection and the WHO collaborating centre on the oral manifestations of the immunodeficiency virus (EC-Clearinghouse, 1993) included OHL in its

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classification as a Group I lesion, that is, an oral lesion strongly associated with HIV infection.

Since its initial description, OHL has since been reported to occur in all risk groups for HIV infection. In rare cases, OHL has been identified in patient groups who are immunosuppressed as a result of immunosuppressive mechanisms other than HIV infection.

In the 13 years since OHL was first reported, many studies have investigated the lesion with respect to aetiology and pathogenesis, histologic features and relevance to HIV disease progression. There have not been, however, any Australian studies which have described, in detail, the behaviour and characteristics of OHL in Australian patients.

In the following Chapter, the literature relating to OHL is reviewed. The first section of the review examines clinical aspects of OHL, in particular its prevalence in HIV-infected populations, its clinical appearance and behaviour, its relationship to HIV disease and finally, its occurrence in HIV-negative patients. The second part of the literature review relates to the histological features of OHL, including the light microscopic and ultrastructural appearances of the lesion. The majority of the literature relating to OHL has concentrated on its aetiology and pathogenesis. This is covered in the third section of the review. The last section of the literature

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review is concerned with diagnostic criteria and treatment modalities which have been described in the literature.

In the Australian context, there are questions relating to OHL which need to be addressed. It is unknown whether the characteristics of the lesion are the same as has been described in the literature. In addition, the role of OHL as an indicator of HIV progression is unknown in Australian populations and there are still questions relating to the aetiology and pathogenesis of the lesion.

This analysis of OHL in a South Australian HIV-infected population was carried out to gain more information on this lesion in the Australian setting. The research project was designed, as described in Chapter 3, in two sections, a retrospective and prospective analysis.

The retrospective analysis of OHL involved collecting information from the case notes of HIV-infected patients who had attended the Medically Compromised Patient Unit of the Adelaide Dental Hospital over an 8 year period. Information was recorded which included the presence of OHL, length of time of HIV infection, medication, AIDS-defining illnesses and laboratory parameters such as CD4+ T-lymphocyte count.

In the prospective analysis, 20 patients with clinical evidence of OHL were examined. Exfoliative cytology specimens of OHL lesions were examined

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using light microscopy and transmission electron microscopy. Identification of EBV in the lesions was also achieved using immunohistochemistry.

Chapter 4 presents the results from the retrospective and prospective analyses of OHL in this group of patients. These results are discussed and appraised in relation to results from other studies of OHL in Chapter 5. Finally, in Chapter 6 the conclusions which could be drawn from this analysis are outlined.

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## Chapter 2

### LITERATURE REVIEW

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## **2. LITERATURE REVIEW**

### **2.1 Clinical features of Oral Hairy Leukoplakia**

Oral hairy leukoplakia (OHL) has been extensively studied since its initial description by Greenspan *et al* (1984). Knowledge of the features and behaviour of this lesion, such as its prevalence in the HIV-infected population and its clinical appearance, is important in order to be able to study its relevance to HIV disease.

#### **2.1.1 Prevalence of Oral Hairy Leukoplakia**

OHL was initially detected in San Francisco in a group of homosexual men who were infected with HIV (Greenspan *et al*, 1984). Since then, it has been found to occur in all risk groups infected with HIV. These groups include intravenous drug users (Ficarra *et al*, 1988; Barrone *et al*, 1990), haemophiliacs (Rindum *et al*, 1987; Greenspan *et al*, 1989A), blood transfusion recipients (Greenspan *et al*, 1989A) and, occasionally, children infected with HIV (Laskaris, Laskaris and Theodoridou, 1990; Leggott, 1992; Itin *et al*, 1994).

OHL has also been reported, albeit rarely, in HIV-negative patients, for example in iatrogenically immunosuppressed patients (Birek *et al*, 1989; Greenspan *et al*, 1989B; Macleod, Lang and Soames, 1990; Schmidt-Westhausen, Gelderblom and Reichart, 1990; Kanitakis *et al*, 1991;

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Schmidt-Westhausen *et al*, 1991; Epstein, Sherlock and Wolber, 1993; Schmidt-Westhausen *et al*, 1993).

There are relatively large discrepancies in the reported prevalence of OHL in HIV-infected populations around the world.

In a group of 75 Dutch patients infected with HIV, 16% had OHL. However, in this group, the prevalence of OHL in patients with AIDS was 21% (Schulten, ten Kate and van der Waal, 1989). Another European study found OHL to occur in 15% of patients infected with HIV (Porter *et al*, 1989).

In 334 HIV-1 seropositive African women, 0.4% were diagnosed with OHL (Wanzala *et al*, 1989). Ramírez-Amador, Gonzalez and de la Rosa (1990) reported OHL occurring in 43% of a cohort of Mexican patients infected with HIV whereas Ceballos-Salobreña, Aguirre-Urizar and Baga-Sebastian (1996), reported an OHL prevalence of 16.2% in an HIV-infected Spanish population.

The variability in the prevalence of OHL is clearly based on factors such as diagnostic criteria, characteristics of the patient cohort (for example, age, stage of HIV infection, access to health care) as well as clinical expertise in diagnosis.

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### 2.1.2 Clinical appearance of Oral Hairy Leukoplakia

The possible prognostic significance of OHL in HIV infection and the fact that it may be an early indicator of infection with HIV emphasises the importance of distinguishing OHL from other fixed white lesions that can occur in the mouth, particularly those involving the tongue.

A clinical differential diagnosis of white lesions of the tongue can include leukoplakia, geographic tongue, frictional keratosis (for example, tongue biting), lichen planus, and hyperplastic candidiasis (Green *et al*, 1989). In addition to these diagnoses, early squamous cell carcinoma should also be considered.

OHL has relatively distinctive clinical features that need to be identified for diagnosis. As its name suggests, OHL is a white lesion and is typically found on the lateral borders of the tongue. The surface of the lesion varies from smooth and flat, to a surface that is corrugated or “hairy” in appearance (Greenspan *et al*, 1984; Schiødt *et al*, 1987; Williams *et al*, 1991). The surface features of the OHL lesions may be influenced by the site at which they occur on the tongue. Schiødt *et al* (1987) found that the smooth flat lesions were more likely to occur on the ventral surface of the tongue whilst the corrugated or “hairy” lesions were usually found on the lateral surface of the tongue and occasionally, in severe cases, extended onto the dorsal surface.

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OHL is often found bilaterally (Schiødt *et al*, 1987; Schulten *et al*, 1992). The size of the lesions is variable. In the study by Schiødt *et al* (1987), it was found that OHL lesions ranged in size from 14 to 4200 mm<sup>2</sup>.

Apart from in the oral cavity, OHL has not been found on other mucosal surfaces. There have been reports, however, of OHL occurring in other areas in the mouth other than on the lateral border of the tongue. Involvement of the buccal mucosa (Greenspan *et al*, 1984; Kabani *et al*, 1989; Ficarra *et al*, 1992), floor of the mouth, palate, retromolar areas, tonsillar region and posterior pharyngeal mucosa have all been reported (Kabani *et al*, 1989).

Although originally considered to be a lesion that is relatively stable without periods of remission, studies have shown that spontaneous resolution can occur followed by recurrence (Katz *et al*, 1991). This may reflect factors such as changing CD4+ T-lymphocyte count or viral load.

### **2.1.3 Correlation between Oral Hairy Leukoplakia and HIV disease progression**

The length of time from seroconversion to the development of severe immunosuppression is of interest to both patients and their health care providers. The use of clinical markers has become a convenient way of monitoring a patient's immune status. Clinical markers have also served, in some instances, as end points for clinical drug trials (Cooper *et al*, 1993).

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Oral lesions such as OHL and oral candidiasis are common in patients with HIV infection and have been investigated as to their prognostic significance. OHL has also been shown by some studies to indicate increased likelihood of HIV disease progression (Greenspan *et al*, 1984; Coates *et al*, 1992) and it is included in the Centers for Disease Control and Prevention (CDC) classification of HIV infection (Appendix I).

As previously mentioned, the prevalence of OHL appears to be higher in patients with AIDS compared with those patients who do not have any AIDS defining conditions (Schulten *et al*, 1989). Many studies have recorded the prevalence of OHL and concurrent indicators of immune function such as CD4+ T-lymphocyte counts, HIV viral replication and p24 antigenaemia (Glick *et al*, 1994; Kolokotronis *et al*, 1994; Lifson *et al*, 1994; Ravina *et al*, 1996), along with rates of progression to AIDS or death (Greenspan and Greenspan, 1992).

OHL has been found to develop soon after HIV seroconversion and in patients with a range of CD4+ T-lymphocyte counts (Lifson *et al*, 1994).

The prognostic value of OHL was initially reported by Greenspan *et al* (1984). In this initial description of OHL it was noted that 9 out of the 37 patients in the study had developed an AIDS defining illness, such as *Pneumocystis carinii* pneumonia, within a 12 month period since diagnosis of OHL lesions. In addition to this, one patient in the study died from AIDS-

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related illness. Further studies by Greenspan *et al* (1987), reinforced the predictive value of OHL for the development of AIDS. Survival analyses predicted that patients with OHL had a probability of AIDS developing of 48% within 16 months and 83% within 31 months.

Glick *et al* (1994) found that when OHL occurred in HIV-infected patients, it could be identified at 24 months (median time) before the diagnosis of an AIDS-defining illness and 41 months before death. Glick *et al* (1994), calculated that the presence of OHL meant that the risk of having a CD4+ T-lymphocyte count below 200 cells/ $\mu$ L was 70.3%.

According to Glick *et al* (1994) and Begg *et al* (1996), the more oral manifestations of HIV infection that are present in a patient, the higher the probability of that patient having a low CD4+ T-lymphocyte count and a poorer prognosis.

Kolokotronis *et al* (1994) concluded that the presence of OHL combined with immunological factors such as low circulating CD4+ T-lymphocytes and loss of serum anti-p24 antibodies may indicate an increased likelihood for progression to AIDS. OHL on its own, however, was not found to be associated with an increased level of immunosuppression because the presence of OHL did not increase as CD4+ T-lymphocyte counts decreased (Kolokotronis *et al*, 1994). Ravina *et al* (1996) also found that the presence

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of OHL was associated with compromised immunological factors and higher viral replication which was indicated by p24 antigenaemia.

Although they are relatively scarce, such studies indicate that the use of OHL as a clinical marker may be as useful as markers such as CD4+ T-lymphocyte levels in predicting progression to AIDS.

OHL may also be a useful clinical marker to help determine whether or not anti-retroviral therapy should be started. According to Schiødt *et al* (1990), the use of clinical indicators are important in situations (for example, in underdeveloped countries) where expensive diagnostic tests such as viral load are not feasible.

#### **2.1.4 Oral Hairy Leukoplakia in HIV-negative patients**

HIV infection is not the only cause of immunodeficiency. Other causes may include primary immunodeficiencies, lymphoproliferative diseases and iatrogenic suppression of the immune system by immunosuppressive medication required to prevent rejection of transplanted organs. Radiotherapy and chemotherapy used in the treatment of neoplastic disease can also contribute to immunosuppression. Consequently, OHL may be a clinical feature that is seen in other types of immunodeficiency.

OHL has been reported in patients who have had renal transplants (Greenspan *et al*, 1989B; Macleod *et al*, 1990; Kanitakis *et al*, 1991), heart

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transplants (Schmidt-Westhausen *et al*, 1990; Schmidt-Westhausen *et al*, 1991), liver transplants (Schmidt-Westhausen *et al*, 1993) and bone marrow transplants (Birek *et al*, 1989; Epstein *et al*, 1993). With the exception of one report (Epstein *et al*, 1993), each case of OHL was shown to be associated with Epstein-Barr virus.

Epstein *et al* (1993) studied lesions resembling OHL in ten patients who had undergone bone marrow transplantation. EBV could be demonstrated in lesions of only three of these patients and two out of the three also demonstrated human papilloma virus (HPV) by *in situ* hybridisation. The presence of histological features such as acanthosis, koilocytosis and hyperparakeratosis could not discriminate between lesions associated with EBV or HPV.

Syrjänen *et al* (1989) reported a case of OHL in a patient undergoing chemotherapy for acute myeloblastic leukaemia. The lesion was detected prior to bone marrow transplantation. EBV was identified in the lesion by the use of *in situ* hybridisation.

Other instances of OHL that were not associated with HIV infection have been described in patients taking systemic steroids. Reports have included an asthmatic patient (Zakrzewska, Aly and Speight, 1995), a patient with Behçet's syndrome (Schjødt, Nørgaard and Greenspan, 1995) and two

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patients who had been treated with topical steroids for oral vesiculobullous disease (Lozada-Nur, Robinson and Regezi, 1994).

The asthmatic case reported by Zakrzewska *et al* (1995) is interesting because the lesion suspected to be OHL was associated with a severe episode of pseudomembranous candidiasis which was thought to be precipitated by the corticosteroid therapy. The "OHL" was treated with Nystatin initially, followed by ketoconazole which also proved to be ineffective. Although EBV was identified in the tongue lesions by *in situ* hybridisation, the lesions described as OHL ultimately responded to antifungal treatment with fluconazole.

Lesions which respond to antifungal treatment are generally not considered to be OHL. While it may be possible that the candidiasis in the previous case report (Zakrzewska *et al* 1995) was a cofactor in the development of OHL, it has been demonstrated that *Candida albicans* is not associated with all cases of OHL (Reichart *et al*, 1989). It can be speculated, however, that *Candida* could be a co-factor in more cases of OHL than have previously been reported.

OHL has been reported, albeit rarely, in patients who have been shown to be immunocompetent (McMillan *et al*, 1989; Eisenberg, Krutchkoff and Yamase, 1992; Felix *et al*, 1992; Lozada-Nur *et al*, 1994).

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Felix *et al* (1992) reported OHL occurring in an elderly man who had no evidence of HIV infection or clinical immunodeficiency. The patient's CD4+ T lymphocyte count was only 364 cells/ $\mu$ L, a level that is consistent with the development of OHL in patients who have HIV infection. The percentage of CD4+ T lymphocytes cells (out of the total lymphocyte count), however, was 44%, which is within the normal range. This case possibly supports the view of Eisenberg *et al* (1992) who surmised that OHL is not necessarily indicative of immunosuppression, but rather, represents a localised and transient infection of the epithelium.

Iatrogenic immunosuppression is relatively common in the general population, it is surprising, therefore, that the prevalence of OHL in these patients is not proportional. In order to account for this, Greenspan *et al* (1989B) proposed that existing leukoplakias in patients receiving immunosuppressive drugs are initially devoid of EBV. The immunosuppression allows EBV to be expressed and to replicate within these lesions. Although this is one explanation which accounts for the rarity of the lesion in HIV negative patients, there is no evidence that OHL should develop differently in HIV-infected patients. Apart from misdiagnosis in HIV negative patients, this may indicate that other factors are involved besides a suppressed immune system.

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## **2.2 Histopathology of Oral Hairy Leukoplakia**

The histopathological features of OHL have been well documented. The initial report of the lesion by Greenspan *et al* (1984) described OHL as having a similar histologic appearance to that of flat warts of the skin. Since 1984 numerous studies have been performed on OHL lesions using both light and electron microscopy.

### **2.2.1 Light microscopic features of Oral Hairy Leukoplakia**

Greenspan *et al* (1984) described the histologic features of OHL in 35 specimens taken from 30 patients. All of the biopsies, taken from the lateral border of the tongue, showed hyperparakeratotic acanthotic epithelium. In some areas, the keratin formed projections which gave the appearance of hairs on the epithelial surface. The cells of the prickle cell layer demonstrated features resembling koilocytes which are characterised by ballooning changes in their cytoplasm and pyknotic nuclei with perinuclear halos. The subepithelial connective tissue showed a minimal or, in most cases, no inflammatory cell infiltrate.

Some authors have objected to the use of the terms “koilocytosis” and “koilocytes” in reference to the ballooning, vacuolated keratinocytes found in the prickle cell layer of the epithelium of OHL (Kanas *et al*, 1988A; Syrjänen *et al*, 1989). According to Kanas *et al* (1988A) these terms should be restricted to lesions which are caused by human papilloma virus. Syrjänen *et al* (1989) described the differences between true koilocytes found in HPV

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infection and those in OHL. True koilocytes have enlarged nuclei that result in an increased nuclear to cytoplasmic ratio. In OHL the nuclei of these cells are small. Kanas *et al* (1988), compared koilocytes found in flat condylomatous lesions of the cervix (FCLC), which are associated with HPV, and the cells found in OHL using light microscopy and immunohistochemistry. In addition to increased nuclear to cytoplasmic ratio, the cells in OHL were less extensively vacuolated compared with FCLC. The latter also showed features of nuclear pleomorphism such as multinucleated nuclei.

Other studies (Schjødt *et al*, 1987; Kanas *et al*, 1988; Itin and Ruffli, 1990) have reported similar histologic features of OHL to that described by Greenspan *et al* (1984). Schjødt *et al* (1987) presented a more detailed description of the histologic spectrum of OHL. Again, parakeratosis, acanthosis, vacuolated cells and hair-like projections of keratin were the prominent features of these lesions. Although there were *Candida* hyphae present in the epithelium of 43% of Schjødt's specimens, no leukocytes were found in the epithelium or in relation to the hyphae. As would be expected, the histologic appearance of the OHL corresponded to its clinical appearance. For example, the lesions that were flat clinically usually lacked the characteristic keratin projections at the epithelial surface. Vacuolated cells were found mainly in the superficial part of the prickle cell layer.

OHL lesions are typically reported as not showing any signs of dysplasia or

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pre-malignancy. However, apart from epithelial hyperplasia which is a recognised histological feature of OHL, minor atypia has been reported including hyperchromatism and increased mitoses of the basal layer (Glick and Pliskin, 1990).

Scuibba *et al* (1989) found that many of the superficial cells of OHL lesions had dense aggregations of nuclear chromatin adjacent to the nuclear membrane. It was suggested that this appearance was similar to the Cowdry type inclusions which are a typical feature of cells infected with herpes viruses. Other studies have also described these inclusions in superficial cells of OHL lesions by examination of exfoliative cytology specimens (Fraga-Fernández and Vicandi-Plaza, 1992; Migliorati *et al*, 1993).

The presence of other cell types in OHL, particularly Langerhans cells, has also been studied (Daniels *et al*, 1987; Cruchley *et al*, 1989; Riccardi *et al*, 1990). Langerhans cells play an important role in the cell mediated immune system and are involved in the processing and presentation of antigens (Barret, Crutchley and Williams, 1996).

Regional variation of Langerhans cells in the oral mucosa has been reported in various studies (Daniels, 1984; Cruchley *et al*, 1989). The lateral border of the tongue normally has small areas, ranging from 0.2 - 0.5mm in diameter, where Langerhans cells are absent (Daniels *et al*, 1987). Cruchley

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*et al* (1989) found that the lateral border of the tongue was an area in the oral cavity, along with the hard palate and floor of the mouth, that normally has reduced numbers of Langerhans cells when compared with other areas in the oral cavity.

Daniels *et al* (1987) found that Langerhans cells are absent or considerably reduced in numbers in OHL lesions, and postulated that this may be a factor in the development of OHL by allowing viral replication within the cells of the epithelium because of a reduced local immune response. Corso, Eversole and Hutt-Fletcher (1989), also found a marked reduction in the number of cells that stained for S-100 in OHL. This was determined by comparing cell counts per high-powered field in 10 cases of OHL with cell counts in 10 cases of normal lateral tongue mucosa. It is unknown, however, whether the absence of Langerhans cells precedes the development of OHL or occurs subsequent to the development of the lesion.

Patterns of keratin expression in OHL have been studied using immunohistochemistry and *in situ* hybridisation in order to elucidate the behaviour of the lesion and compare it with other white lesions that occur in the mouth (Williams *et al*, 1991; Langford *et al*, 1992; Su *et al*, 1993).

Su *et al* (1993) found comparable levels of keratin 14, a major cytokeratin that is found in the basal layer of stratified squamous epithelium, between normal control oral mucosa and OHL lesions using immunohistochemistry.

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*In situ* hybridisation, however, demonstrated a decrease in the level of keratin 14 mRNA in OHL. This may be a consequence of the increased sensitivity of *in situ* hybridisation compared with immunohistochemistry or simply reflect the increased stability of cytokeratin proteins compared with mRNA (Su *et al*, 1993).

Langford *et al* (1992) found that cytokeratins 10 and 11 were increased in the epithelium of the lateral border of the tongue of HIV positive patients compared with levels in epithelium from HIV negative patients. These cytokeratins are considered markers for keratinisation and are not normally expressed in non-keratinising epithelium (Langford *et al*, 1992). This study found that irrespective of the clinical presence of OHL, there is an increase in keratinising cells in the lateral border of the tongue in HIV positive patients. In addition, it was reported that there appeared to be no correlation between the types of cytokeratins expressed in the epithelium of the lateral border of the tongue and the clinical presence of OHL lesions.

Williams *et al* (1991) found that expression of keratins 6, 16 and 19 were markedly reduced in OHL. Keratin 19 has been associated with a premalignant potential in epithelia because its presence indicates delayed terminal differentiation of epithelial cells. Keratins 6 and 16 are related to epithelia with a high turnover, their absence indicating decreased turnover of the epithelium, thereby resulting in acanthosis which is a characteristic histologic feature of OHL. Other keratins such as 5 and 14 were expressed

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similarly between OHL and controls. The combination of these facts relating to cytokeratin expression possibly indicates a low premalignant potential for OHL which is in accordance with the histologic features of the lesion.

It should be noted that the “characteristic” features of OHL can also be seen in some cases in normal tongue epithelium. Andersen, Philipsen, Reichart (1990) looked at the normal anatomy of the lateral border of the tongue and found that features mimicking hyperplasia, acanthosis, and keratin projections could be demonstrated depending on which way the tissue was sectioned. Andersen *et al* (1990) also reported cells that could be interpreted as koilocyte-like cells in association with filiform papillae.

### **2.2.2 Electron microscopic features of Oral Hairy Leukoplakia**

Several ultrastructural studies of OHL have been completed since 1984 (Greenspan *et al*, 1984; Belton and Eversole, 1986; El-Labban *et al*, 1988; Reed, Fowler and Brannon, 1988; Zhang *et al*, 1988; Sciubba *et al*, 1989; Greenspan *et al*, 1989; El-Labban *et al*, 1990). The initial ultrastructural descriptions of OHL by Greenspan *et al* (1984) concentrated on the presence of viral particles both within the nuclei of the prickle cells and intercellularly. These viral particles had the appearance of a herpes-group virus. This feature was confirmed by subsequent investigations (Reed *et al*, 1988; Zhang *et al*, 1988; Greenspan *et al*, 1989C).

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Virions have been found in various stages of development and scattered throughout the nucleus (Belton and Eversole, 1986). They have also been seen budding from the nuclear envelope which is thickened at these locations (Belton and Eversole, 1986).

Other ultrastructural features of the epithelium correlate with those observed using light microscopy.

Ballooned keratinocytes or koilocyte-like cells are a characteristic feature in the prickle cell layer of OHL. Ultrastructural studies indicate that they contain only a few organelles including degenerated mitochondria and abundant intermediate sized keratin fibrils (Zhang *et al*, 1988). These authors noted two characteristic features occurring within the cytoplasm of the virus infected keratinocytes. The first consisted of 35nm tubules that were arranged in bundles 1µm in length. The other structure was composed of “undulating and convoluted membranes”, 25nm in width, associated with the nuclei of the koilocytes. It was suggested that these structures may represent tubuloreticular precursors. Tuboreticular structures have been identified in various tissues in patients with AIDS-related complex and AIDS along with other immunological and neurodegenerative diseases. It is possible that they may be an indicator of interferon production which is consistent with a viral aetiology for OHL.

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El-labban *et al* (1988) reported two types of crystalline inclusions within the cytoplasm of the epithelial cells of OHL. One structure consisted of microtubules associated with an electron dense body. The other was an elongated multivesicular structure. The vesicles were of similar size to the herpes-type virions that were also present within the cells. It was suggested that these represented an abnormal form of microtubule within the epithelial cells or that the multivesicular structures may, in some way, be related to the viruses within the cell.

Foci of condensed chromatin are also found in keratinocytes along with infestation of the epithelium with *Candida*. *Candida* organisms have been found extracellularly, within the cytoplasm and, in some cases, within the nucleus (Belton and Eversole, 1986).

Ultrastructural studies of biopsies confirmed that Langerhans cells are absent in these lesions (Zhang *et al*, 1988; Riccardi *et al*, 1990), a feature that is consistent with the findings of light microscopic examination. Furthermore, no evidence of inflammatory cells were noted within the lesions.

Sciubba *et al* (1989) described the ultrastructural morphology of the superficial cells of OHL lesions. The nuclei of the superficial cells were flattened, had a "ground glass" appearance and clusters of chromatin were distributed radially inside the nuclear envelope. Although Sciubba *et al*

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(1989) eliminated the possibility of the presence of human papillomavirus (HPV) in OHL lesions by using Southern blot analysis, the presence of EBV was demonstrated. There was also no morphological evidence for the occurrence of HPV within the lesions.

Ultrastructural examination of exfoliative cytology specimens has also been performed (Kratochvil *et al*, 1990; Epstein *et al*, 1995). In these studies, herpes-type virions were observed within the cells and in the intercellular spaces. Kratochvil *et al* (1990) also observed bacterial colonisation and fungal hyphae associated with the keratinocytes. In both studies the cells had few organelles, a feature consistent with their derivation from superficial layers of the epithelium. The ease of collecting exfoliative cytology specimens and the demonstration of herpes-type virions within the cells makes this a useful, non-invasive technique for the diagnosis of OHL.

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## 2.3 **The aetiology and pathogenesis of Oral Hairy Leukoplakia**

The identification of an aetiological agent for the development of OHL has been a goal since the lesion was first described. OHL is often found in association with *Candida* (Glick and Pliskin, 1990). The strong association of the lesion with the Epstein-Barr virus has resulted in the need for the identification of this virus for the definitive diagnosis of OHL (Williams *et al*, 1992).

### 2.3.1 ***Candida* and Oral Hairy Leukoplakia**

Infections with *Candida* in the mouth have become increasingly more common as a result of immunodeficiency due to HIV infection. *Candida albicans* is the most common species of *Candida* that is found in the oral cavity (Scully, El-Kabir and Samaranayake, 1994).

*Candida* has frequently been isolated in OHL. In the initial reported cases of OHL, *Candida* was found in 26 out of 37 cases (Greenspan *et al*, 1984). Other studies have also found a high prevalence of *Candida* associated with OHL (Schiødt *et al*, 1987) and ultrastructural studies have demonstrated *Candida* present in the superficial layers of the epithelium (Zhang *et al*, 1988).

The question of whether or not *Candida* plays a role in the aetiology and pathogenesis of OHL has been considered because of the frequent finding of fungal organisms within the lesions.

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Changes in the epithelium are consistent findings associated with oral *Candida* infection. An example where epithelial changes are observed is chronic hyperplastic candidiasis. The histologic features of this condition include a parakeratotic, hyperplastic and acanthotic epithelium, features also found in OHL lesions. Clinically, the lesions appear as white or speckled areas which do not rub off (Reed *et al*, 1990). The organisms are confined to the upper layers of the epithelium. It has been postulated that the thickening of the epithelium is a physiologic protective response to mechanical and chemical damage caused by the fungal organisms (Nagai, Takeshita and Saku, 1992).

Increases in epithelial cell mitotic activity by products of *C. albicans* have also been demonstrated (Reed *et al*, 1990). These authors proposed that *Candida* products could have a direct effect on epithelial cells, although the exact factor responsible was not isolated nor identified. *C. albicans* produces a wide variety of enzymes which may have an effect on epithelial cells.

Clinically, the role of *Candida* as the aetiologic agent in the development of OHL lesions is not supported. Topical or systemic antifungal medication does not result in a resolution of OHL. Hence, it is unlikely that *Candida* is a sole aetiologic agent in the development of OHL. It is more probable that the conditions that are created by the presence of OHL produce a suitable environment for the opportunistic infection of the epithelium by *Candida*.

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### 2.3.2. Viruses and Oral Hairy Leukoplakia

The initial description of OHL reported that the ultrastructural appearance of the lesion demonstrated two types of virus (Greenspan *et al*, 1984). Human papillomavirus was identified using immunocytochemistry and a herpes-type virus was seen at the ultrastructural level. Greenspan *et al* (1984), suggested that OHL may be a result of a dual infection by the two viruses.

Since this initial study, the evidence for human papilloma virus in OHL has been conflicting. The herpes-type virus has been consistently found in OHL lesions and subsequently demonstrated to be Epstein-Barr virus (Greenspan *et al*, 1985).

#### 2.3.2.1 Human Papilloma Virus

Human papilloma viruses (HPV) belong to the family Papovaviridae of which there are over 70 members (Miller, 1995). Each type of HPV appears to have a preference for infecting specific epithelial sites. "High risk" types of HPV that are implicated as the aetiological agents of cervical squamous cell carcinomas are 16, 18, 31, 45 and 54 (Laimins, 1996). HPV types 6 and 11 have been associated with genital warts, lesions that are considered to have a low malignant potential (Kumar *et al*, 1992; Laimins, 1996).

HPV has also been implicated in numerous oral lesions including oral viral warts, focal epithelial hyperplasia (Heck's disease), oral squamous cell carcinoma, lichen planus and OHL (Chang *et al*, 1991).

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The evidence for HPV in hairy leukoplakia is conflicting. Greenspan *et al* (1984), in their initial report of OHL, found evidence of HPV. It was also demonstrated in a subsequent study in which HPV was detected by electron microscopy and immunocytochemistry in 73% of biopsy specimens (Greenspan *et al*, 1985).

Eversole *et al* (1986) found no evidence of HPV by ultrastructural examination of OHL lesions. However immunocytochemistry demonstrated HPV in 14% of koilocyte nuclei.

Adler-Storthz *et al* (1992) found HPV DNA corresponding to HPV types 16 and 18 in ten out of eighteen biopsies of OHL by the use of polymerase chain reaction amplification. In three of the cases the type of HPV was of an unknown type and not 6, 11, 16 or 18. Overall, the predominant type of HPV that was found in this study was type 16. Interestingly, however, HPV was also found regularly in clinically normal buccal mucosa in this HIV positive patient sample. This may indicate that the presence of HPV is not an important factor in the development of OHL, but merely a reflection of the patient's immune status. Felix *et al* (1993) carried out a similar study and found no evidence of HPV 16.

Resnick *et al* (1988) attributed their inability to detect HPV in OHL lesions to sampling factors, stating that the detection of HPV from verrucae can also

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vary between tissue specimens. This could account for the variation in the detection of HPV in OHL lesions in different studies.

#### **2.3.2.2 Epstein-Barr Virus**

Epstein-Barr virus has been reported to infect up to 90% of the world's population (Thomas *et al*, 1991; Luxton *et al*, 1993). It is the aetiologic factor in a spectrum of diseases including infectious mononucleosis, nasopharyngeal carcinoma and Burkitt's lymphoma. Infection with EBV usually occurs during childhood as a subclinical infection. Although EBV can be reactivated in many people, the reactivation "infection" is most often subclinical (Baskin *et al*, 1995)

Like other members of the herpes virus family (Appendix II), after an initial primary infection, the EBV establishes a persistent, lifelong infection in the host. These healthy seropositive people occasionally shed low levels of EBV particles in oropharyngeal secretions (Thomas *et al*, 1991). The reservoirs of latently infected cells are thought to be epithelial cells and circulating B cells. Wolf, Haus and Wilmes (1984) demonstrated EBV persistence in the parotid gland. Normal tongue epithelium (that is, not associated with OHL or HIV) does not appear to be a natural reservoir for EBV in immunocompetent people even if serology testing indicates past EBV infection (Miller *et al*, 1994).

Two types of Epstein-Barr virus are recognised, type A and type B. Various

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strains of each type of EBV can also be found. *In vitro*, types A and B differ in their ability to transform B lymphocytes. Type B EBV infected B lymphocytes grow poorly in culture (Kieff, 1995).

EBV has been detected in biopsy specimens of OHL using electron microscopy (Reed *et al*, 1988; Sugihara *et al*, 1990), immunocytochemistry (Kanas *et al*, 1988; Mabruk *et al*, 1996; Murray *et al*, 1996), polymerase chain reaction (Adler-Storthz *et al*, 1992; Felix *et al*, 1993) and *in situ* hybridisation (De Souza *et al*, 1990; Cubie, Felix and Wray, 1991; Mabruk *et al*, 1994; Mabruk *et al*, 1996). EBV DNA has also been found in clinically normal tongues sampled at autopsy from AIDS patients (Mabruk *et al*, 1995).

Walling *et al* (1995), examined patients with OHL lesions not associated with HIV infection and consistently found EBV coinfection and recombination. Raab-Traub and Webster-Cyriaque (1997) detected multiple strains of EBV within OHL lesions. These authors found that recombination of EBV occurred during viral replication resulting in the generation of new EBV variants. These reports suggested that viral determinants may be more important factors in the development of OHL than immune status.

In OHL, EBV appears to be confined to the upper epithelial cell layers (Murray *et al*, 1996). There is currently no evidence to show a latent infection of EBV within the basal cell layers of the epithelium (Niedobitek *et*

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*al*, 1991; Crutchley *et al*, 1997). It is suggested by some authors that the EBV is sustained within the more differentiated layers of the epithelium by continual reinfection as they could not find any evidence of latent infection within the basal or suprabasal layers. This distribution of viral infection corresponds to the location of receptors for EBV in the epithelium (Corso *et al*, 1989). This receptor is the same as the receptor for the C3d fraction of complement and it is also expressed by B-lymphocytes (Sixbey *et al*, 1987).

The epithelial phenotype may play a role in the development of OHL lesions. Corso *et al* (1989) found that only the upper and middle cell layers of parakeratinised epithelia express the EBV/C3d receptor and that this receptor was absent in orthokeratinised gingival and hard palate epithelia. Although the latter observation has been offered as an explanation for the absence of OHL development on the hard palate and gingiva, the validity of such an argument is debatable when considering that orthokeratinised epithelium is an uncommon finding in the oral cavity.

EBV has been shown to cause changes in epithelia and may play a role, like HPV, in carcinogenesis (Miller *et al*, 1994). EBV has been shown to prevent epithelial cell differentiation (Dawson, Rickinson and Young, 1990) and also to cause hyperplastic lesions in mice (Wilson *et al*, 1990). These features may provide support for the role of EBV in the hyperplasia observed in OHL lesions.

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Risk factors that influence EBV replication in oral epithelial cells have been investigated. Boulter *et al* (1996) found that antifungal treatment and avoidance of cigarette smoking may reduce the likelihood of development of OHL in HIV positive individuals. They also observed that EBV replication reduced as CD8+ T-lymphocytes (suppressor cells) increased. This indicates that the aetiology and pathogenesis of OHL is possibly multifactorial rather than the result of a sole aetiologic agent.

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## 2.4 The diagnosis and management of Oral Hairy Leukoplakia

The accurate diagnosis of OHL, is important for the following reasons:

- OHL, in some cases, may be an early indicator of HIV infection (Lifson *et al*, 1994)
- The development of OHL in a patient with known HIV infection may have prognostic implications for that patient (Greenspan and Greenspan, 1992; Kolokotronis *et al*, 1994; Ramírez-Amador, Esquivel-Pedraza and Ponce de León , 1996)
- Staging of HIV disease is influenced by the presence or absence of oral manifestations including OHL (Cruz *et al*, 1996)

The EC-Clearinghouse update of the classification and diagnostic criteria of oral lesions in HIV infection outlined both presumptive and definitive criteria for the diagnosis of HIV infection (EC-Clearinghouse, 1993).

Presumptive criteria for the diagnosis of OHL are the presence of bilateral white or grey lesions on the lateral border of the tongue. Definitive criteria for the diagnosis of OHL are the demonstration of the presence of Epstein-Barr virus within the lesions or, in cases where this is not possible, a lack of response to antifungal treatment. An immunodeficient status also provides weight to a diagnosis of OHL.

In most instances, presumptive criteria are usually adequate for the

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identification of this lesion (except perhaps when definitive OHL criteria are required for specific studies, such as a clinical end point in drug trials).

Various techniques for the definitive diagnosis of OHL (that is, detection of EBV) have been investigated. These have included the use of electron microscopy of exfoliative cytology specimens (Kratochvil *et al*, 1990; Sugihara *et al*, 1990; Fraga-Fernandez and Vicandi-Plaza, 1992; Migliorati *et al*, 1993; Miller *et al*, 1994; Epstein *et al*, 1995), immunohistochemistry (Zhang *et al*, 1988) and *in situ* hybridisation (Mabruk *et al*, 1996).

OHL is an oral manifestation of HIV infection which usually requires no treatment. It is generally asymptomatic and there has been no reported evidence of any premalignant potential of the lesion. Patients may occasionally complain of slight discomfort or of a “burning” sensation (Schöfer *et al*, 1987; Greenspan and Greenspan, 1989D; Greenspan and Greenspan, 1996). This burning sensation, in some cases, may also be a feature of a *Candida* infection superimposed on the OHL. Up to 50% of lesions have been found to harbour *Candida albicans* (Glick and Pliskin, 1990). This can be resolved with antifungal treatment.

Treatment may occasionally be requested by patients, for example, for widespread lesions which may pose an aesthetic concern.

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Various methods have been used to treat OHL including both systemic and topical medication.

The beneficial effect of systemic antiviral medication on OHL supports the view that the main aetiologic agent for OHL is viral. Medication such as aciclovir is successful in eliminating or reducing the size of the lesions (Schöfer *et al*, 1987; Glick and Pliskin, 1990; Scully, Porter and Luker, 1991; Barr, 1994). However, once this treatment is stopped, recurrence of the lesion is a common event (Schöfer *et al*, 1987; Resnick *et al*, 1988; Greenspan and Greenspan, 1996). Greenspan and Greenspan (1989), used a drug, desiclovir, which is similar to aciclovir, with some success. However recurrence of the lesions was also a feature of this treatment when cessation of the drug occurred. OHL lesions have also been shown to resolve when patients have started to take antiretroviral medication such as Zidovudine (AZT) (Phelan and Klein, 1988; Greenspan and Greenspan, 1989; Barr, 1994).

Side effects are occasionally encountered with aciclovir such as nausea, vomiting, diarrhoea and headache (Mims Annual, 1994). This may contraindicate the use of this and similar drugs to treat OHL (Glick and Plisken, 1990; Gowdy *et al*, 1995). In addition, resistance to aciclovir can occur (Epstein and Scully, 1991; Mims Annual, 1994; Gowdy *et al*, 1995) complicating the treatment of other, more serious opportunistic infections in immunocompromised patients.

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Various topical medications have been employed to reduce OHL or relieve symptoms associated with the lesion. Topical retinoids, such as vitamin A acid (0.1%), applied twice a day has been used in some trials, however the lesions recurred soon after cessation of the medication (Schöfer *et al*, 1987; Gowdy *et al*, 1995). Retinoids act as dekeratinising agents by causing changes in patterns of epithelial cell differentiation (Mims Annual, 1994).

Another topical medication employed for the treatment of OHL is podophyllum resin (Lozada-Nur and Costa, 1992; Gowdy, Lee and Carpenter, 1995). This compound is an extract of *Podophyllum peltatum*, a plant found in the northern and middle United States. It has been used in the past for the treatment of dermatologic diseases caused by human papillomavirus infection such as verrucae and condyloma acuminatum. It inhibits cell mitosis in metaphase because of its high affinity for a protein found in microtubules of the mitotic spindle (Lozada-Nur and Costa, 1992; Gowdy *et al*, 1995). For the treatment of OHL, podophyllum resin is applied directly to the lesion for a period of 30 to 60 seconds after which the patients rinse their mouths with water. Lozada-Nur and Costa (1992), found remissions in OHL for up to 28 weeks with very few side effects encountered.

In the majority of patients OHL is a lesion that only requires periodic monitoring as to its presence. Unless requested by patients, active treatment of the lesion is not indicated.

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## 2.5 Conclusions

OHL has been extensively studied since its first description in 1984 by Greenspan *et al.* The range of clinical and histological appearances of this lesion are well documented. Despite this information, however, several questions remain relating to various aspects of the lesion including:

- the exact role that EBV plays in the pathogenesis of OHL
- the possible role of co-factors in the development of OHL
- the reason why OHL lesions are located predominantly on the lateral border of the tongue

In addition, the use of OHL as a clinical indicator of HIV disease progression is an area that needs further investigation. Anecdotal evidence derived from the patients infected with HIV who attend the Adelaide Dental Hospital suggest that the presence of OHL may not be as accurate a reflection of HIV disease progression as that previously described in some studies from the United States and Europe. This fact, however, has not been demonstrated experimentally.

There have not been any detailed Australian studies which have described the behaviour and characteristics of OHL in Australian HIV-infected patients.

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This study of OHL in HIV-infected patients at the Adelaide Dental Hospital was undertaken in order to:

- collect information regarding the occurrence of OHL in an HIV infected Australian population
- collect information correlating the presence of OHL to other systemic conditions and laboratory parameters such as CD4+T-lymphocyte count. It is hypothesised that the presence of OHL is not strongly correlated with CD4+T-lymphocyte counts in South Australian patients, and accordingly, its presence is not an indicator of poor prognosis
- detect the presence of EBV in OHL lesions of an Australian population using ultrastructural immunogold labelling of exfoliative cytology specimens.

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## Chapter 3

### MATERIALS AND METHODS

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### **3. MATERIALS AND METHODS**

This research project was designed in two sections:

*Retrospective analysis of oral hairy leukoplakia:*

A retrospective analysis of 197 patients infected with HIV who had attended the Medically Compromised Patient Unit of the Adelaide Dental Hospital. Information such as estimated time since conversion, date of diagnosis of HIV infection, CD4+ T-lymphocyte counts, current medication and previous or concurrent AIDS-defining medical conditions was acquired from entries in the patients' case notes and were correlated to the presence or absence of OHL.

*Prospective analysis of oral hairy leukoplakia:*

A prospective analysis of a group of 20 patients with OHL who attended the Medically Compromised Patient Unit of the Adelaide Dental Hospital. This section of the study was aimed to correlate features occurring concurrently with OHL such as CD4+ T-lymphocyte count, medication and concurrent systemic medical conditions.

The clinical appearance of the lesions consistent with OHL was described. In addition, this section of the project aimed to provide definitive criteria for the diagnosis of OHL, that is, the demonstration of Epstein-Barr virus (EBV) in the lesions.

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The project was undertaken with the approval of the University of Adelaide  
Committee on the Ethics of Human Experimentation, approval number  
H/29/94.

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### **3.1 Retrospective analysis of Oral Hairy Leukoplakia**

Data was collected from patient records of the Adelaide Dental Hospital. The information was obtained from records of 197 HIV positive patients attending the Medically Compromised Unit from January 1986 to February 1995.

Patients were referred to the unit for dental management from a variety of sources, principally private medical practitioners, the Royal Adelaide Hospital and the Flinders Medical Centre.

The information that was recorded at each patient's visit included estimated time since HIV seroconversion, the date of diagnosis, CD4+ T-lymphocyte counts, current medication and any AIDS-defining illnesses that they may have had. All of this information was provided by the patients and by consultation with their referring medical practitioners.

For the purpose of this study, data derived from records was manually transferred to proformas (Appendix III) and subsequently analysed as follows:

- prevalence and site of OHL
- presence of OHL and length of time since infection with HIV
- presence of OHL and AIDS-defining illness
- presence of OHL and concurrent medication

- 
- presence of OHL and CD4+ T-lymphocyte count
  - presence of OHL and oral *Candida* infection

OHL was defined according to the EC-Clearinghouse-WHO criteria (EC-Clearinghouse, 1993). Medical conditions were classed as AIDS-defining conditions according to the 1993 Revised Classification System for HIV infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults (MMWR, 1992). Statistical analysis of the data was done using the chi squared test.

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### **3.2 Prospective analysis of Oral Hairy Leukoplakia**

A total of 20 patients were involved in the study. They were selected on the basis that lesions resembling OHL were clinically apparent. OHL was defined according to the presumptive diagnostic criteria outlined in the EC-Clearinghouse classification of oral manifestations of HIV infection (EC-Clearinghouse, 1993).

One patient was included who had a lesion resembling OHL on his hard palate. OHL has been reported to occur in areas other than the tongue (Kabani *et al*, 1989).

Patients were given an information sheet (Appendix IV) and asked to consent to participate in the study (Appendix V).

#### **3.2.1 Clinical examination**

Patients with OHL were examined and details relating to the lesions recorded. This included the site and size of the lesions. Other clinical information was obtained such as CD4+ T-lymphocyte count, medication and concurrent systemic medical conditions.

Clinical photographs of the OHL lesions were taken with a Pentax SFXn camera, using Kodachrome® 64 ASA (Kodak Eastman, USA), 35mm film.

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### 3.2.2 Exfoliative cytology smears

Exfoliative cytology smears were taken from the areas of the tongue involved by the OHL lesions. A total of five smears were taken from each lesion. The patient's tongue was held using a piece of gauze whilst the lesion was gently scraped using a blunt spatula.

The spatula was then run along a glass microscope slide which was allowed to briefly air dry before being placed in 10% formalin for fixation. The slides were fixed for 60 minutes.

One smear was stained with Haematoxylin and Eosin (H and E) according to the procedure outlined in Appendix VI.

Two smears were stained with Periodic acid-Schiff (PAS) to determine the presence of fungal organisms in the lesions. This was done according to the procedure outlined in Appendix VII.

The remaining two smears were used for immunohistochemical studies in order to determine the presence of EBV in the lesions. This was required for a definitive diagnosis of OHL according to EC-Clearinghouse-WHO criteria (EC-Clearinghouse, 1993). The exfoliative cytology smears were labelled with two monoclonal antibodies specific for EBV, latent membrane protein (LMP-1, Dako-EBV, CS 1-4) and the diffuse early antigen (EA-D, NovoCastra, NCL-EADE31). The positive controls consisted of paraffin

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sections from a specimen of a previously confirmed EBV-positive case of Hodgkin's Lymphoma. Immunohistochemical labelling of the exfoliative cytology smears was carried out by Dr K. Smith, Institute of Medical and Veterinary Sciences, Adelaide, according to the procedure that is outlined in Appendix VIII.

#### **3.2.2.1 Light microscopy**

H and E stained, PAS stained and immunohistochemistry specimens were all examined using the an Olympus CHK-F microscope. They were photographed using the PM-10ADS Olympus Automatic Photographic system. The film used was Kodachrome<sup>®</sup> 64 ASA (Kodak Eastman, USA), 35mm film.

#### **3.2.2.2 Electron microscopy**

Initially, cytology samples from the lesions were investigated using electron microscopy to determine the morphology of the cells and the presence of viruses within the lesion. Plain electron microscopy and immunogold labelling of ultrathin sections was carried out by Dr. P. Smith, Institute of Medical and Veterinary Sciences, Adelaide. Further smears were taken for future immunogold labelling studies in order to attempt to identify the viruses that were found within the epithelial cells.

For electron microscopy, smears were taken from the tongue according to the procedure outlined previously. Instead of smearing glass microscope

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slides, however, the spatulas were rinsed with a solution of 1% glutaraldehyde in 0.05M sodium cacodylate buffer. The suspension of cells within the fixative was prepared for electron microscopy according to the procedure outlined in Appendix IX.

After embedding, 0.5µm survey sections were cut and dried on a glass slide before being stained with 0.1% Toluidine Blue. Ultrathin sections were then cut using a Sorvall Porter-Blum Ultramicrotome. They were then picked up on a copper grid and stained with alcoholic uranyl acetate and lead citrate.

For immunogold labelling studies using a monoclonal antibody specific for the diffuse early antigen (EA-D, NovoCastra, NCL-EADE31), the spatulas were rinsed in 0.5% glutaraldehyde in phosphate buffered saline (PBS) and the suspension was fixed for 1 hour before preparation and embedding in L. R. White resin (Appendix X). The procedure for immunogold labelling of the exfoliative cytology smears is outlined in Appendix XI.

Immunogold labelling of the exfoliative cytology specimens was carried out on 2 specimens in this study as a pilot investigation to ascertain the effectiveness of the technique as a basis for future immunogold studies. As a consequence no controls were used in this instance.

All of the ultrastructural examinations of the sections was done using the JEOL 1200 EXII electron microscope (80 kV).

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For the purpose of this study, data derived from clinical and histological examination was manually transferred to proformas (Appendix XII). Data was subsequently analysed as follows:

- general features of OHL
- association between OHL and concurrent systemic medical conditions and medication
- association between the size of OHL lesions and CD4+ T-lymphocyte count
- light microscopic and transmission electron microscopic examination of exfoliative cytology specimens from OHL lesions

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## **Chapter 4**

### **RESULTS**

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## **4. RESULTS**

### **4.1 Retrospective analysis of Oral Hairy Leukoplakia**

Data was gathered from records of 197 patients who had attended the Medically Compromised Unit of the Adelaide Dental Hospital.

The group of 197 patients was composed of 186 (94.4%) males and 11 (5.6%) females. The mean age of the group was 36.9 years (range 19 - 67 years). One hundred and eighty one patients were male homosexuals, thirteen patients (2 male, 11 female) had a known history of intravenous drug use and three patients (all male) had haemophilia.

Thirty nine patients died during the period January 1986 to February 1995.

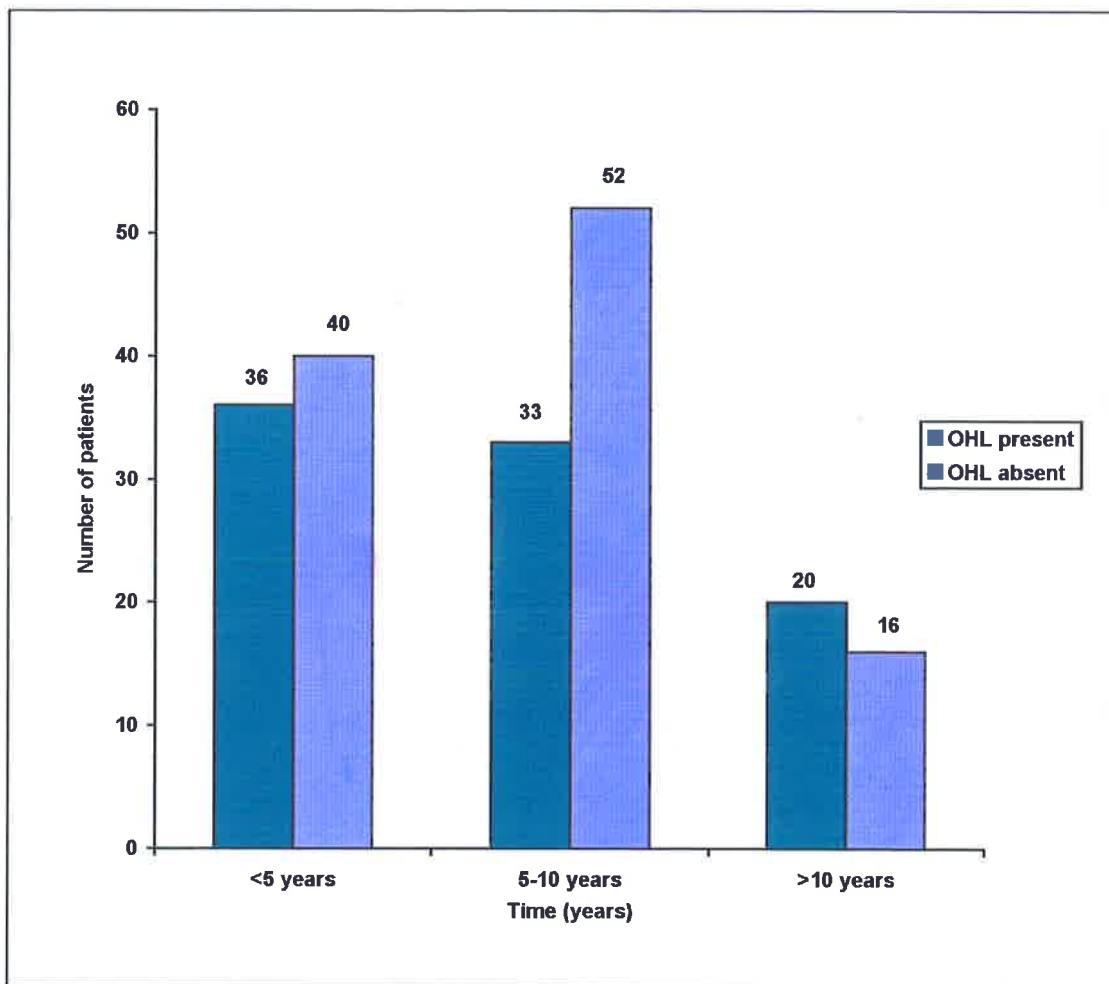
#### **4.1.1 Prevalence and site of Oral Hairy Leukoplakia**

Clinical evidence of OHL was seen on the lateral border of the tongue in 89 patients (45.2%). In 40 (44.9%) of these cases, OHL was bilateral.

In 3 patients, OHL was also reported in the case notes to occur on the buccal mucosa. These three patients also had bilateral OHL lesions on their tongues.

#### 4.1.2 Presence of Oral Hairy Leukoplakia and the length of time since infection with HIV

Seventy six patients had been infected with HIV for less than or equal to 5 years, and, of these, 36 (47.4%) had OHL present. Eighty five patients had been infected with HIV for more than 5 years and less than 10 years previously, and, of these, 33 (38.8%) had OHL present. Thirty six patients had been infected with HIV for more than 10 years, and, of these, 20 (55.6%) had OHL present ( $X^2=3.116$ ,  $0.10 < P < 0.05$  (Chi squared analysis)) (Figure 1).



**Figure 1:** The presence of oral hairy leukoplakia according to the length of time since infection with HIV in study subjects.

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#### 4.1.3 Presence of Oral Hairy Leukoplakia and AIDS-defining illness

One hundred and sixty one patients had no AIDS-defining medical condition recorded in their dental records. Of these, 70 (43.5%) had OHL lesions present.

Thirty six patients had an AIDS-defining illness recorded in their case notes (Table 1). These AIDS-defining illnesses included, *Pneumocystis carinii* pneumonia (PCP), *Mycobacterium avium* complex (MAC), Tuberculosis (TB), Cytomegalovirus infection (CMV), Herpes simplex virus infection (HSV), Cryptococcal infection, Aspergillus infection, Kaposi's sarcoma (KS) and Lymphoma. Seventeen patients (51.5%) patients in this group had OHL ( $X^2=0.074$ ,  $P>0.5$  (Chi square analysis)) (Figure 2).

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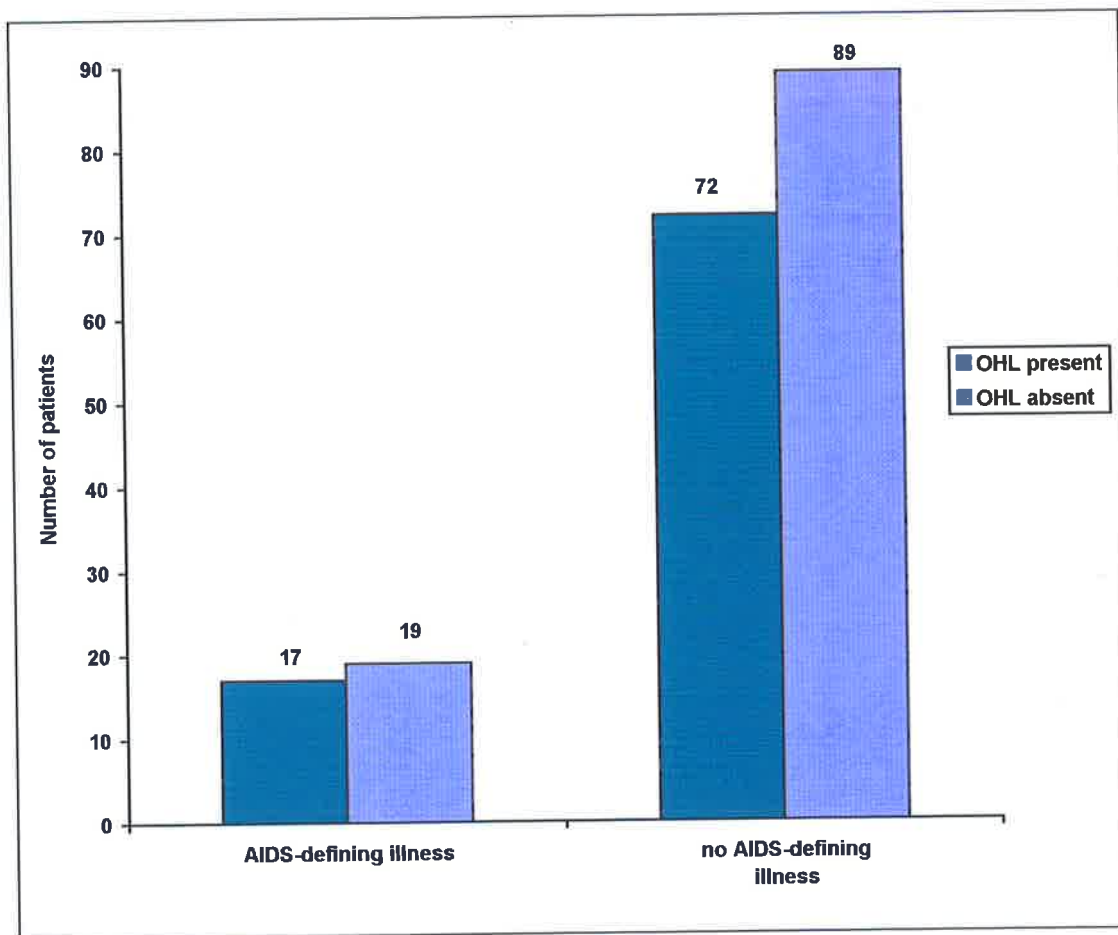


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<b>AIDS-Defining Illnesses</b>	<b>OHL present</b>	<b>OHL absent</b>
Pneumocystis carinii pneumonia	4	8
Kaposi's sarcoma	4	4
Mycobacterium avium complex	3	4
Cytomegalovirus infection	1	3
Cryptococcal infection	3	1
Aspergillus infection	3	1
Herpes simplex infection	3	2
Tuberculosis	0	1
Lymphoma	0	2

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**Table 1:** AIDS-defining illnesses that occurred within the group and the number of study subjects with OHL and without OHL according to each illness.



**Figure 2:** The proportion of patients with oral hairy leukoplakia with AIDS-defining illnesses and without AIDS-defining illnesses

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#### 4.1.4 Presence of Oral Hairy Leukoplakia and concurrent medication

One hundred and fifty eight patients were taking medication (Table 2). This medication included antiviral drugs (Zidovudine (AZT), Didanosine (ddI), Zalcitabine (ddC), Stavudine (d4T), Aciclovir and Ganciclovir), antifungal drugs (Fluconazole, Ketoconazole and Itraconazole) and antibacterial drugs (Ethambutol, Rifampicin, Clofazamine, Clarithromycin, Trimethoprim and Sulfamethoxazole combinations and Pentamidine). Of these, 77 (48.7%) had OHL ( $X^2=4.076$ ,  $0.02 < P < 0.05$  (Chi squared analysis)) (Figure 3).

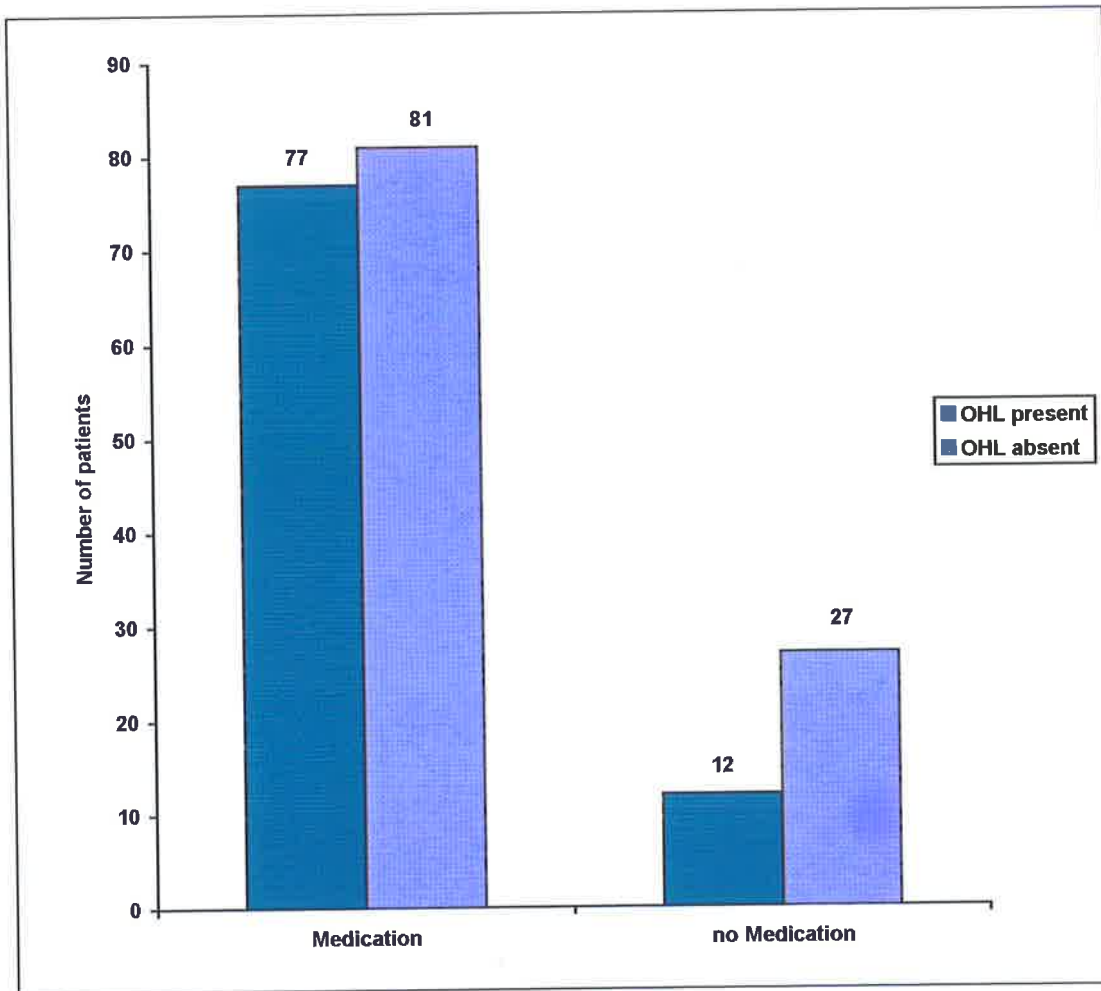
There were 39 patients who were not taking any medication, of these 30.8% had OHL which is low compared with the overall prevalence of OHL (45.2%) in the study

One hundred and thirty patients were taking antiviral medication. Of these, 91 were taking AZT and of these, 27 (29.7%) had OHL compared to 62 (58.5%) patients who were not taking AZT and had OHL ( $X^2=16.420$ ,  $0.001 < P < 0.01$  (Chi squared analysis)) (Figure 4).

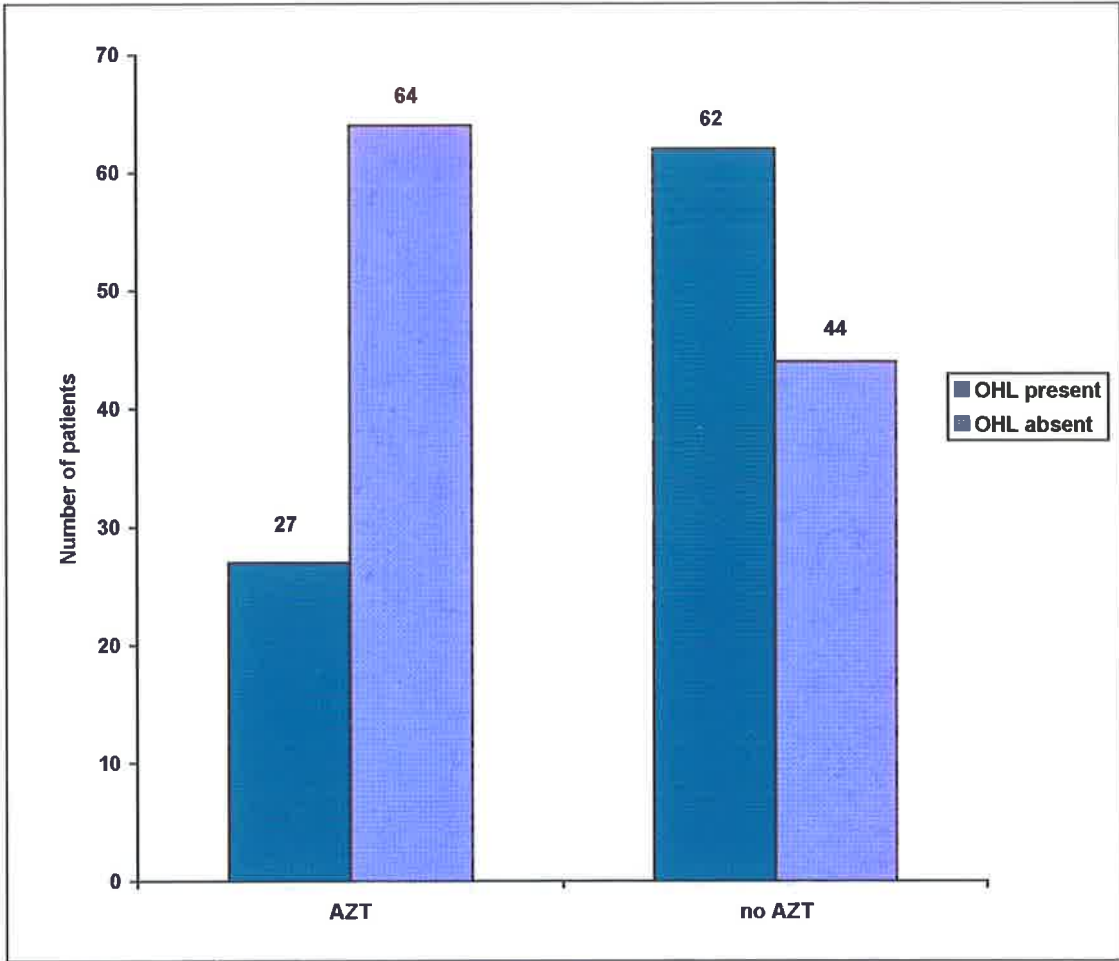
Forty nine patients were taking aciclovir, of these, 14 (28.6%) had OHL compared with 75 (50.7%) patients who were not taking aciclovir and had OHL ( $X^2=7.262$ ,  $0.001 < P < 0.01$  (Chi squared analysis)) (Figure 5).

<b>Medication</b>	<b>OHL present</b>	<b>OHL absent</b>
<b>Antiretroviral medication</b>		
Zidovudine (AZT)	27	64
Didanosine (ddl)	16	21
Zalcitabine (ddC)	3	12
Stavudine (d4T)	1	0
<b>Other antiviral medication</b>		
Aciclovir	14	35
Ganciclovir	2	1
<b>Antifungal medication</b>		
Fluconazole	18	17
Ketoconazole	2	10
Itraconazole	4	2
<b>Antibacterial medication</b>		
Ethambutol	2	5
Rifampicin	1	3
Clofazamine	1	2
Clarithromycin	1	2
Trimethoprim/sulfamethoxazole combinations	19	37
Pentamidine	4	3

**Table 2:** The range of medication taken by study subjects and the numbers of patients with OHL present according to each type of medication



**Figure 3:** The proportion of subjects with oral hairy leukoplakia according to whether or not they are taking medication



**Figure 4:** The presence of oral hairy leukoplakia and its association with AZT

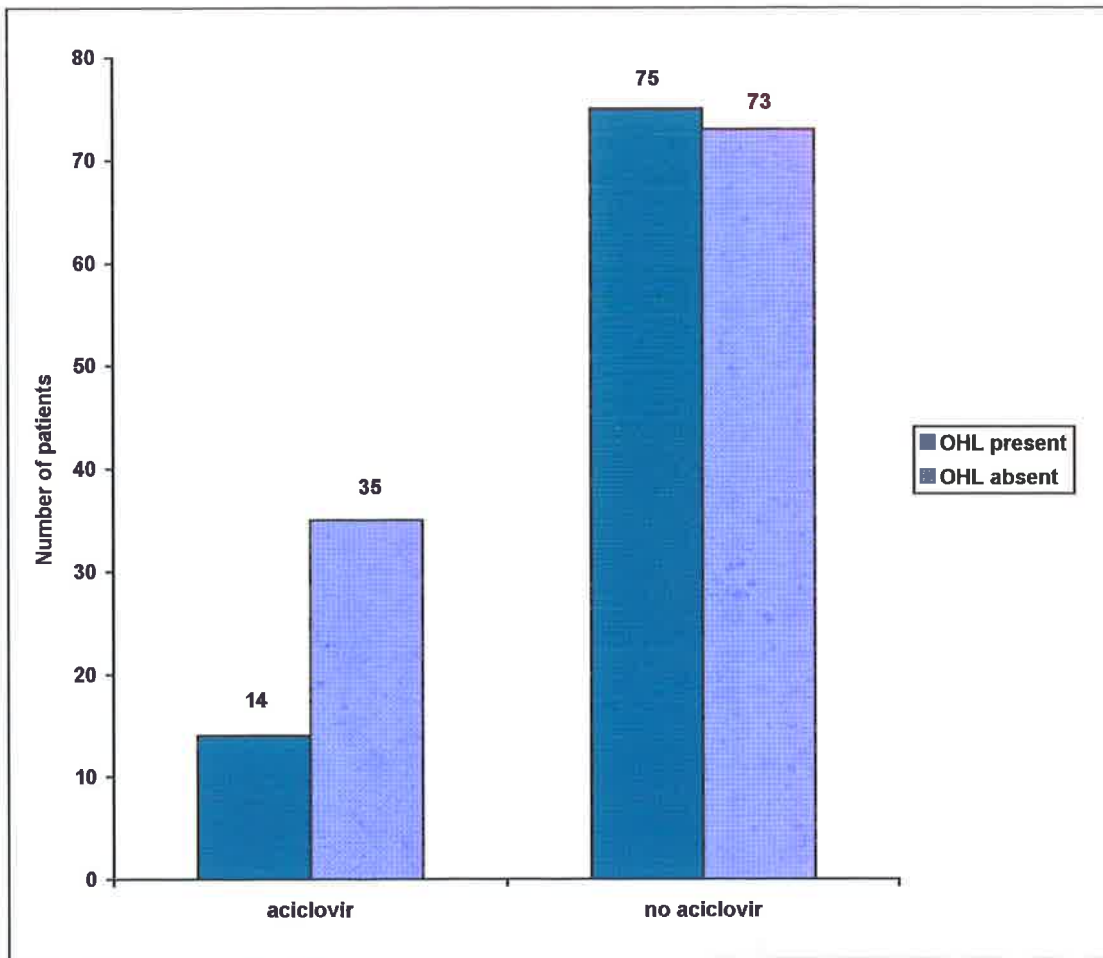


Figure 5: The presence of oral hairy leukoplakia and its association with aciclovir

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#### 4.1.5 Presence of Oral Hairy Leukoplakia and CD4+ T-lymphocyte count

CD4+ T-lymphocyte counts were recorded in case notes of 150 of the 197 patients. The numbers of CD4+ T-lymphocytes in patients with OHL ranged from 0 - 1200 cells/ $\mu$ L (mean 294.01 cells/ $\mu$ L). In patients without OHL, the CD4+ T-lymphocyte counts ranged from 0-2200 cells/ $\mu$ L (mean 376.99 cells/ $\mu$ L).

The patients were divided into 3 groups according to CD4+ T-lymphocyte count, namely CD4+ T-lymphocyte counts <200 cells/ $\mu$ L, 200-500 cells/ $\mu$ L and >500 cells/ $\mu$ L. Fifty three patients had CD4+ T-lymphocyte counts less than 200 cells/ $\mu$ L, of these 27 (50.9%) had OHL. Fifty six patients had CD4+ T-lymphocyte counts between 200 and 500 cells/ $\mu$ L, of these 26 (46.4%) had OHL. Forty one patients had CD4+ T-lymphocyte counts above 500 cells/ $\mu$ L, of these 15 (36.6%) had OHL ( $X^2=1.966$ ,  $0.1 < P < 0.5$  (Chi squared analysis)) (Figure 6).

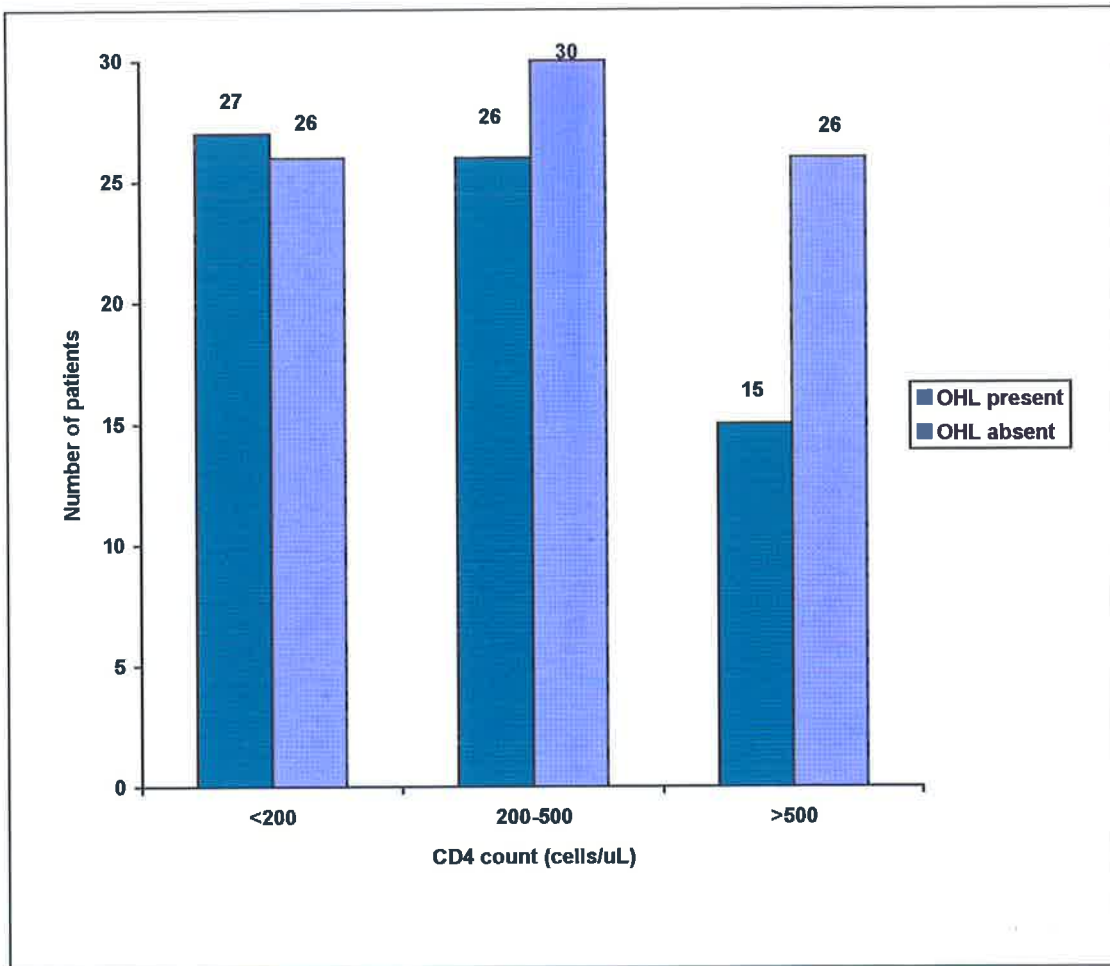
The estimated length of time since HIV seroconversion was less than 5 years in 50 patients. In this group, the mean CD4+ T-lymphocyte count was 303.14 cells/ $\mu$ L in patients with OHL, compared with 400.18 cells/ $\mu$ L in patients without OHL. Eighteen patients had CD4+ T-lymphocyte counts below 200 cells/ $\mu$ L, of these 11 (61.1%) had OHL. Sixteen patients had CD4+ T-lymphocyte counts between 200 and 500 cells/ $\mu$ L, of these 9

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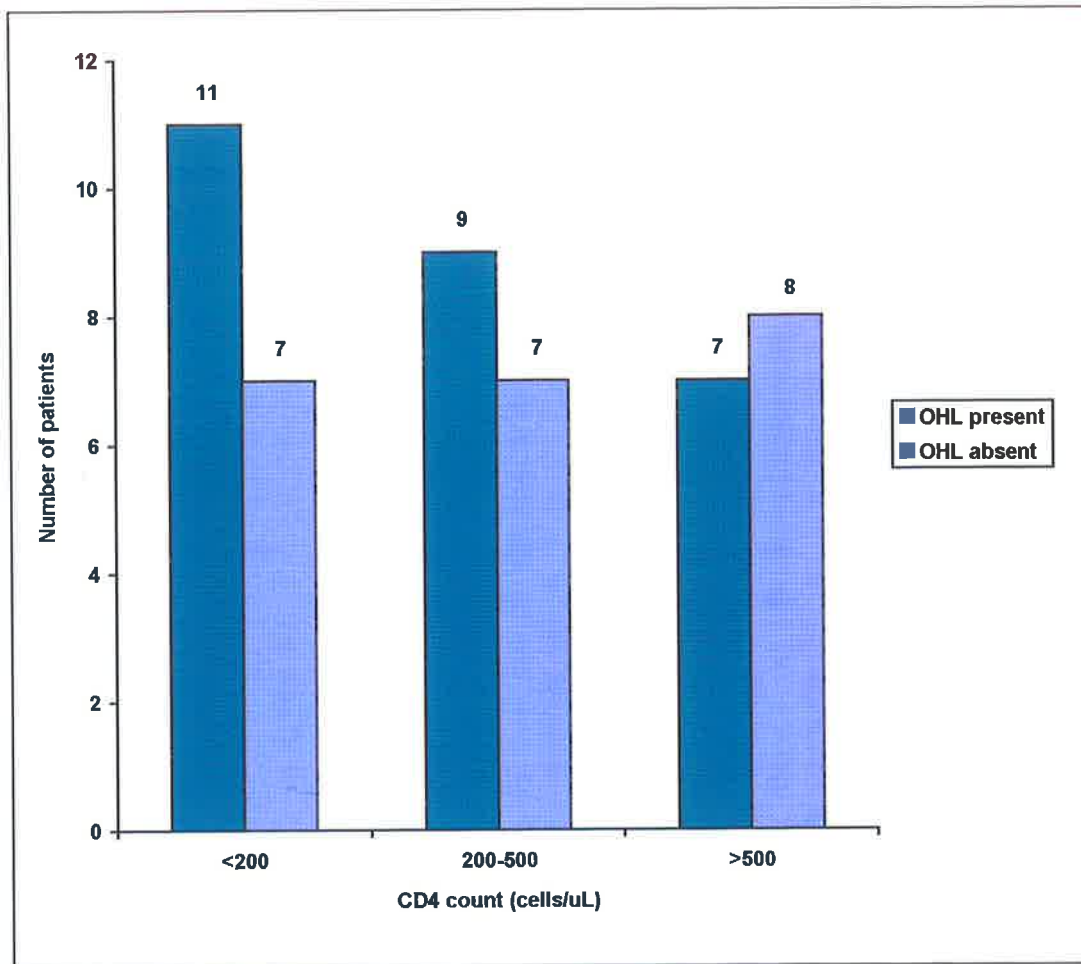
(56.3%) had OHL. Sixteen patients had CD4+ T-lymphocyte counts above 500 cells/ $\mu$ L, of these 8 (50%) had OHL ( $X^2=0.425$ ,  $P>0.5$  (Chi squared analysis)) (Figure 7).

The estimated length of time since HIV seroconversion was between 5 and 10 years in 70 patients. In this group, the mean CD4+ T-lymphocyte count was 404.03 cells/ $\mu$ L in patients with OHL compared to 385.21 cells/ $\mu$ L in patients without OHL. Twenty three patients had CD4+ T-lymphocyte counts below 200 cells/ $\mu$ L, of these 7 (30.4%) had OHL. Twenty six patients had CD4+ T-lymphocyte counts between 200 and 500 cells/ $\mu$ L, of these 10 (38.5%) had OHL. Twenty one patients had CD4+ T-lymphocyte counts greater than 500 cells/ $\mu$ L, of these 6 (28.6%) had OHL ( $X^2=1.122$ ,  $P>0.5$  (Chi squared analysis)) (Figure 8).

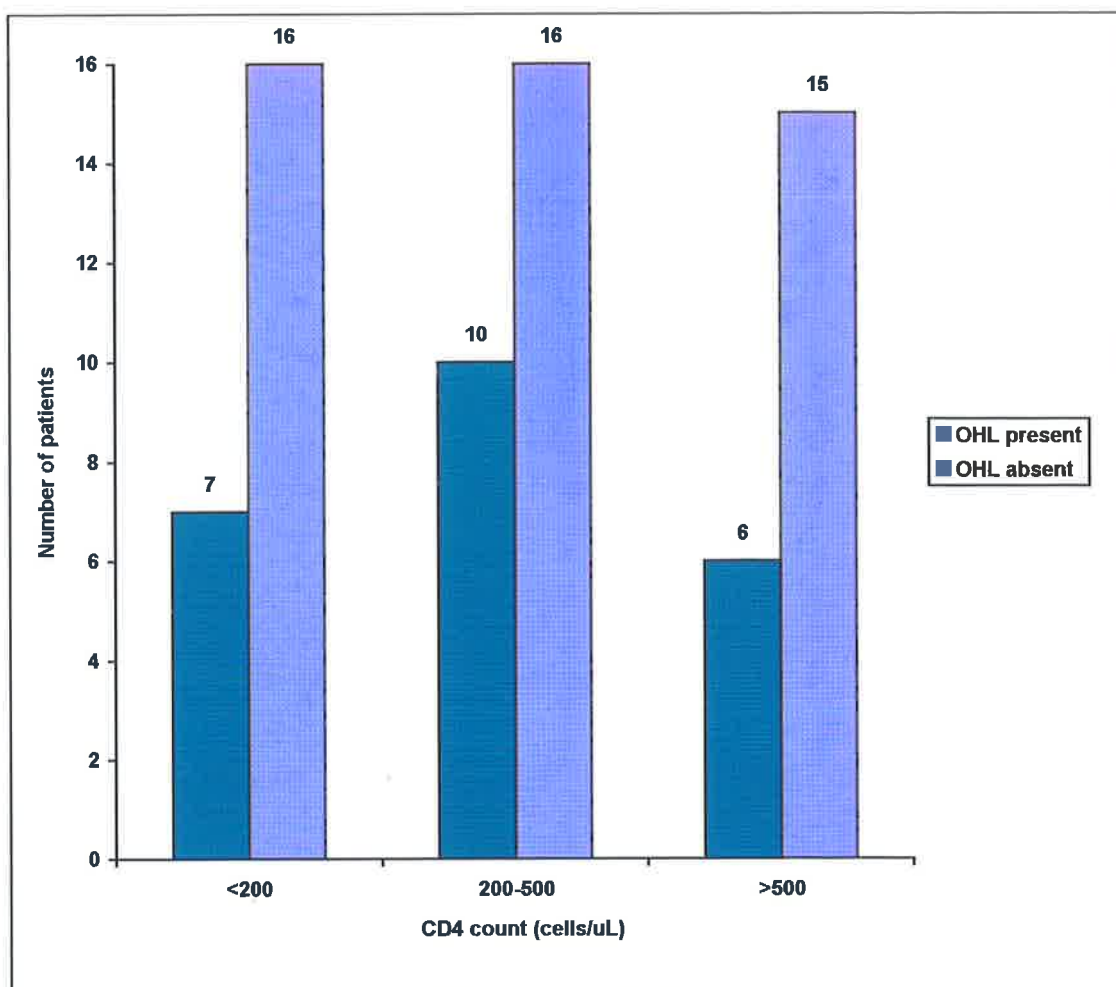
The estimated length of time since HIV seroconversion was greater than 10 years in 30 patients. In this group, the mean CD4+ T-lymphocyte count was 174.85 cells/ $\mu$ L in patients with OHL compared to 345.60 cells/ $\mu$ L in patients without OHL. Twelve patients had CD4+ T-lymphocyte counts below 200 cells/ $\mu$ L, of these 9 (75%) had OHL. Fourteen patients had CD4+ T-lymphocyte counts between 200 and 500 cells/ $\mu$ L, of these 7 (50%) had OHL. Four patients had CD4+ T-lymphocyte counts greater than 500 cells/ $\mu$ L, of these 1 (25%) had OHL ( $X^2=3.608$ ,  $0.1<P<0.5$  (Chi squared analysis)) (Figure 9).



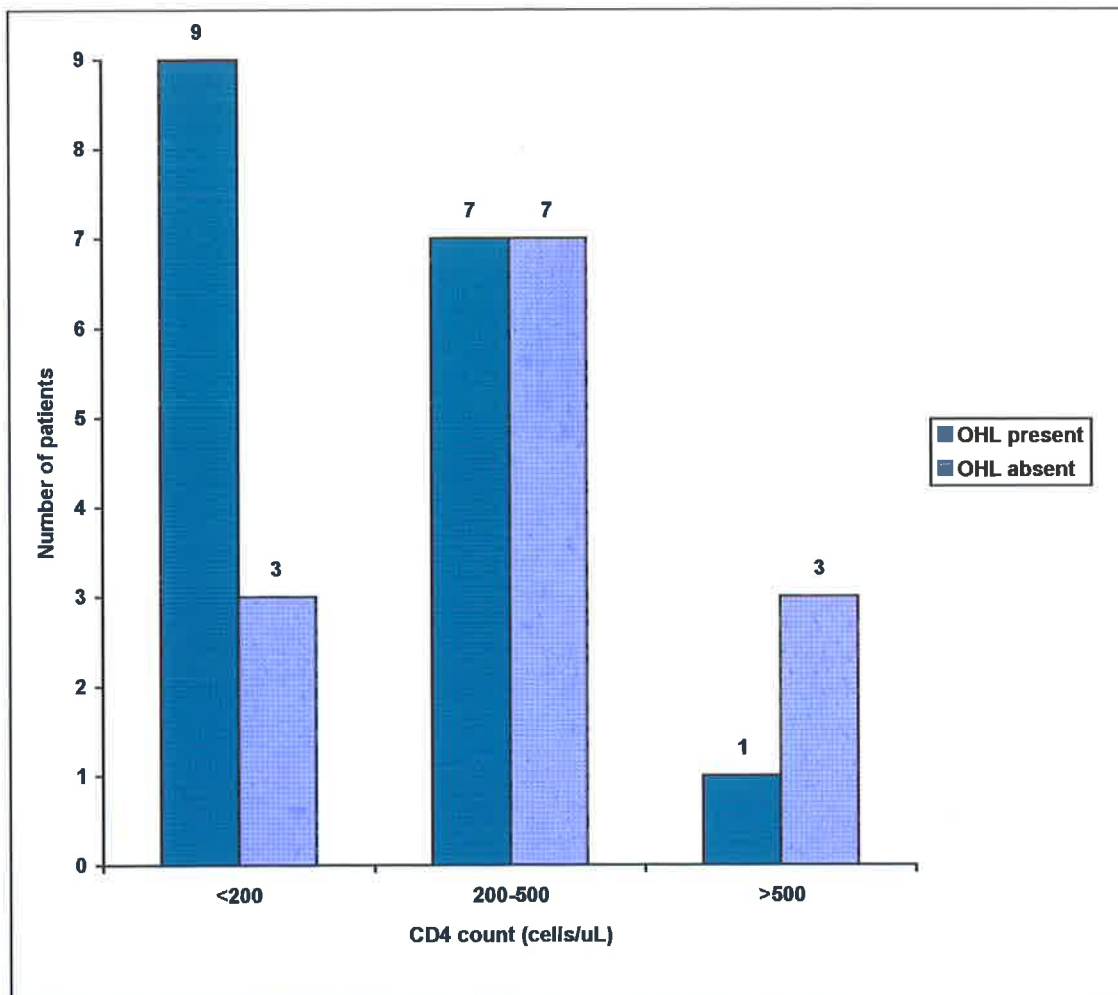
**Figure 6:** The presence of oral hairy leukoplakia and its association with CD4+ T-lymphocyte counts in study subjects



**Figure 7:** The presence of oral hairy leukoplakia and its association with CD4+ T-lymphocyte count in study subjects who had HIV infection for less than 5 years



**Figure 8:** The presence of oral hairy leukoplakia and its association with CD4+ T-lymphocyte count in study subjects who had HIV infection for between 5 and 10 years



**Figure 9:** The presence of OHL and its association with CD4+ T-lymphocyte count in study subjects who had HIV infection for greater than 10 years

#### 4.1.6. Presence of Oral Hairy Leukoplakia and *Candida*

A total of 53 (26.9%) patients in the sample of 197 had some form of oral *Candida* infection. Out of this group of patients, 28 (52.8%) had OHL present, whilst 25 patients (47.2%) had no evidence of OHL. ( $X^2=1.714$ ,  $0.1 < P < 0.5$  (Chi squared analysis)) (Figure 10).

The prevalence of oral *Candida* infection in the group of 89 patients with OHL was 31.5% ( $X^2=0.624$ ,  $P > 0.5$  (Chi squared analysis)).

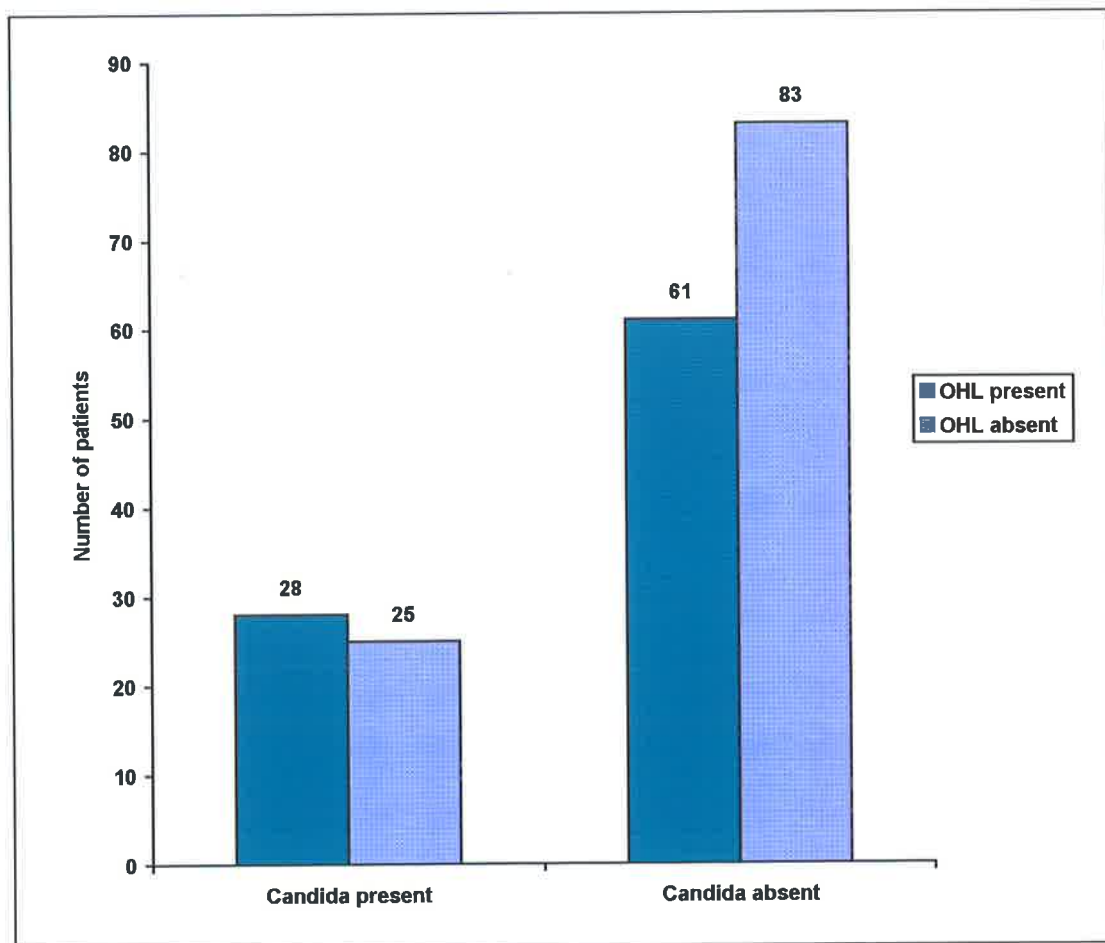


Figure 10: Subjects with or without oral hairy leukoplakia and the presence of oral *Candida* infection

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## **4.2 Prospective analysis of Oral Hairy Leukoplakia**

The group of 20 patients in this section of the project comprised 19 males and 1 female. All patients were infected with HIV, 4 patients (3 male and 1 female) had a history of intravenous drug use. The remaining patients were homosexual men. The average CD4+ T-lymphocyte count for this group of patients was 324 cells/ $\mu$ L (range 14 - 1000 cells/ $\mu$ L). All patients had white, non-removable lesions on their tongues that were consistent with oral hairy leukoplakia.

### **4.2.1 General features of Oral Hairy Leukoplakia**

The clinical features of OHL that were seen in this group of 20 patients were variable. Seven patients had bilateral lesions occurring on the tongue. The average area of the tongue covered by the lesions was 509mm<sup>2</sup> (3-2400mm<sup>2</sup>).

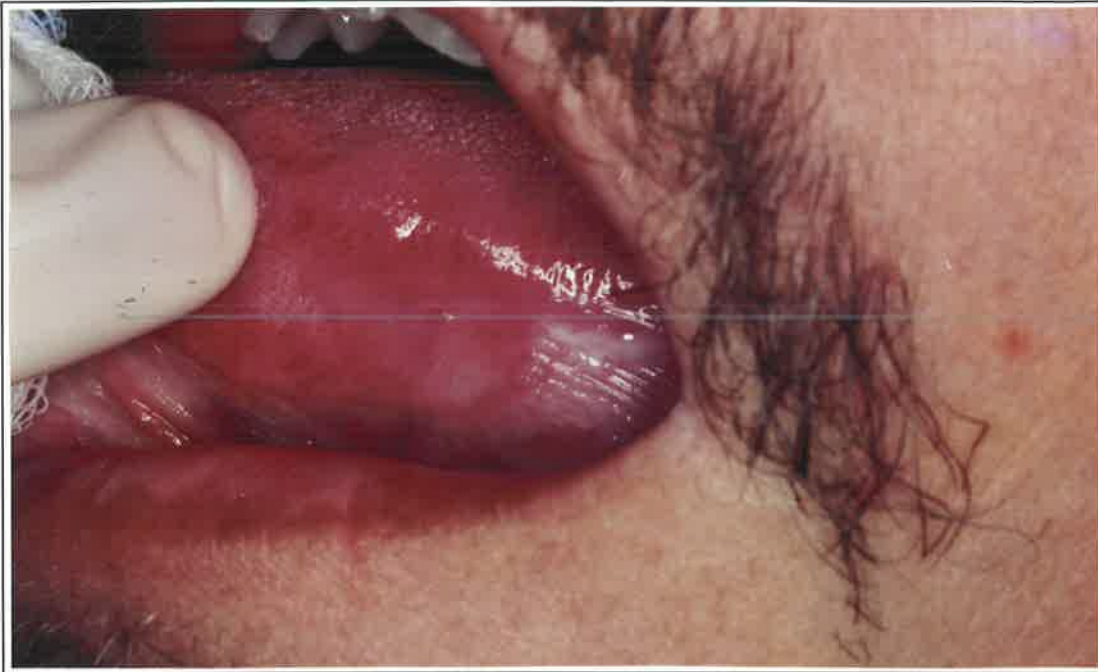
In some cases the OHL that was observed was mild. This form of OHL was characterised by a slight thickening of the lateral border of the tongue forming a slightly corrugated surface. More severe forms of OHL observed presented as white, "shaggy" lesions which often extended beyond the lateral border of the tongue including, in some cases both the dorsal surface of the tongue and floor of the mouth. The range of clinical appearances of OHL that was observed in the study subjects is illustrated in Figures 11 to 15.



**Figure 11:** Oral hairy leukoplakia in a 30 year old HIV infected male. It presented as a small white corrugated area on the lateral border of the tongue



**Figure 12:** Oral hairy leukoplakia in a 37 year old HIV infected male. It presented as a white corrugated lesion which extended towards the ventral surface of the tongue where it had a smoother appearance



**Figure 13:** Oral hairy leukoplakia occurring in a 25 year old HIV infected male. The lesion presented on the ventral aspect of the lateral border of the tongue and had a smooth flat, thickened appearance



**Figure 14:** Oral hairy leukoplakia occurring in a 35 year old HIV infected female. The lesion presented as a white, corrugated lesion on the lateral border of the tongue



**Figure 15:** Oral hairy leukoplakia in a 33 year old HIV infected male. It presented as a shaggy white lesion with a corrugated appearance in some areas. It was located mainly on the lateral border of the tongue, extending in some areas onto the dorsal and ventral aspects of the tongue. Fungal organisms were found in association with the oral hairy leukoplakia in this patient.

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One patient in the study had a white, non-removable lesion present on his hard palate. Although not a typical site for the presentation of OHL, this lesion was unresponsive to antifungal therapy and resolved in response to antiretroviral treatment. It was therefore included as a possible atypical presentation of OHL.

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#### 4.2.2 Relationship between the size of Oral Hairy Leukoplakia lesions and concurrent systemic medical conditions and medication

None of the patients, at the time of having cytology smears taken from their tongues, had any concurrent systemic medical conditions.

Eight patients were not taking any medication. Ten patients were taking some form of antiretroviral medication (AZT, ddI, ddC, 3TC, d4T). Five patients were taking aciclovir. The mean area of the lesions for patients who were taking antiviral medication was approximately 333.3mm<sup>2</sup>, whilst the area of OHL lesions in patients not on any medication was approximately 277.78mm<sup>2</sup>.

Six patients were also taking prophylactic medication for *Pneumocystis carinii* pneumonia (Sulfamethoxazole/Trimethoprim combinations such as Bactrim or Septrim). One patient was taking fluconazole and one patient was taking methadone.

#### 4.2.3 Association between the size of Oral Hairy Leukoplakia lesions and CD4+ T-lymphocyte count

The average CD4+ T-lymphocyte count for the group of 20 patients was 323.65 cells/μL (range 14-1000 cells/μL). The average size of the lesions according to CD4+ T-lymphocyte count was 338.83mm<sup>2</sup>, 372.5mm<sup>2</sup> and 363.25mm<sup>2</sup> for CD4+ T-lymphocyte counts <200 cells/μL, 200-500 cells/μL and >500 cells/μL respectively.

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#### **4.2.4 Exfoliative cytology smears**

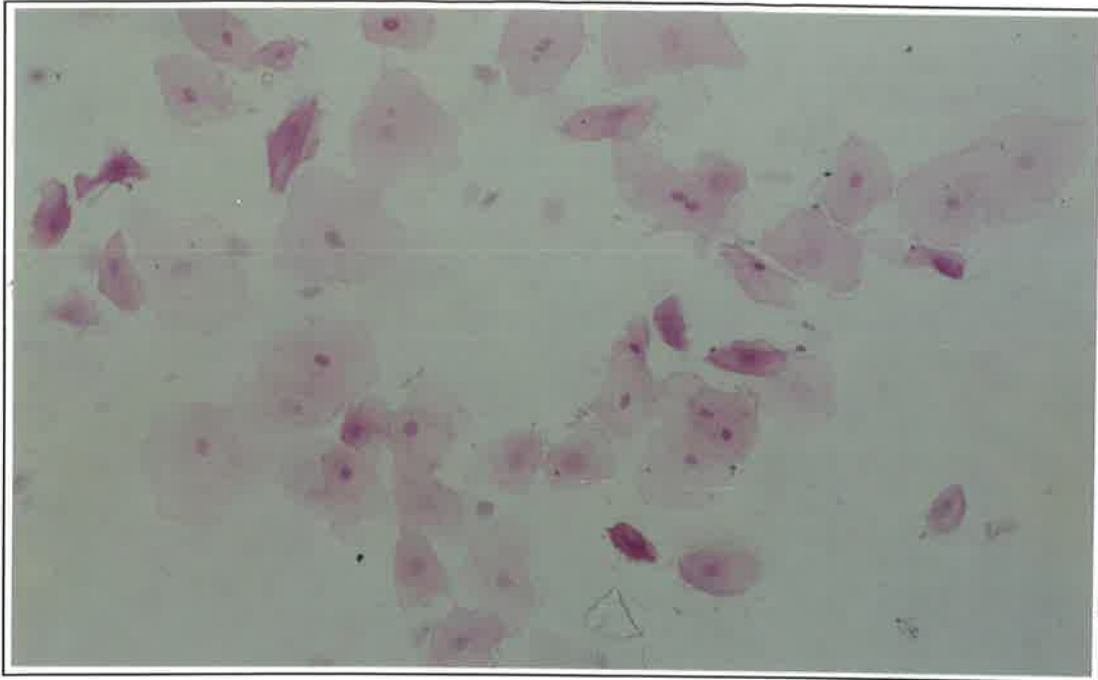
Exfoliative cytology smears taken from the lateral border of the tongue were examined using both light and electron microscopy.

##### **4.2.4.1 Light microscopy**

Tongue smears were initially stained with Haematoxylin and Eosin (H and E). This was done for two reasons, initially it was to determine whether adequate numbers of cells could be obtained by scraping the tongue for future electron microscopic analysis. Additionally H and E demonstrated cytological features of the superficial epithelial cells. Additional smears were stained with Periodic acid - Schiff (PAS) to detect the presence of fungal organisms.

##### **4.2.4.1.1 Haematoxylin and Eosin Stain**

Haematoxylin and eosin staining of the cytology smears demonstrated a large number of cells in each specimen (Figure 16). All smears exhibited relatively bland cytologic features consisting mainly of superficial squamous cells with small, dark staining nuclei. Nuclear to cytoplasmic ratios were consistent between cells and between different patients. In occasional cells, margination of the nuclear material was observed. No cytoplasmic or nuclear inclusions were seen in any of the smears.

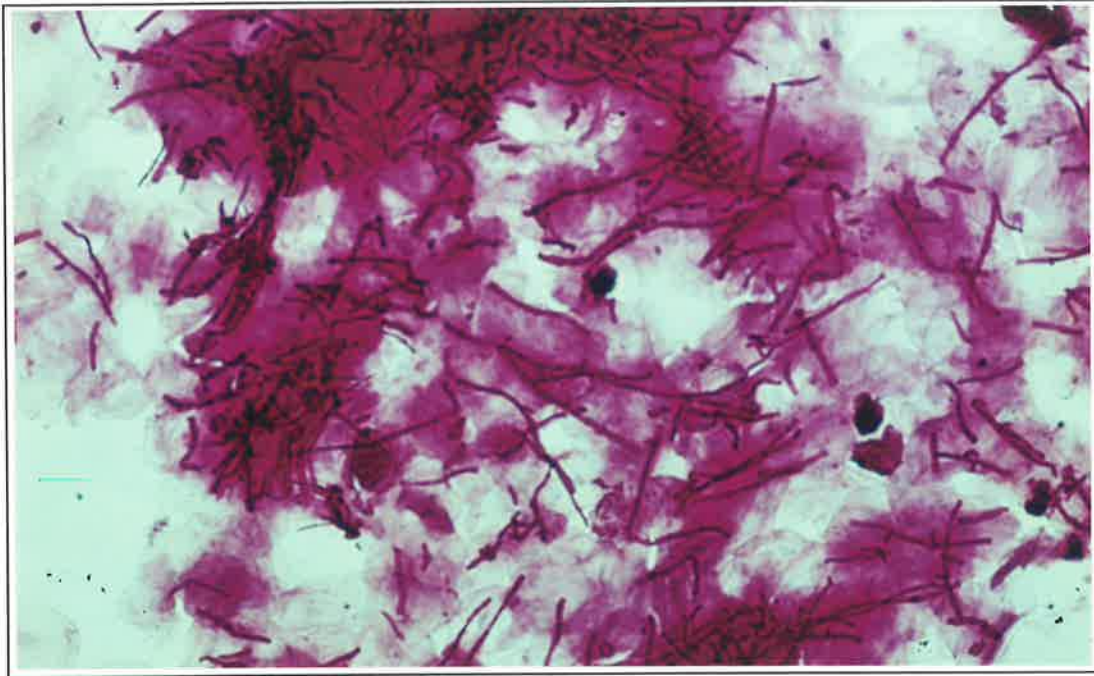


**Figure 16:** Exfoliative cytology smear from the lateral border of the tongue. The features exhibited in this smear are typical of those observed in all smears from all subjects with oral hairy leukoplakia (H&E) Original magnification x200

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#### 4.2.4.1.2 Periodic Acid-Schiff Stain

The squamous cells stained with PAS stained an intense purple colour. In 5 (25%) of the 20 patients, fungal pseudohyphae and spores were seen (Figure 17).

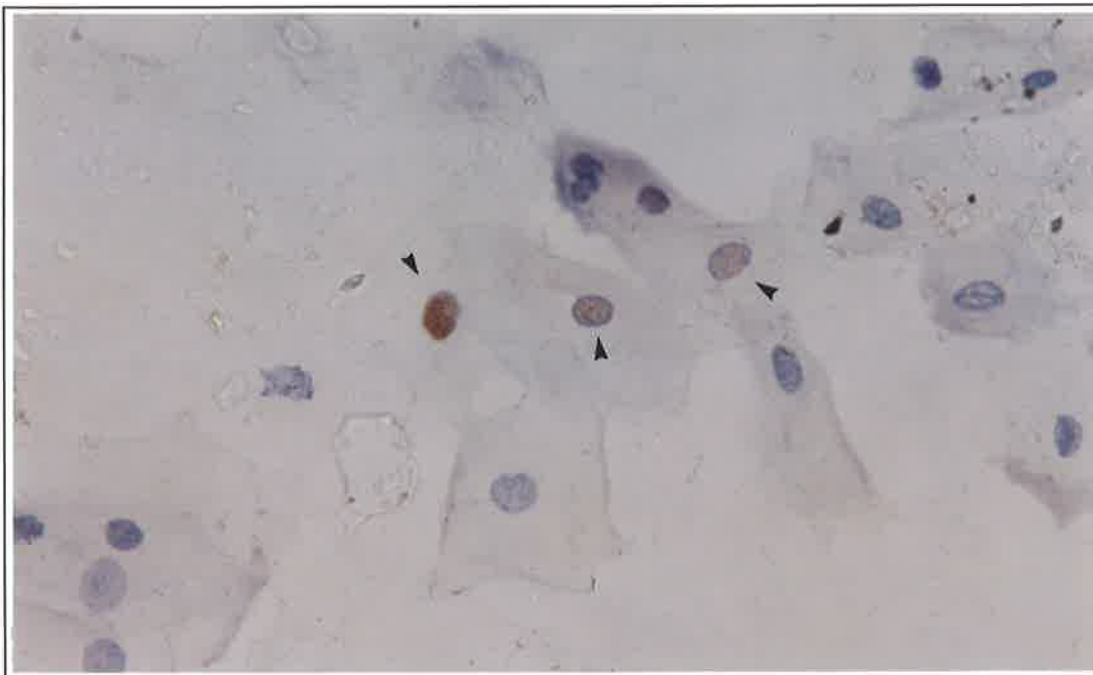


**Figure 17:** Exfoliative cytology smear from oral hairy leukoplakia on the lateral border of the tongue demonstrating fungal organisms (PAS) Original magnification x200

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#### 4.2.4.1.3 Immunohistochemistry

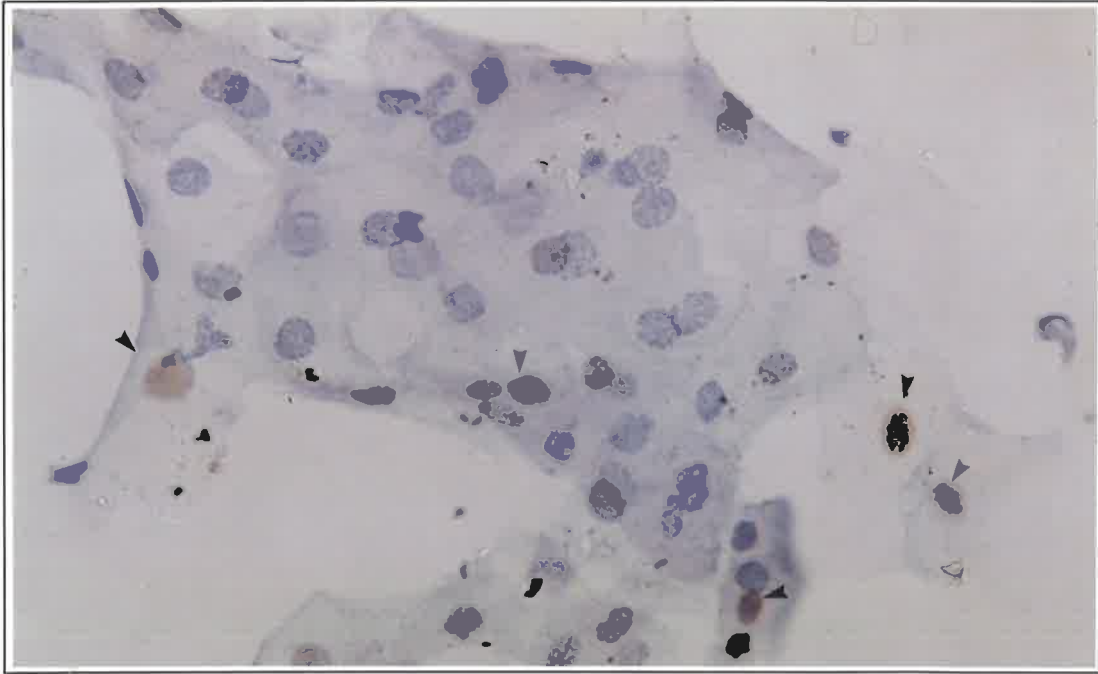
Exfoliative cytology smears were available from thirteen of the twenty subjects. All of the smears were negative for the latent membrane protein (LMP-1) antigen. Eight smears had positive labelling for the diffuse early antigen (EA-D) (Figure 18). Three smears were negative for the EA-D antigen and two smears had insufficient numbers of cells present to provide conclusive evidence. Only a small fraction of the cells in the positive smears demonstrated positive labelling for EBV using the EA-D antibody. In some smears, the cells were relatively numerous (Figure 19). In other smears, only occasional positive cells were observed (Figure 20).



**Figure 18:** Exfoliative cytology smear from oral hairy leukoplakia on the lateral border of the tongue. Squamous epithelial cells that have positive labelling for EBV EA-D antigen are demonstrated (arrows) Original magnification x400



**Figure 19:** Exfoliative cytology smear from oral hairy leukoplakia on the lateral border of the tongue. A group of EBV EA-D antigen positive cells is demonstrated (arrows). Original magnification x400



**Figure 20:** Exfoliative cytology smear from oral hairy leukoplakia on the lateral border of the tongue. Occasional EBV EA-D antigen positive cells are demonstrated (arrows). Original magnification x400

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#### **4.2.4.2 Transmission electron microscopy**

Investigation of the ultrastructural features of the superficial squamous cells associated with OHL was carried out to determine whether the technique of scraping the tongue provided adequate material for TEM examination.

##### **4.2.4.2.1 Cellular morphology**

Twelve patients had material collected for ultrastructural investigation. In the case of 3 of these patients insufficient material was collected for TEM, and the samples were discarded.

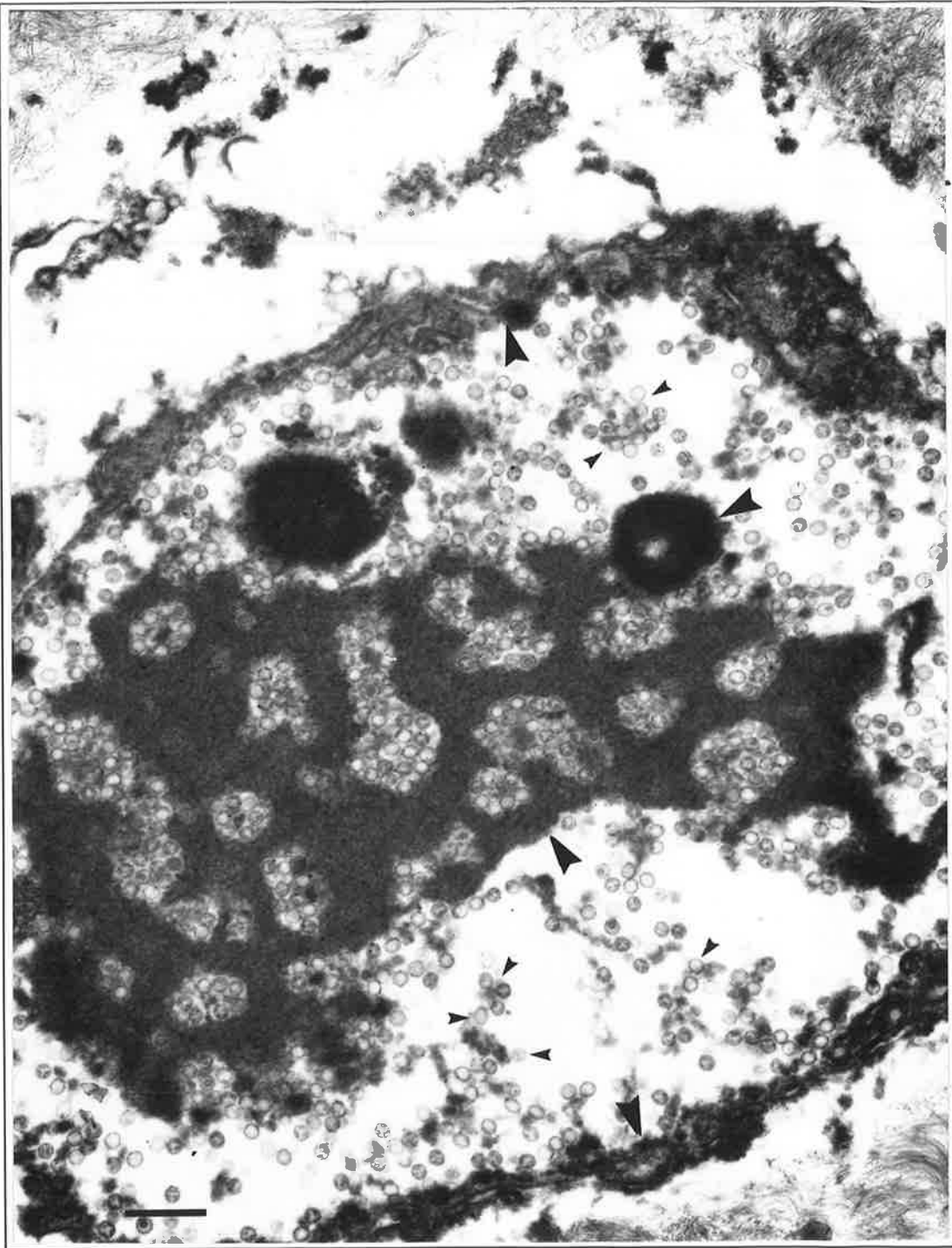
In the remaining 9 specimens, characteristic features were seen. The cells had a fibrillar cytoplasm relatively devoid of organelles consistent with superficial squamous epithelial cells.

The nuclei of the cells had degenerated and all of the squamous cells in the cytology specimens demonstrated “clumping” and margination of the chromatin in the nuclei to some degree (Figure 21). In some cases the nuclear membrane appeared to be absent.

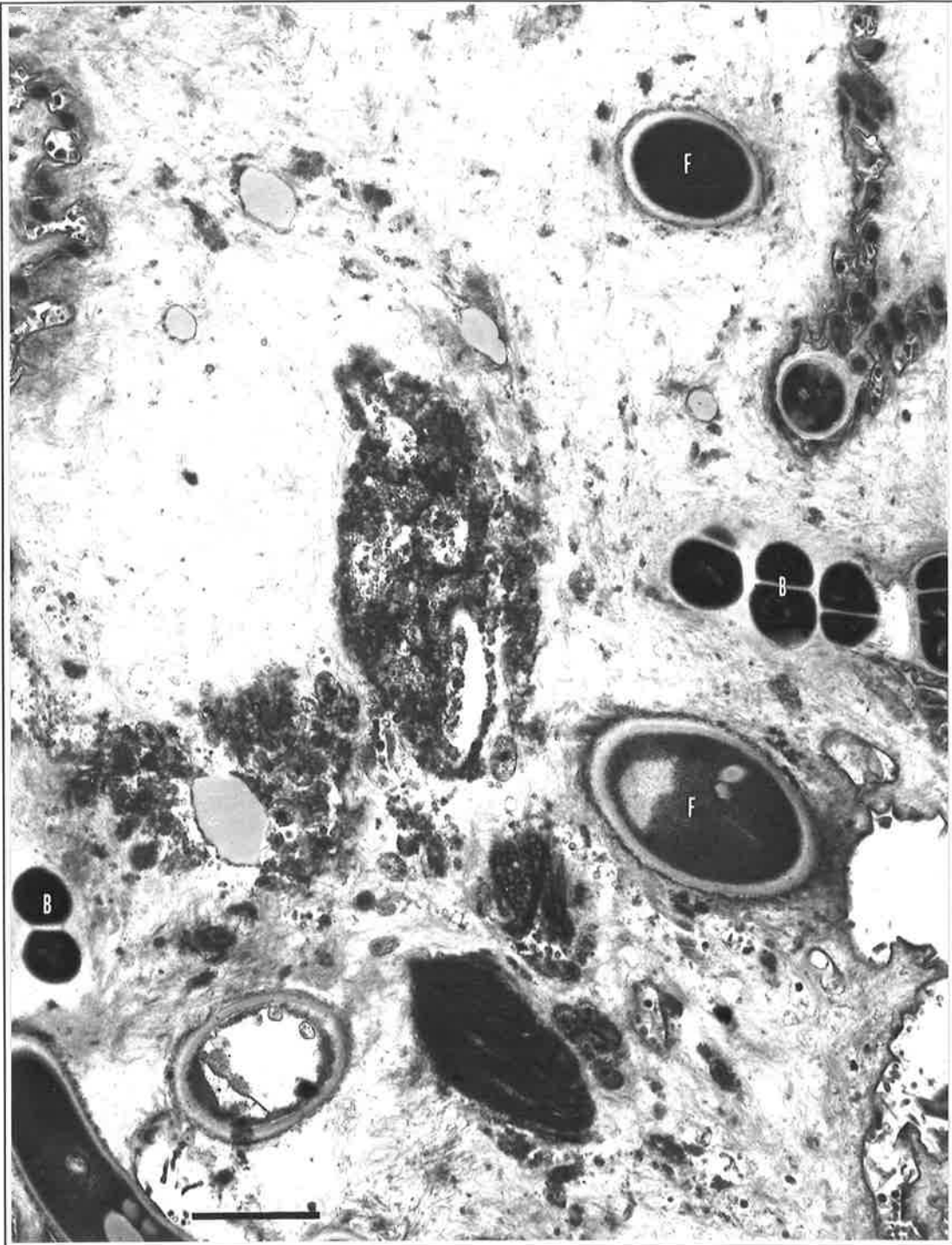
Fungal organisms were demonstrated in four of the specimens by ultrastructural examination. Fungal elements were observed both intracellularly and extracellularly (Figure 22).

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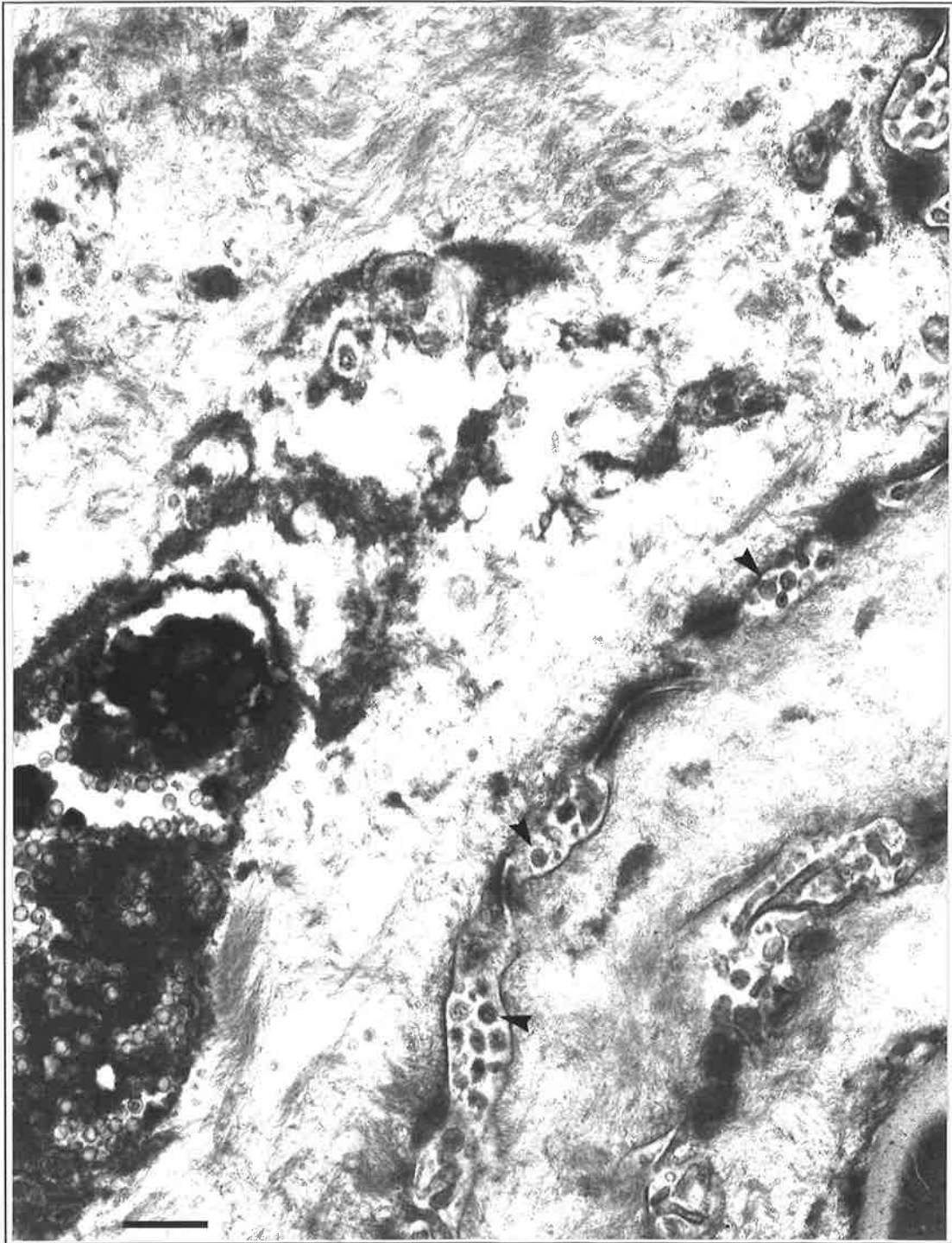
Numerous herpes-type virus particles were seen in the nuclei of the some of the epithelial cells from 7 of the study subjects. When present, they made up the bulk of the contents of the nuclei and appeared to be at various stages of development. Virus particles were also observed intercellularly (Figure 23). The herpes-type virus particles had a mean diameter of 92nm (range 80 - 107nm) (Figure 24).



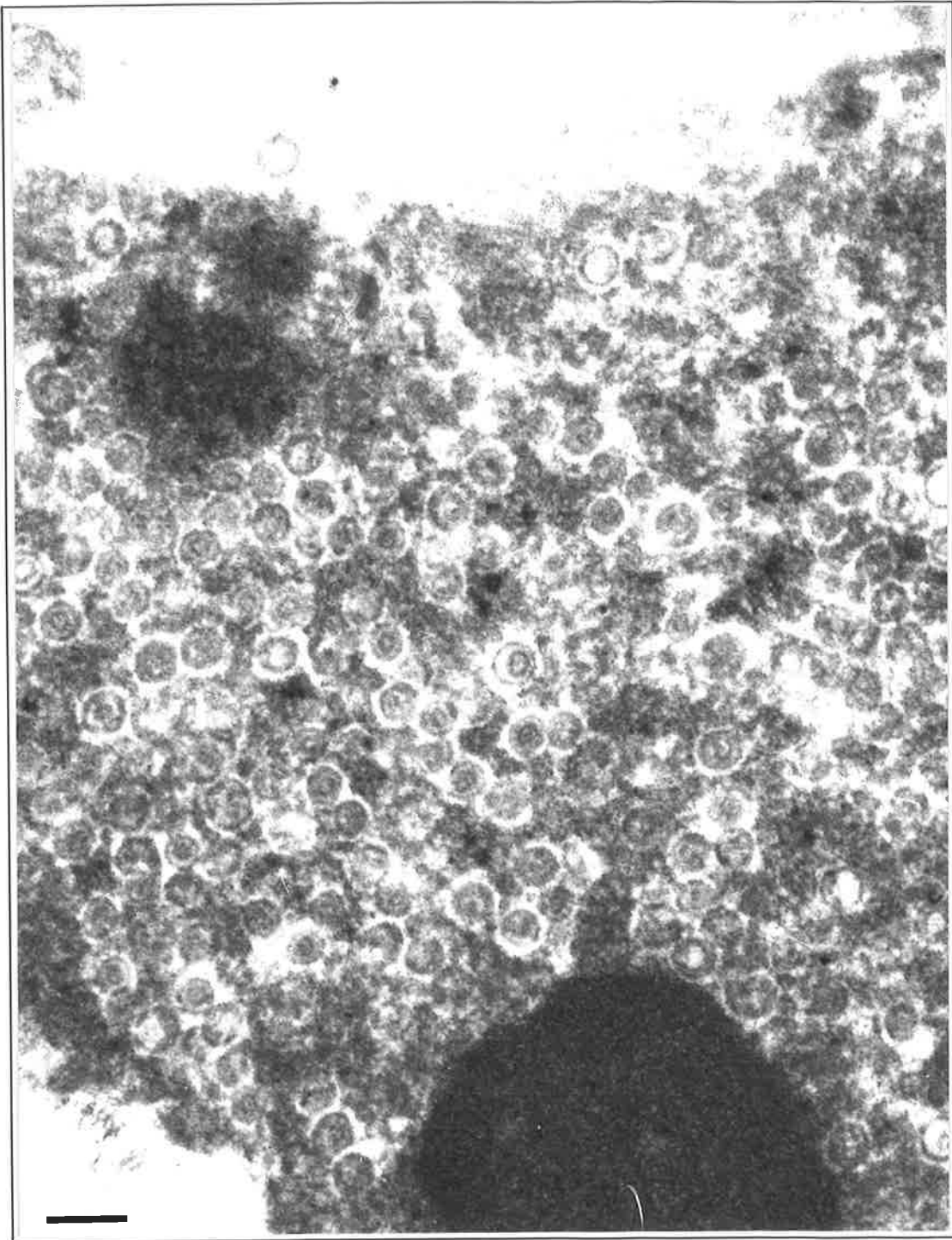
**Figure 21:** TEM of a squamous epithelial cell nucleus from an exfoliative cytology specimen demonstrating numerous herpes type virus particles (small arrows) and margination and clumping of the nuclear chromatin (large arrows). Bar represents 500nm



**Figure 22:** TEM of squamous cells from an exfoliative cytology specimen demonstrating fungal organisms (F) and bacteria (B). Bar represents 2 $\mu$ m



**Figure 23:** TEM of squamous epithelial cells from an exfoliative cytology specimen demonstrating herpes-type virus particles in the intercellular spaces (arrows). Bar represents 500nm

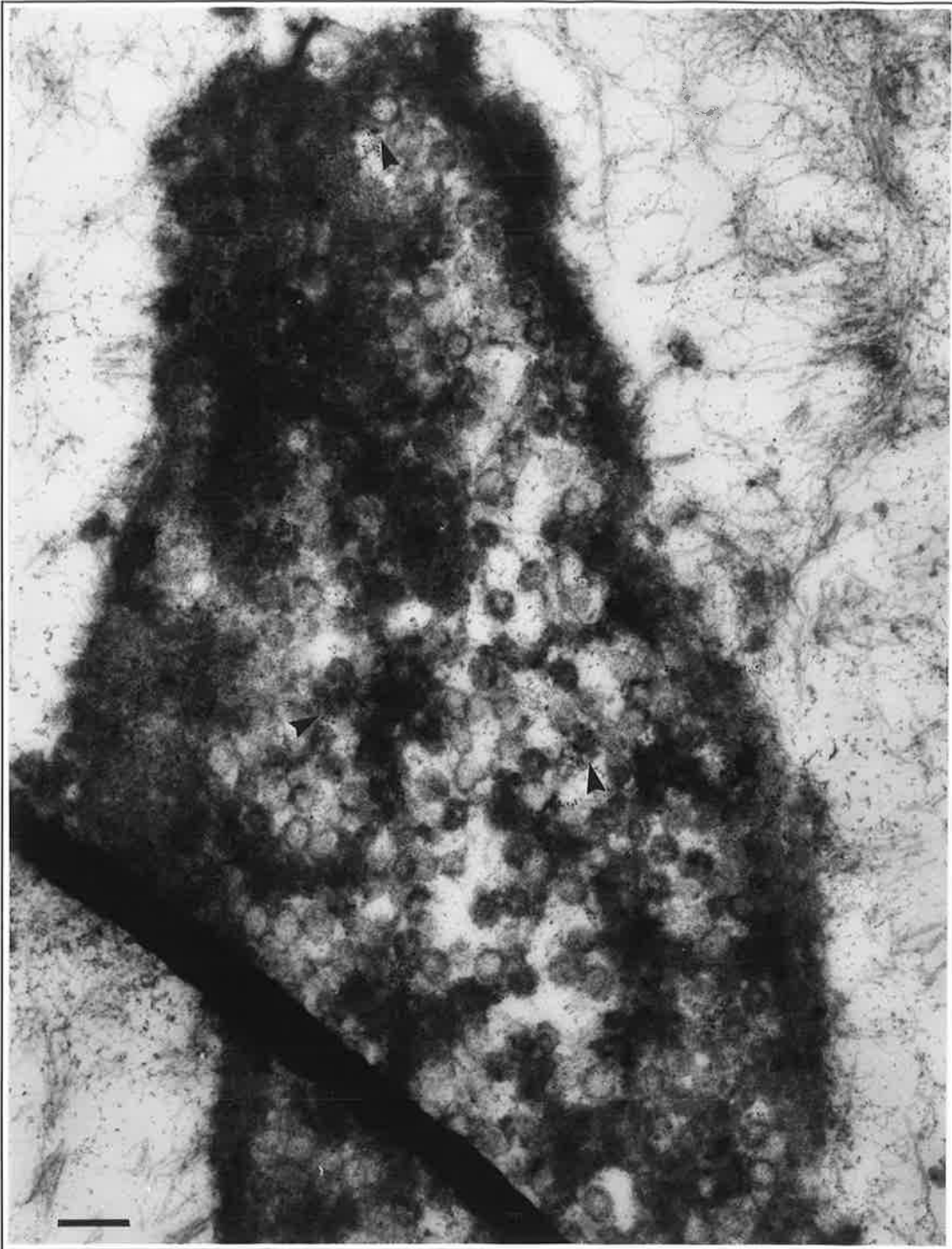


**Figure 24:** TEM of a squamous epithelial cell from an exfoliative cytology specimen demonstrating numerous herpes-type virus particles within the cell nucleus. Bar represents 200nm

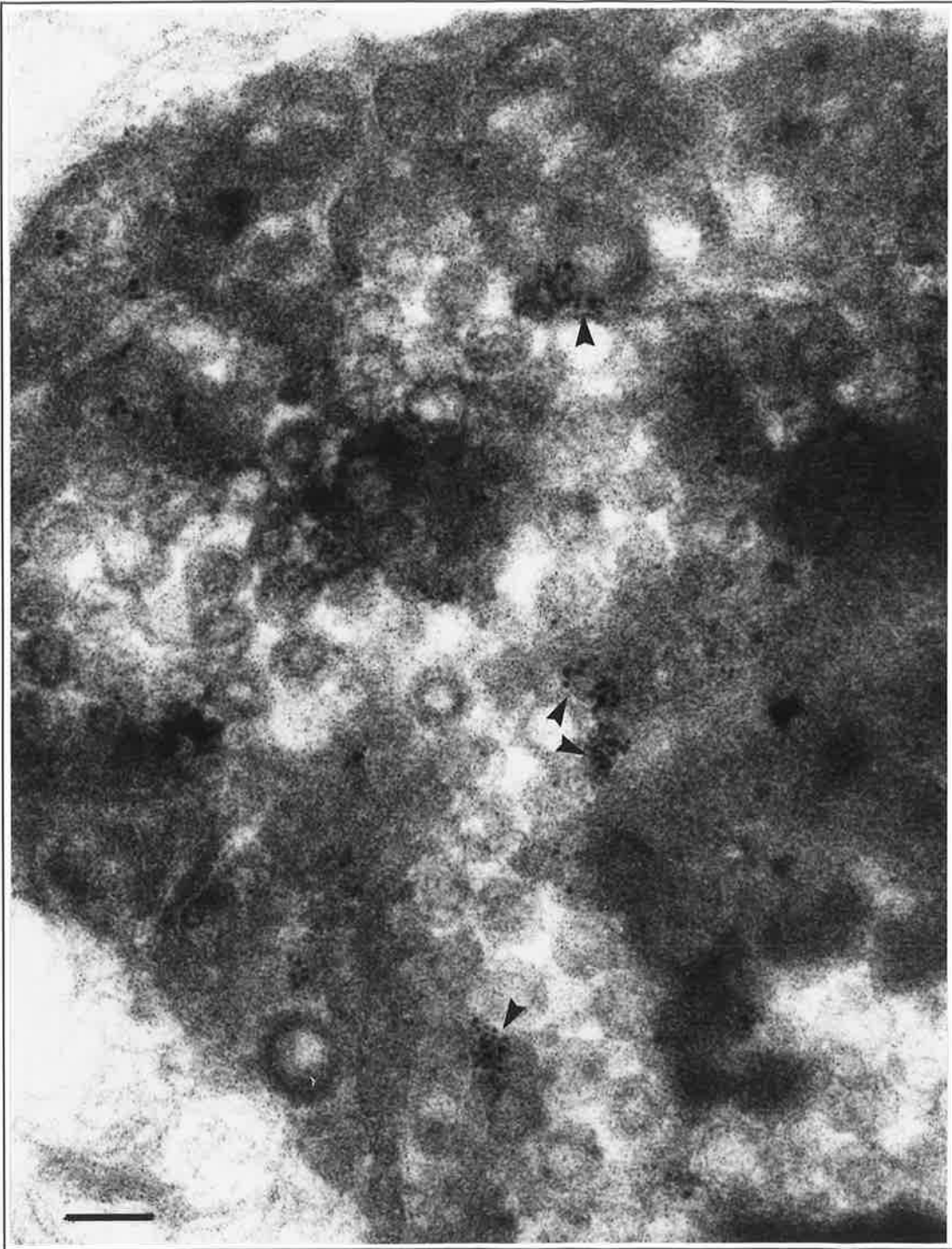
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#### 4.2.4.2.2 Ultrastructural immunogold labelling

Ultrastructural immunogold labelling of squamous epithelial cells from exfoliative cytology samples from OHL lesions was performed on 2 specimens using the monoclonal antibody specific for the EBV diffuse early antigen (EA-D). This was done as a pilot investigation to ascertain the effectiveness of the technique with a view to undertake more comprehensive immunogold labelling studies in the future. Both specimens had numerous herpes-type virions present in the nuclei of the epithelial cells. Gold particles were found to be restricted to the nuclei of the epithelial cells and in some cases found to be aggregated around virus particles (Figures 25 and 26).



**Figure 25:** TEM of a squamous epithelial cell from an exfoliative cytology specimen demonstrating herpes-type virus particles and aggregations of gold particles in the nuclei of the cell (arrows). Bar represents 200nm



**Figure 26:** TEM of a squamous epithelial cell nucleus from an exfoliative cytology specimen demonstrating herpes-type virus particles and aggregations of gold particles (arrows). Bar represents 100nm

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## **Chapter 5**

### **DISCUSSION**

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## **5. DISCUSSION**

The availability of South Australian data relating to the oral manifestations of HIV infection is limited. A study of the social impact of oral conditions among HIV-infected dental patients in South Australia was reported by Coates *et al* (1996). This study concentrated on clinical dental indices such as the number of decayed, missing and filled teeth (DMFT) and the community periodontal index of treatment needs (CPITN).

The present study provides additional, more specific information on oral hairy leukoplakia in relation to its behaviour and the association of OHL with systemic features of HIV disease. Retrospective and prospective sections of this study are discussed separately.

### **5.1 Retrospective analysis of Oral Hairy Leukoplakia**

Since its initial description by Greenspan *et al* (1984), OHL has been studied with respect to its relationship to various clinical parameters associated with HIV infection. These clinical features have included the length of time since HIV seroconversion, CD4+T-lymphocyte count, medication and AIDS-defining illness.

OHL attracted attention early on in the global HIV epidemic because of its potential significance as a prognostic factor for HIV infection. Its presence, as suggested by Greenspan *et al* (1984), was thought to herald the

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progression to AIDS or even death. Other international studies have since found OHL to be a marker of immunosuppression and progression of HIV disease (Greenspan *et al*, 1987; Kolokotronis *et al*, 1994; Begg *et al*, 1996; Ceballos-Salobreña *et al*, 1996; Ramírez-Amador *et al*, 1996).

It should be noted that the information from the retrospective analysis in the present study is based solely on the information contained in the patients' casenotes. The information in the clinical records, although comprehensive, was subject to variation. At least 3 dentists were involved in the management of HIV-infected patients throughout the period January 1986 to February 1995. In addition, the medical information was based on that obtained from a variety of sources (for example, different medical practitioners) which may have further influenced the information in the patient records.

Also, the diagnosis of OHL was based on presumptive criteria as outlined by the EC-Clearinghouse, WHO criteria (EC-Clearinghouse, 1993). This diagnostic criteria requires that the lesions are white and present on the lateral border of the tongue. In some cases the lesions can extend onto the ventral and dorsal surfaces of the tongue and, in some cases, involve the buccal mucosa.

Definitive diagnosis of OHL requires the demonstration of EBV within the lesions, or, where this is not possible, a lack of response to antifungal

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treatment can add weight to a presumptive diagnosis. It was not always evident by examination of the casenote entries whether antifungal treatment was employed to differentiate lesions.

### **5.1.1 Intraoral location of Oral Hairy Leukoplakia**

OHL was originally described by Greenspan *et al* (1984) as a fixed, white lesion that occurred on the lateral border of the tongue. In the present study, the clinical findings in relation to the predominant site of occurrence of OHL were in accord with the descriptions of a number of investigators from a variety of countries.

Schiødt *et al* (1987) found that in a sample of 50 patients, OHL occurred exclusively on the lateral border of the tongue. In that group of patients, 86% had OHL occurring bilaterally. In the present study, bilateral OHL was not observed as frequently as this. Only 44.9% of our patients had OHL occurring bilaterally on their tongues. There are two explanations for this difference, firstly, it may be that there were inaccuracies in the diagnosis of OHL in both the present study and in the study by Schiødt *et al*. Secondly, the presence of OHL in patient records may have been under-reported. Apart from the possibility of a real difference in the clinical presentation of OHL between the two studies, the differences may be a reflection of the difference in sample sizes. The present study may give a more accurate picture of the clinical presentation of OHL because of the greater number of subjects in the study.

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Other studies have observed more extensive involvement of the oral cavity by OHL (Kabani *et al*, 1989; Ficarra *et al*, 1992). These studies have described OHL occurring on the buccal mucosa, floor of the mouth, palate and dorsal surface of the tongue. OHL was found on the buccal mucosa in 3 patients in the present study. Although an interesting clinical feature from a diagnostic point of view, it has been shown that the severity of OHL (that is, the extent of intraoral involvement), as opposed to its presence does not seem to be predictive for immunosuppression or for the development of AIDS in patients infected with HIV (Schjødt *et al*, 1987).

#### **5.1.2 Prevalence of Oral Hairy Leukoplakia and time since infection with HIV**

The most common oral manifestations of HIV infection, namely OHL and oral candidiasis, have been found to occur sooner after HIV seroconversion than the development of AIDS. Lifson *et al* (1994) found that within 5 years after seroconversion, 42% of the patients in their study developed OHL.

This retrospective analysis of OHL in South Australian HIV-infected patients found that 5 years after estimated seroconversion, the cumulative prevalence of OHL was 47.4%, a finding consistent with the previous study by Lifson *et al* (1994). Although not statistically significant, the present study also found a slight increase in the prevalence of OHL after a period of infection with HIV that was greater than or equal to 10 years, in this instance OHL was found in 55.6% of patients.

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### 5.1.3 Presence of Oral Hairy Leukoplakia and AIDS-defining illnesses

Extensive OHL has been reported in two studies where it occurred concurrently with an AIDS-defining illness. Kabani *et al* (1989) described extensive OHL in a patient four months after they developed KS and PCP. Southam *et al* (1992) reported a case where OHL occurred in the epithelium actually overlying KS lesions in the oral cavity.

The present study examined current or previous AIDS-defining illnesses relative to the prevalence of OHL. Only 36 out of the 197 patients were identified as having had an illness that was AIDS defining. In this group of patients the prevalence of OHL was 52.8%. The prevalence of OHL in the 161 patients without any reported AIDS-defining condition was 43.5%. There was no statistical significance in the prevalence of OHL between these two groups.

In relation to single AIDS-defining illnesses it was not possible to draw any conclusions regarding their relationship to the presence of OHL because of the low patient numbers.

### 5.1.4 Presence of Oral Hairy Leukoplakia and systemic medication

One hundred and fifty eight patients were taking medication of some kind either for the treatment of HIV infection, the treatment of opportunistic infections or for the prophylaxis of opportunistic infections.

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Opportunistic infections for which prophylactic therapy is required include PCP, MAC, and CMV infections. It is important to note that these opportunistic infections are generally seen in patients with advanced immune deficiency, that is, where CD4+ T-lymphocyte counts are less than 200 cells/ $\mu$ L (Stewart, 1993; Peiperi, 1995).

The prevalence of OHL in patients who were taking medication was 48.7%. This figure is only very slightly higher than the overall prevalence of 45.2% for the sample as a whole. However, although slight, this higher prevalence may be a reflection of more advanced immune deficiency in some of these patients.

Some studies have reported a resolution of OHL following the commencement of antiviral medication (Newman and Polk, 1987; Resnick *et al*, 1988; Phelan and Klein, 1988). This finding would be expected because of the close association of OHL with Epstein-Barr virus and its possible role in the pathogenesis of the lesion.

Newman and Polk (1987) described a patient with severe OHL which resolved after undergoing treatment with an antiviral drug (9-(1,3-dihydroxy-2-propoxymethyl) guanine) that was being trialed for use as a treatment for CMV retinitis. This drug is now used routinely for the treatment of CMV retinitis and is called ganciclovir. Three of the patients in the present study were taking ganciclovir. In one patient's records it was noted that their OHL

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resolved at the time of commencing ganciclovir therapy. The behaviour of the OHL was not recorded in the records of the second patient, whilst the third did not have OHL.

Resnick *et al* (1988) described the regression of OHL following the use of aciclovir in 5 out of 6 patients who had clinical and histological evidence of OHL. The use of intravenous aciclovir as a potential treatment for OHL has also been reported, resulting in resolution of OHL until cessation of the treatment (Schöfer *et al*, 1987). Although the response OHL to various drugs is interesting as far as the behaviour of the lesion is concerned, treatment of OHL is not generally required because of the asymptomatic nature of the lesion.

Forty nine patients in the present study were taking aciclovir. The prevalence of OHL in this group was 28.1% which is statistically significant compared to the prevalence of OHL in patients who were not taking aciclovir. Given the role of EBV in the pathogenesis of OHL, it follows that aciclovir should have some impact on the prevalence of the lesion. This drug has been shown to stop EBV production by inhibiting the EBV DNA polymerase enzyme (Elion, 1982).

Antiretroviral drugs have also been reported to result in resolution of OHL. Phelan and Klein (1988) presented a case report of a patient who had a 10 month history of continuous OHL. Within six weeks of treatment with AZT

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for HIV infection, the OHL had completely resolved. AZT was the only medication that this patient had taken. OHL did not recur in this patient on cessation of AZT.

Ninety one patients in the present study were taking AZT and again the prevalence of OHL was reduced compared to the overall prevalence in the total sample population. The prevalence of OHL in patients taking AZT was 29.7% compared to an prevalence of OHL of 57.5% in patients who were not taking AZT. This was statistically significant ( $0.001 < P < 0.01$ , Chi squared analysis). Other antiretroviral drugs taken by patients in this study were ddI and ddC. These medications belong to the same class of drug as AZT (that is, nucleoside analogues) and the prevalence of OHL was also reduced in patients taking these medications. However patient numbers were too low to draw any conclusions.

The reduced prevalence of OHL in these patients taking antiretrovirals may be a result of direct action by the drug. This is especially likely in the case of aciclovir and ganciclovir which are drugs directed at herpes viruses. EBV is a member of the herpes virus family. Alternatively, the patients' immune function may be improved by antiviral medication thereby enabling a more effective immune response to EBV, thus reducing viral replication. Although these drugs definitely have a beneficial effect on OHL lesions, the results of the present study indicate that there must be other factors that lead to the development of OHL. It is therefore supposed that the pathogenesis of OHL

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is multifactorial and that viruses, namely EBV are probably co-factors in the development of OHL.

Thirty-nine patients were not taking any medication and the prevalence of OHL in this group was 30.8%. The lower prevalence of OHL than that found in the group of patients taking medication may reflect the fact that the patients not on medication were healthier in terms of their immune function.

#### **5.1.5 Presence of Oral Hairy Leukoplakia and CD4+T-lymphocyte counts**

CD4+T-lymphocyte counts have been used to monitor the health of patients with HIV infection to give an indication of HIV disease progression. CD4+ T-lymphocyte counts also help predict the development of opportunistic infections (Stewart, 1993A; Dwyer *et al*, 1996). OHL also plays a role in the staging of HIV infection. It is included in the Centers for Disease Control and Prevention (CDC) classification of HIV infection (Appendix I).

Reichart *et al* (1989) found in a study of 95 HIV-infected patients who had clinical and histological evidence of OHL that the average CD4+ T-lymphocyte count was 149 cells/ $\mu$ L (range 10-470 cells/ $\mu$ L). Glick *et al* (1994) found that, in patients with OHL, the mean CD4+ T-lymphocyte count was 143.3 cells/ $\mu$ L. The same study reported that the presence of OHL in HIV positive patients had a positive predictive value of 70.1% that the CD4+ T-lymphocyte count would be less than 200 cells/ $\mu$ L. Begg *et al* (1996)

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found a significant association between a low level of CD4+ T-lymphocytes and OHL in intravenous drug users.

A chronological model of HIV disease progression has been illustrated by Stewart (1993) in which CD4+ T-lymphocyte counts and opportunistic infections associated with HIV are correlated. According to this model, oral manifestations of HIV infection are more likely to occur at an intermediate level of immunodeficiency where CD4+ T-lymphocytes are between 200-500 cells/ $\mu$ L.

In the present study, it was interesting to note that the range of CD4+ T-lymphocyte counts was very broad both in the group of patients without OHL (0-2200 cells/ $\mu$ L ) and in the group of patients with OHL (0-1200 cells/ $\mu$ L). This was also reported by Lifson *et al* (1994). The mean CD4+ T-lymphocyte count for patients with OHL in the present study was 294.01 cells/ $\mu$ L which is in accordance with the model proposed by Stewart (1993). The mean CD4+ T-lymphocyte count in patients without OHL was 376.99 cells/ $\mu$ L.

Ravina *et al* (1996) found that patients who had OHL demonstrated a greater decline in CD4+ T-lymphocytes over an eight year period compared with HIV-infected patients without OHL. This is also inferred by the results of the present study by determining CD4+ T-lymphocyte counts and presence

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of OHL after placing the patients into groups according to the length of time since estimated seroconversion.

At less than 5 years since estimated seroconversion, the CD4+ T-lymphocyte count for patients with OHL was 303.14 cells/ $\mu$ L compared with 400.18 cells/ $\mu$ L in patients without OHL. When the time since estimated seroconversion was greater than 10 years, the CD4+ T-lymphocyte counts in patients with OHL was 174.85 cells/ $\mu$ L compared to 345.60 cells/ $\mu$ L in patients without OHL. The exception that was found in this study was that in the group of patients who had seroconverted between 5 and 10 years previously, the CD4+ T-lymphocyte counts were reversed, that is, they were higher in patients with OHL (404.03 cells/ $\mu$ L) than in patients without OHL (385.21 cells/ $\mu$ L).

The results from this study do not demonstrate any statistically significant relationship between the presence of OHL and CD4+ T-lymphocyte count. This supports the original hypothesis that in this Australian group of patients the presence of OHL was not associated with a lower CD4+ T-lymphocyte count. Further detailed longitudinal studies of patients would provide more accurate information relating to this. Also, it would be interesting to observe how the presence of OHL relates to HIV viral load.



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### 5.1.6 Presence of Oral Hairy Leukoplakia and oral *Candida* infection

*Candida albicans* is the most common species of *Candida* that is found in the oral cavity (Scully *et al*, 1994). Oral candidiasis is the most frequently found oral fungal disease and is seen in both acute HIV infection and in later stages of the disease process (Greenspan and Greenspan, 1996).

Ramírez-Amador *et al* (1996) found the prevalence of oral candidiasis in HIV-infected populations to be approximately 20%. Other studies have also reported similar results (Kolokotronis *et al*, 1994). The overall prevalence of oral candidiasis in the present study was 27%. Lifson *et al* (1994) found that the prevalence of oral candidiasis increased as the length of time from HIV seroconversion increased. This was also the situation with OHL.

Greenspan *et al* (1984) reported that *Candida* was associated with OHL in 26 out of 37 patients in their initial description of OHL. Schiødt *et al* (1987) found that 50% of patients with OHL in their study had evidence of *Candida*. Other studies have also found a high proportion of *Candida* associated with OHL (Kanas *et al*, 1988; Kratochvil *et al*, 1990; Schulten *et al*, 1991).

Although in the present study it was impossible to determine whether cases of oral candidiasis were directly associated with OHL, the prevalence of oral candidiasis was increased in the group of patients who had OHL compared to the overall prevalence of oral candidiasis. In patients with OHL, the prevalence of oral candidiasis was 31.5% compared to 27% overall. This

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possibly reflects the fact that OHL provides a suitable environment for *Candida* organisms to colonise.

Conversely, in the 53 patients who had oral candidiasis, 53% had OHL present also. This prevalence of OHL is higher than that in the overall population in this study. This may indicate that *Candida* may, in some way, help to promote the development of OHL, although studies have shown that OHL is not affected by antifungal medication. Alternatively, it may reflect the fact that patients with oral candidiasis have reduced immune function making them more susceptible to opportunistic infections such as fungal infections and OHL.

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## 5.2 **Prospective analysis of Oral Hairy Leukoplakia**

The definitive diagnosis of OHL, according to the EC-Clearinghouse classification of oral lesions in HIV infection, requires demonstration of Epstein-Barr virus in the lesions (EC-Clearinghouse, 1993). Direct examination of OHL lesions in this part of this study provided the opportunity to satisfy these criteria.

### 5.2.1 **Clinical appearance and location of Oral Hairy Leukoplakia**

In 19 patients, lesions were present on the lateral border of the tongue which had clinical appearances consistent with OHL as reported in the literature (Greenspan *et al*, 1984; Greenspan *et al*, 1990; Itin and Ruffli, 1992). In the majority of patients the lesions were unilateral, a feature that does not concur with the findings of Schiødt *et al* (1987) who reported that OHL usually occurred bilaterally.

One patient presented with an unusual fixed, white lesion on their palate. This lesion did not respond to antifungal treatment and was included as a possible atypical presentation of OHL. OHL has been previously reported involving the soft palate (Kabani *et al*, 1989). An alternative aetiological agent for this lesion could be human papillomavirus (HPV). HPV has been implicated in a variety of oral lesions including viral warts, focal epithelial hyperplasia and squamous cell carcinoma.

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### 5.2.2 Association between the size of Oral Hairy Leukoplakia lesions and concurrent medication

The reported beneficial effect of antiviral medication such as AZT and aciclovir on OHL (Resnick *et al*, 1988; Phelan and Klein, 1988) was not evident in this small group of patients. Nine patients were taking combinations of antiretroviral medication (eg. AZT, ddI, ddC, 3TC, d4T, indinavir), four of these patients were also taking aciclovir, whilst one patient was taking aciclovir alone.

The size of OHL lesions has been shown to vary within individual patients. In some cases, lesions have been observed to completely disappear and then recur from one examination to the next (Lifson *et al*, 1994). This could possibly be due to changes in HIV viral load or possibly even medication. In the present study, the area of the tongue covered by the lesions was recorded and compared between the groups of patients on antiviral medication and those not on medication. The average area of the tongue covered by OHL was 277.8mm<sup>2</sup> in patients who were not taking any antiviral medication, whilst the area involved in patients on antiviral medication was slightly higher, 333.3mm<sup>2</sup>. If antiviral medication was to have an influence on the development of OHL, one would expect an absence of OHL or decreased size of lesions in those patients taking antiviral medication compared to those not taking antiviral medication. However, the number of patients was small which limits the interpretation of the results. Alternatively, the size of OHL lesions may not be influenced by the antiviral medication at all and simply reflect that those patients who required this medication,

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whether to treat the HIV or opportunistic diseases, have more advanced HIV disease or are more immunocompromised.

### **5.2.3 Relationship between the size of Oral Hairy Leukoplakia lesions and CD4+ T-lymphocyte count**

The commonly accepted notion is that OHL is a lesion that is associated with intermediate immunodeficiency, that is, CD4+ T-lymphocyte count between 200-500 cells/ $\mu$ L (Stewart, 1993). The average CD4+ T-lymphocyte count in this group of 20 patients had lesions was consistent with this at 323.65 cells/ $\mu$ L. The range of CD4+ T-lymphocyte counts in this group, however, was varied, ranging from 4 - 1000 cells/ $\mu$ L. This concurred with the results of the retrospective analysis where a large range in the CD4+ T-lymphocyte count was also observed in patients where OHL lesions were present.

It was interesting to determine whether CD4+ T-lymphocyte count influenced the size of the OHL lesions. Essentially, no difference was observed in the size of OHL lesions between patients with  $<200$  cells/ $\mu$ L, 200-500 cells/ $\mu$ L or  $>500$  cells/ $\mu$ L, the areas being 338.83mm<sup>2</sup>, 372.5mm<sup>2</sup> and 363.25mm<sup>2</sup> respectively. This supports the initial hypothesis that CD4+ T-lymphocyte counts are not associated with the presence of OHL. In addition, other studies have reported that the severity of OHL has not been associated with progression of HIV disease, that is, the development of AIDS (Schjødt *et al*, 1987; Kabani *et al*, 1989).

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#### 5.2.4 Cytology of Oral Hairy Leukoplakia

Examination of exfoliative cytology smears using light microscopy and electron microscopy demonstrated features similar to those found in other international studies of OHL.

##### 5.2.4.1 Light Microscopy

There have been numerous reports of the histological features of OHL documented (Greenspan *et al*, 1984; Schiødt *et al*, 1987; Kanas *et al*, 1988; Greenspan *et al*, 1989). The characteristic histology of OHL is that of epithelial hyperplasia with hyperparakeratosis and acanthosis. In addition to this, ballooning degeneration of epithelial cells is seen in the upper part of the prickle cell layer resulting in a koilocyte-like appearance. Inflammation is minimal or rarely seen in association with the lesions.

Because of its benign nature, biopsies of OHL are not always indicated because presumptive diagnosis is usually adequate from a clinical management perspective. In addition it is often difficult to get a patient to consent to a biopsy especially in this group of patients who are often subjected to numerous other invasive diagnostic procedures. Exfoliative cytology smears, therefore, are a convenient non-invasive method to examine OHL lesions.

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#### 5.2.4.1.1 Haematoxylin and Eosin stain

Migliorati *et al* (1993) examined the possibility of diagnosing OHL using exfoliative cytology smears stained with Papanicolaou (PAP) stain. The specimens in the study by Migliorati *et al* demonstrated numerous epithelial cells and bacteria. The cells had round to oval basophilic nuclei. In some cases the nuclei demonstrated prominent margination of nuclear chromatin, referred to as nuclear beading. These features were deemed to be characteristic of OHL as they were not observed in any of the control groups.

Epstein *et al* (1995) also observed these nuclear changes in PAP stained cytologic smears of OHL. The latter study found, that of 30 specimens, 16 showed margination of nuclear chromatin.

The present study, in which cytologic smears were stained with H and E, large numbers of pale staining squamous epithelial cells were observed. The cells contained basophilic round to oval nuclei as seen in the previous studies. Margination of nuclear chromatin material or nuclear beading was not readily apparent in the majority of cells that were seen. Occasional cells showed evidence of this type of nuclear change.

Some studies have reported the presence of Cowdry-type inclusion bodies in the cells which are characteristic in cells infected by herpes viruses

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(Migliorati *et al*, 1993). However they were not evident in the cytology smears examined in the present study.

#### 5.2.4.1.2 Periodic Acid - Schiff stain

*Candida* has regularly been found in association with OHL since its initial description by Greenspan *et al* (1984). In that initial report of OHL, *Candida* was found in 26 out of 37 cases (70%). Similar high prevalences of *Candida* association with OHL have been found in other studies (Schiødt *et al*, 1987; Kanas *et al*, 1988). The prevalence of *Candida* organisms found in association with OHL in the present group of patients was only 25% on examination of PAS-stained cytologic smears. This low prevalence could possibly be attributed to the small sample size in this part of the present study. A limitation that was evident in the use exfoliative cytology specimens were variations in sampling. It was found that patients with fungal organisms present by light microscopy did not always have fungal organisms present according to ultrastructural examination of the cytology smears (Section 5.2.4.2.1) and *vice versa*.

#### 5.2.4.1.3 Immunohistochemistry

EBV has been consistently found in association with OHL since its identification in the lesions by Greenspan *et al*, (1985). It has since become the definitive criterion that is required for the diagnosis of OHL (EC-Clearinghouse, 1993).

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In this study, two monoclonal antibodies specific for EBV were used to label the epithelial cells from exfoliative cytology specimens. These antibodies were for the latent membrane protein (LMP-1) and diffuse early antigen (EA-D). None of the smears from 13 patients labelled with LMP-1 demonstrated any evidence of positive labelling.

The presence of LMP-1 in OHL lesions is contentious. It has been postulated that the expression of this antigen may play a role in the development of the characteristic histology that is seen in OHL as it has been shown to inhibit human epithelial cell differentiation and induce epithelial hyperplasia (Dawson *et al*, 1990; Wilson *et al*, 1990). There are few studies which have investigated the immunohistochemical profile of OHL (Kanas *et al*, 1988; Zhang *et al*, 1988; Murray *et al*, 1996). Niedobitek *et al* (1991) found no evidence of LMP-1 expression in OHL lesions using *in situ* hybridisation. Thomas *et al* (1991), who also used *in situ* hybridisation, did find evidence of LMP-1 in 2 out of 11 OHL biopsies. Murray *et al* (1996) concluded that the expression of this protein is not necessary for EBV replication in OHL lesions.

The expression of EA-D in OHL specimens has been previously demonstrated by Rabanus *et al* (1991). In the present study, positive labelling for this protein was observed in 8 out of the 13 specimens studied. Two specimens had insufficient numbers of epithelial cells present and the remaining 3 appeared to be negative. EA-D is involved in the induction of

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EBV replication and synthesis of viral proteins (Rabanus *et al*, 1991). The negative labelling for EBV EA-D in these 3 lesions may be a reflection of sampling problems. In the ultrastructural examination of the epithelial cells from OHL lesions (Section 5.2.4.2.1) it was found that some, but not all of the cells demonstrated the presence of herpes-type virus particles in their nuclei. This feature was also demonstrated in the positive immunohistochemical smears, some, but not all of the cells demonstrated positive labelling for EA-D in their nuclei. It is conceivable that, due to sampling factors, cells positive for EA-D were not represented in the negative specimens. It should be noted that one of the negative specimens was that taken from a flat, fixed white lesion on the hard palate. This lesion was investigated on the premise that it represented an atypical example of OHL. The palatal lesion possibly represented an example of a flat viral wart caused by human papillomavirus infection. The remaining two negative specimens in this study may also have represented an alternative pathology to OHL.

#### **5.2.4.2 Transmission electron microscopy**

Numerous ultrastructural investigations of OHL have been undertaken. The most striking feature that has been observed is the presence of viral particles consistent with the herpes virus family. Greenspan *et al* (1984) were the first to observe and report the presence of herpes-type virus particles within the nuclei of prickle cells and also within the intercellular spaces in biopsies of OHL.

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#### 5.2.4.2.1 Cellular morphology

Apart from the abundant herpes type viral particles which have been described in various studies (Greenspan *et al*, 1984; Belton and Eversole, 1986; El-Labban *et al*, 1988; Ficarra *et al*, 1988; Kanas *et al*, 1988; Reed *et al*, 1988; Zhang *et al*, 1988; Greenspan *et al*, 1989; Kratochvil *et al*, 1990; Epstein *et al*, 1995) other features have been described in ultrastructural investigations of OHL.

Belton and Eversole (1986) found *Candida albicans* organisms present in the superficial layers of the epithelium. The fungal hyphae were found to be present extracellularly and intracellularly. This was subsequently reported by other studies (El-Labban *et al*, 1988; Zhang *et al*, 1988).

Fungal organisms were observed in four exfoliative cytology specimens from patients in the present study. Interestingly none of these patients had observable fungal organisms on examination of their PAS stained cytology smears using light microscopy. In addition, one patient who had a PAS stained smear positive for *Candida* had no evidence of fungal organisms ultrastructurally. This demonstrates a drawback in the use of exfoliative cytology in that adequate tissue or cell samples may not be obtained by this method compared to a biopsy.

Some studies have reported crystalline inclusions and tubular structures within the epithelial cells (Belton and Eversole, 1986; El-Labban *et al*, 1988;

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Zhang *et al*, 1988). Zhang *et al* (1988) described two structures occurring in the virus-infected cells. These included 35nm diameter tubules arranged in parallel bundles. This feature had previously been reported by Belton and Eversole (1986). A similar structure was reported by El-Labban *et al* (1988). Another structure was also observed which was composed of undulating convoluted membranes present close to the nucleus of the cell. The nature and role of these structures are unclear. El-Labban *et al* (1988) also described a multivesicular structure within the cytoplasm of the “koilocyte-like” cells of OHL. This was characterised by clusters of membrane-bound vesicles which had an average diameter of 100nm. The authors of this study postulated that this structure may represent an abnormal form of microtubule due to HIV infection or may be directly related to EBV infection because similar structures were observed in the first report of cultured cells from Burkitt’s lymphoma.

Our findings were not consistent with those of the aforementioned studies. This is possibly because the cells that were obtained by scraping the tongue are very superficial and have lost features such as their organelles as they matured through the thickness of epithelium. This accounts for the fact that the majority of cells in the present study had only a fibrillar cytoplasm with little evidence of organelles.

Nuclear changes, as reported in other studies (Ficarra *et al*,1988; Zhang *et al*, 1988; Epstein *et al*, 1995) such as margination and condensation of

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chromatin and punched out appearances of nuclei were observed in the present study.

The use of ultrastructural examination of exfoliative cytology specimens for the diagnosis of OHL has been investigated as an alternative to biopsying lesions. Kratochvil *et al* (1990) found that the predominant feature of the epithelial cells scraped from OHL lesions was the presence of abundant virus particles within the cells and in the intercellular spaces. Seven out of the nine patients in our study had herpes-type virus present in the epithelial cells scraped off of OHL lesions. The absence of virus present in the cells of the remaining patients could be due to sampling difficulties. The present study tends to confirm the usefulness of TEM examination of cytology samples in the diagnosis of OHL.

In the study by Epstein *et al* (1995) electron microscopy demonstrated 24 out of 30 specimens to be positive for viral particles and nuclear damage, compared with 16 specimens that showed signs of nuclear damage (for example, nuclear beading) with light microscopy. They concluded, therefore, that EM was a more sensitive and reliable method for diagnosing OHL than light microscopy.

The limitation of regular TEM examination of the squamous cells from OHL lesions is that the virus particles cannot be positively identified on the basis of morphology alone. The viruses present in epithelial cells from OHL

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lesions have features that are consistent with those of members of the herpes virus family (Miller, 1995). At present there are eight known viruses within this family that infect humans. These include Herpes simplex types 1 and 2, Varicella-zoster virus, Cytomegalovirus, Epstein-Barr virus, Human herpes viruses types 6 and 7 and the recently described Human herpes virus 8 which has been shown to have a close association with Kaposi's sarcoma (Epstein *et al*, 1995; Orenstein *et al*, 1997).

In this study, distinct herpes-types virus particles were observed but, as already indicated, the morphologic appearance of virus particles is not sufficient for identification of specific viruses. Ultrastructural immunolabelling studies on virus particles from OHL lesions are few in number (Zhang *et al*, 1988; Rabanus *et al*, 1991). Zhang *et al* (1988) demonstrated positive identification of EBV in OHL lesions using two monoclonal antibodies which were directed against the EBV capsid and membrane antigen. Rabanus *et al* (1991) investigated the subcellular distribution and life cycle of EBV in keratinocytes from OHL lesions. Three monoclonal antibodies were used in that study which were directed against EBV membrane antigen (gp350/220), EBV capsid antigen (VCA) and EBV early antigen D (EA-D). The study by Rabanus *et al* (1991) found that EA-D was found principally in the areas of chromatin that had a "punched out" appearance whilst VCA was located in close approximation to EBV capsids which appeared to be at an advanced stage of maturation. Membrane antigen was detected most abundantly on enveloped EBV capsids.

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Epstein *et al* (1995) attempted immunohistochemistry using an immunogold labelling technique on specimens that were processed for ultrastructural examination. The technique in that study was not found to be effective and subsequently discontinued. In that report, however, there was no reference to the type of antibody used nor of the immunogold labelling technique that was attempted. In the present study immunogold studies are proceeding in order to provide further evidence that the virus is EBV.

In the present study, an immunogold labelling technique was used on exfoliative cytology specimens from 2 patients as a pilot investigation. The antibody used in this study was a monoclonal antibody specific for the EBV diffuse early antigen (EA-D) which was also an antibody employed by Rabanus *et al* (1991). In the present study, this antigen was restricted to the nuclei of the squamous epithelial cells as indicated by the presence of the gold particles. In some instances the gold particles seemed to be in close association with herpes-type virus particles, a feature not observed by Rabanus *et al* (1991). In the latter study, the EA-D antigen was found in the cell nuclei but appeared to be in close association with the areas of "punched out" chromatin. The pattern of immunogold labelling obtained with this monoclonal antibody in the present study requires further investigation.

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## Chapter 6

### CONCLUSIONS

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## 6. CONCLUSIONS

On the basis of this retrospective and prospective analysis of oral hairy leukoplakia it is concluded that:

- OHL is a common lesion in this South Australian group of HIV infected patients. In the 197 patients studied, 45.2% had the presence of OHL reported in their records. This prevalence is comparable to that found by international studies on the prevalence of OHL in association with HIV infection.
- The viral aetiology of OHL, in particular the role of Epstein-Barr virus, is supported, as in other studies, by:
  - the demonstration of the presence of numerous herpes-type virions in the nuclei of squamous epithelial cells
  - the demonstration of EBV infection in squamous epithelial cells by immunohistochemical labelling of exfoliative cytology specimens
  - the lower prevalence of OHL in patients who were taking antiviral medication, in particular, aciclovir and AZT
- In this study, the presence of OHL, was not correlated with low CD4+T-lymphocyte counts nor was its prevalence increased in patients who had concurrent or previous AIDS-defining illness and who were therefore potentially more immunocompromised. The presence of OHL, according

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to these results, does not appear to be an indicator of HIV disease progression or of poor prognosis. The results of this study, however, were not based on longitudinal data. Although no relationship between CD4+ T-lymphocyte count and OHL was established by this project, it would be interesting to conduct a longitudinal study of the oral manifestations of HIV infection, in particular OHL, especially in relation to the new marker of HIV replication, HIV viral load.

This project provided substantial information on an oral manifestation of HIV infection that, although well described in the literature, had not been previously described in an Australian HIV-infected population. Further questions relating to the aetiology and pathogenesis of OHL still need to be addressed.

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## **APPENDICES**

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## APPENDIX I

### 1993 Revised Classification System for HIV Infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults

#### CD4+ T-lymphocyte Categories:

Category 1 -  $\geq 500$  cells/ $\mu$ L

Category 2 - 200-499 cells/ $\mu$ L

Category 3 -  $<200$  cells/ $\mu$ L

#### Clinical Categories:

##### Category A

- asymptomatic HIV infection
- persistent generalised lymphadenopathy
- acute HIV infection

Category B - Symptomatic conditions not included in Category C

Examples include, bacillary angiomatosis; oropharyngeal candidiasis, vulvovaginal candidiasis; cervical dysplasia; cervical carcinoma *in situ*; constitutional symptoms  $>1$  month duration; **oral hairy leukoplakia**; herpes zoster; idiopathic thrombocytopenic purpura; listeriosis; pelvic inflammatory disease; peripheral neuropathy

Category C - AIDS defining illnesses

Candidiasis, bronchi, trachea, lungs, oesophagus; invasive cervical cancer; disseminated or extrapulmonary coccidioidomycosis; extrapulmonary cryptococcosis; chronic intestinal cryptosporidiosis; cytomegalovirus disease (other than liver, spleen or nodes); cytomegalovirus retinitis (with loss of vision); HIV-related encephalopathy; chronic herpes simplex infection; disseminated or extrapulmonary histoplasmosis; chronic intestinal isosporiasis; lymphoma, Burkitt's, immunoblastic, primary brain; disseminated or extrapulmonary *Mycobacterium avium* complex or *M. kansasii*; *M. tuberculosis*; other species of *Mycobacterium*, disseminated or extrapulmonary; *Pneumocystis carinii* pneumonia; recurrent pneumonia; progressive multifocal leukoencephalopathy; recurrent *Salmonella* septicaemia; toxoplasmosis, brain; HIV wasting syndrome

A3, B3 and C3 meet the immunologic criteria for AIDS, whilst C1, C2 and C3 meet the clinical criteria for AIDS.

Adapted from "CDC. "1993 Revised Classification System for HIV Infection and Expanded Surveillance Case Definition for AIDS Among Adolescents and Adults" *MMWR*, 1992;**41**(RR-17);1-35"

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## APPENDIX II

### VIRAL TAXONOMY OF THE FAMILY HERPESVIRIDAE

<u>Subfamily</u>	<u>Genus</u>	<u>Representative species pathogenic for humans</u>
Alphaherpesvirinae	Simplexvirus	Human herpes virus 1 (herpes simplex virus type 1)
		Human herpes virus 2 (herpes simplex virus type 2)
	Varicellovirus	Human herpes virus 3 (Varicella-zoster virus)
Betaherpesvirinae	Cytomegalovirus	Human herpes virus 5 (Cytomegalovirus)
	Roseolovirus	Human herpes virus 6 (Human B lymphotropic virus)
		Human herpes virus 7
Gammaherpesvirinae	Lymphocryptovirus	Human herpes virus 4 (Epstein-Barr virus)
		Human herpes virus 8 (Kaposi's sarcoma associated herpes virus)

(Adapted from Miller M., "Viral taxonomy" *Clin Inf Dis*, 1995; **21**: 279-280)

## APPENDIX III

### RETROSPECTIVE ANALYSIS OF ORAL HAIRY LEUKOPLAKIA no.:

#### 1. Patient details:

Patient UR number:

Date of birth:

Sex:

Date of examination:

#### 2. Information relating to HIV infection:

Time of contraction of HIV:  
(estimated)

Date of diagnosis:

Estimated time since seroconversion:

CD4+ T-lymphocyte count:

Percentage:

Medication:

AIDS defining illnesses:

#### 3. Oral manifestations of HIV infection:

Candida:

OHL:

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## APPENDIX IV

### INFORMATION SHEET

#### THE SIGNIFICANCE OF ORAL HAIRY LEUKOPLAKIA AND THE PREVALENCE OF EPSTEIN-BARR VIRUS IN LESIONS OF A SOUTH AUSTRALIAN POPULATION

Oral Hairy Leukoplakia (OHL) is a white lesion found on the sides of the tongue and occasionally other areas of the mouth. It is found in approximately a quarter of HIV infected patients as well as some organ transplant recipients whose immune systems are affected by their treatment. OHL does not cause any significant problems and does not require treatment. Questions have been raised as to whether or not the presence of OHL may be a useful indicator in the management of HIV infection.

Numerous causes of OHL have been suggested, these include Candida, Human Papillomavirus (HPV) and Epstein-Barr virus (EBV). The exact cause, at this stage, remains unclear.

Your participation in the study involves a normal clinical examination. Also, samples will be collected from OHL lesions that are present by gently scraping the tongue with an instrument. This is entirely painless. These samples will be used to try and detect the presence of Candida and EBV.

Your participation in the project is entirely voluntary, all the data that is collected is recorded onto a computer and is strictly confidential. All information will be analysed anonymously.

The results of the study will help to find out more about oral hairy leukoplakia and its relevance to HIV infection.

Investigators:

Dr. R. M. Logan	ph. 8222 8339
Dr. D.F.Wilson	ph. 8303 3071

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## APPENDIX V

THE UNIVERSITY OF ADELAIDE

<b>CONSENT FORM</b>
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**See also Information Sheet attached.**

1. I \_\_\_\_\_ (please print) hereby consent to  
take part in the research project entitled:  
  
THE SIGNIFICANCE OF ORAL HAIRY LEUKOPLAKIA AND THE PREVALENCE OF  
EPSTEIN-BARR VIRUS IN A SAMPLE SOUTH AUSTRALIAN POPULATION
2. I acknowledge that I have read the Information Sheet entitled:  
  
THE SIGNIFICANCE OF ORAL HAIRY LEUKOPLAKIA AND THE PREVALENCE OF  
EPSTEIN-BARR VIRUS IN A SAMPLE SOUTH AUSTRALIAN POPULATION
3. I have had the project, so far as it affects me, fully explained to my satisfaction by the  
research worker. My consent is given freely.
4. Although I understand that the purpose of this research is to improve the quality of  
medical care, it has also been explained that my involvement may not be of any benefit  
to me.
5. I have been given the opportunity to have a member of my family or a friend present  
while the project was explained to me.
6. I have been informed that, while information gained during the study may be published, I  
will not be identified and my personal results will not be divulged.
7. I understand that I am free to withdraw from the project at any time and that this will not  
affect any medical advice in the management of my health, now or in the future.
8. I am aware that I should retain a copy of this Consent Form, when completed, and the  
relevant Information Sheet.

**SIGNED** ..... **DATE**.....

**NAME OF WITNESS**..... **SIGNED**.....  
(Please print)

**DATE**.....

I, .....have described to.....  
(Please print)

the nature of the procedures to be carried out. In my opinion she/he understood the  
explanation.

**SIGNED**..... **DATE**.....

**STATUS IN PROJECT**.....

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## APPENDIX VI

### Haematoxylin and Eosin Staining Method

(Modified for Cytology Smears)

#### Staining

Haematoxylin - 4 minutes

Wash running tap water - approximately 1minute\*

#### Differentiation

0.5% Hydrochloric acid - 2 dips

#### Blue\*

Running water - 10 minutes\*

Dilute Alkali - 2 dips

#### Eosin - 30 seconds

#### Differentiation

70% alcohol for a few dips if required

#### Dehydration and cleaning\*

Absolute alcohol - 2 minutes

Absolute alcohol - 2 minutes

Xylol or Clearene or HistoClear - 2 minutes

Xylol or Clearene or HistoClear - 2 minutes\*

#### Mounting with coverslips

Mount in Depex

Note that an asterisk (\*) next to procedure means that an overnight pause is possible.

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## APPENDIX VII

### Periodic acid-Schiff staining method

(cytology smears)

1. Decerate sections:

2 minutes xylol  
2 minutes xylol  
2 minutes absolute alcohol  
2 minutes absolute alcohol

2. Sections placed in water (tap)

3. Oxidise in 1% periodic acid - 5 minutes

4. Rinse in tap water, then in distilled water

5. Place in Schiff's reagent - 15 minutes

6. Rinse for 3 changes of distilled water in coplin jar - 2 minutes (each change)

7. Wash in running water for 10 minutes

8. Counterstain in Haematoxylin for 3 minutes

9. Wash in running water

10. Dip in 0.5% Hydrochloric acid in 70% alcohol

11. Wash in running tap water for 5 minutes

12. Alkali - 30 seconds

13. Dehydrate and clear:

2 minutes xylol  
2 minutes xylol  
2 minutes absolute alcohol  
2 minutes absolute alcohol

14. Mount slides in Depex

NB: 1) Control slides are required for PAS staining

2) For cytology smears deceration step can be omitted

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## **APPENDIX VIII**

### **Avidin-biotin peroxidase technique for immunohistochemical labelling of exfoliative cytology specimens**

1. Following formalin fixation, block endogenous peroxidase with 0.5% H<sub>2</sub>O<sub>2</sub> in methanol 30 min
2. Apply required pre-treatment for relevant primary antibody\*
3. Rinse in phosphate buffered saline (PBS) buffer 2 x 3 min
4. Incubate in 3% normal horse serum (NHS) 30 min
5. Drain NHS and incubate with primary antibody (room temperature) overnight
6. Rinse in PBS buffer 2 x 3 min
7. Incubate with secondary antibody<sup>†</sup> 30 min
8. Rinse in PBS buffer 2 x 3 min
9. Incubate with streptavidin peroxidase<sup>‡</sup>, 60 minutes
10. Rinse in PBS buffer 2 x 3 min
11. Apply peroxidase substrate solution, control reaction macroscopically and microscopically ~7 min
12. Rinse in PBS buffer 2 x 3 min
13. Lightly counter stain with Mayer's haematoxylin
14. Dehydrate, clear and mount

\* Primary antibodies

- Latent membrane protein (LMP-1), monoclonal mouse antibody, Dako-EBV, CS 1-4, Code no. M 897, Dilution 1/200
- Diffuse early antigen (EA-D), monoclonal mouse antibody, NovoCastra NCL-EADE31, Dilution 1/50

<sup>†</sup> Secondary antibody - Rabbit antimouse IgG, Dako E0354

<sup>‡</sup> Streptavidin, HRP conjugated, Pierce 21127

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## **APPENDIX IX**

### **Preparation of cell suspension for ultrastructural examination**

#### **Cellular Morphology**

1. Cells suspended in 1% glutaraldehyde in 0.05M sodium cacodylate buffer (pH = 7.3) for 1 hour fixation
2. Cells resuspended in 2% osmium tetroxide in 0.05M sodium cacodylate plus 6% sucrose for 1 hour
3. Cells resuspended in 100% methanol for 1 hour
4. Cells resuspended in Spur's Epoxy resin for 1 hour and embedded in specimen capsule.

Polymerised at 60°C overnight

Each solution changed by centrifugation (1000 rpm) and resuspension.

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## **APPENDIX X**

### Preparation of cell suspension for ultrastructural examination

(Prior to immunogold labelling)

1. Cells are suspended in 0.5% glutaraldehyde in phosphate buffered saline (PBS) for 1 hour fixation at room temperature
2. Cells resuspended in PBS and stored at 4°C

At room temperature:

3. Cells resuspended in 100% methanol for 1 hour
4. Cells resuspended in L.R. White resin for 2 hours
5. Cells are embedded in 10mL resin with 1 drop of accelerant and spun in capsule

Each solution changed by centrifugation (1000rpm) and resuspension.

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## **APPENDIX XI**

### **Immunogold Labelling**

1. Grid floated on a drop of phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) (10 minutes)
2. Grid drained on filter paper (done between each step)
3. Grid floated on a drop of antibody (NovoCastra NCL-EADE31) diluted (1:10) in PBS with 1% BSA plus 1% Tween 20 detergent (4 hours)
4. Rinsed in PBS (5 minutes)
5. Rinsed in PBS (5 minutes)
6. Rinsed in PBS (5 minutes)
7. Grid floated on a drop of PBS with 1% BSA
8. Grid floated on goat-antimouse gold diluted (1:100) in PBS with 1% BSA plus 1% Tween 20 detergent
9. Rinsed in PBS (5 minutes)
10. Rinsed in PBS (5 minutes)
11. Rinsed in PBS (5 minutes)
12. Grid floated on distilled water (to remove salt)
13. Grid allowed to dry

## APPENDIX XII

### PROSPECTIVE ANALYSIS OF ORAL HAIRY LEUKOPLAKIA

no:

#### 1. Patient details:

Patient UR number:

Date of birth:

Sex:

M

Date of examination:

#### 2. Information relating to HIV infection:

Time of contraction of HIV:  
(estimated)

Date of diagnosis:

Estimated time since seroconversion:

CD4+ T-lymphocyte count:

Percentage:

Medication:

AIDS defining illnesses:

3. Oral Hairy Leukoplakia

Site:

Size:

Area:

Photographs (no.):

4. Candida

5. Light Microscopy

Haematoxylin and Eosin:

Features:

Periodic acid- Schiff:

Immunohistochemistry:

6. Electron Microscopy:

Cellular morphology:

Immunogold labelling:

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