
Oral Submucous Fibrosis:
An Immunohistochemical Study of the
Epithelial Growth Fraction, and
p53 Protein Presentation

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Statement of Authorship

The experiments described in this thesis were performed at Westmead Hospital, in the Institute for Clinical Pathology and Medical Research (ICPMR) and the Dental School between February 1992 and June 1993. The clinical work and survey was undertaken at Patan Hospital, Kathmandu, Nepal. The work described is that of the author unless otherwise stated. Processing of tissue for light microscopy was performed by staff of the Department of Anatomical Pathology, ICPMR, and the subsequent examinations were carried out with the assistance of Professor D.M. Walker, from the Department of Oral Surgery. Immunohistochemical staining was performed with the help of Ms Jane Milliken of the Department of Anatomical Pathology, and Ms Janice Matthews of the Dental Clinical School. This work has not been submitted in whole or in part for any other degree.

A handwritten signature in black ink, appearing to read 'S. B. Lee', with a long, sweeping horizontal flourish underneath.

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Chapter 1

Oral Submucous Fibrosis

(A) Review of the Literature

1.1. Introduction

Oral submucous fibrosis (OSF) is a chronic disease of insidious onset affecting the oral mucosa, with associated involvement of pharynx and oesophagus. Patients frequently present with a history of oral pain and intolerance of spicy foods, together with a progressive reduction in oral opening. This condition is prevalent in the Indian Subcontinent.

A condition resembling OSF was described as early as 600 BC by Sushruta, who named it 'Vidari' (cited by Mukherjee and Biswas, 1972). Its features were progressive narrowing of the mouth, depigmentation of the oral mucosa and pain on taking food. In 1952 a similar disease entity was described in five Kenyan women of Indian origin (Schwartz, 1952). This was the first documentation of the disease in recent times.

OSF derives its name from the deposition of fibrous tissue in the submucosal layer of the pharynx, palate, fauces, cheek and lips, which causes varying degrees of disablement. Schwartz, (1952) in Kenya, described the disease under the term *atrophia idiopathica (tropica) mucosae oris*. Since then it has been referred to under a number of names, *idiopathic scleroderma of the mouth* Su, (1954), *idiopathic palatal fibrosis* Rao, (1962), and *sclerosing stomatitis* Behl, (1962). In India the condition was initially described as *diffuse oral submucous fibrosis* Lal, (1953) and as *submucous fibrosis of the palate and pillars* Joshi, (1953).

The disease is characterised by mucosal rigidity of varying intensity due to the fibroelastic transformation of the juxtaepithelial layer, resulting in progressive inability to open the mouth (Fig 1.3 and 1.4). The underlying muscles and the muscles of mastication can also be affected (El-Labban and Canniff, 1985). In addition, there is involvement of the tongue, pharynx and oesophagus, and patients complain of a generalised burning sensation of the mouth. However a more serious complication of this disease is the risk of the development of oral carcinoma (Murti et al 1985).

1.2. Geographic distribution

Oral submucous fibrosis occurs predominantly among Indians and to a lesser extent in other Asiatic people. The majority of the case reports and epidemiological studies have been with patients born in the Indian subcontinent, or whose families have migrated from this region.

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1.3.Prevalence

Most of the documentation of OSF has been compiled in India. In a series of epidemiological surveys, (Table 1.1) the prevalence rate within India varied from 0% (Singhbhum) to 1.2% (Trivandrum).

It is estimated that about 2.5 million people are probably affected by this disease (Canniff and Harvey, 1981)..

The intra-oral site primarily involved was found to be different depending on the region within India (Bhonsle et al, 1987).

Table 1.1. Prevalence of oral submucous fibrosis in India and elsewhere.

Reference	Country	Total Population Surveyed	Prevalence (%)
India:			
Pindborg, Kalapesi, 1965	Bombay	10,000	0.5
Pindborg, Chawla, 1965	Lucknow	10,000	0.5
Pindborg, Bhatt, 1966	Bangalore	10,000	0.18
Zachariah, Mathew, 1966	Trivandrum	5,000	1.2
Pindborg, Mehta, 1968	Eknakulam	10,287	0.4
	Srikakulam	10,169	0.04
	Bhavnagar	10,071	0.2
	Singhbhum	10,048	0
Mehta, Gupta, 1972	Pune	101,761	0.03
Wahi, Mital, 1970	Agra	7,286	0.6
Bhonsle, Murti, 1976	Goa	5,449	0.04
Shear, Lemmer, 1967	South Africa	1,000	0.5
Dockrat and Shear, 1969	South Africa	1,200	0.6
Shiau and Kwan, 1979)	Taiwan	35 cases	-
Canniff, Harvey, 1986	United Kingdom	44 cases	-
Seedat et al, 1988	South Africa	2,058	3.4
van Wyk, Staz, 1977	South Africa	238	1.3

1.4 Incidence

Incidence rates exist only for India, where Gupta (1980) reports an incidence in north-western India of 2.6 and 8.5 per 100,000 per year for males and females respectively, which is less than in the south of India by a factor of about two.

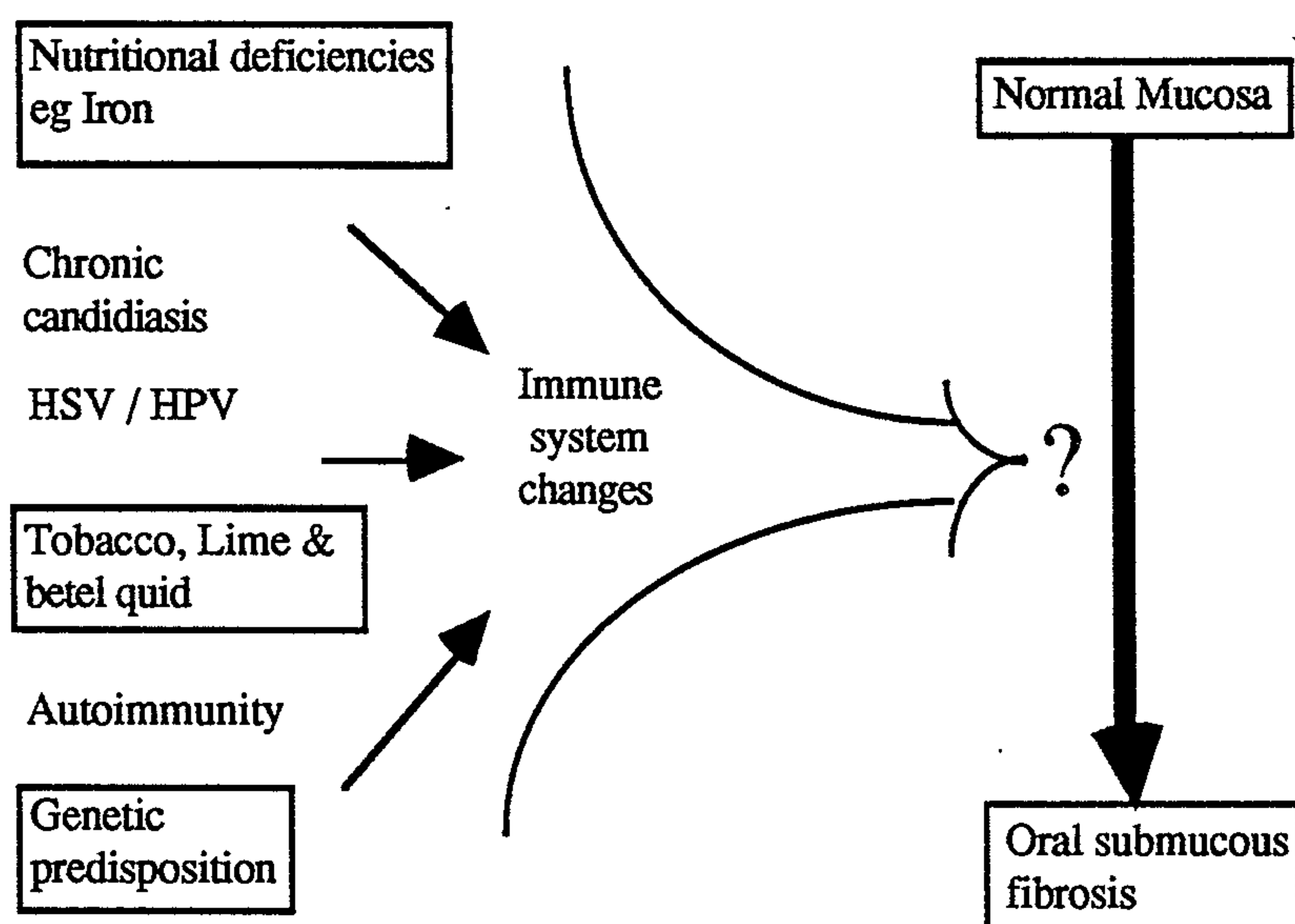
Table 1.2. Annual incidence of oral submucous fibrosis

Reference	Locality	Incidence per 100,000
Pindborg, Bhonsle, 1980	Ernakulam, Kerala	9 males / 20 females
Gupta, Mehta, 1980	Southern India	8 males / 19 females
	North western India	2.6 males / 8.5 females

1.5. Aetiology

A number of theories have been presented as to the development and progression of this disease. Nutritional deficiencies, the use of chillies, and betel nut (*Areca catechu*) chewing have been thought to play a major aetiological role in oral submucous fibrosis.

Figure 1.1. A multifactorial model for the pathogenesis of oral submucous fibrosis



1.5.1. Chillies

One of the main features of this disease is intolerance to spiced foods which frequently form a substantial part of the diet in the Indian Subcontinent.

The possibility that chillies had an aetiological role in the development of OSF was strengthened by the experimental evidence of Sirsat and Khanolkar who after the

application of capsaicin, the active ingredient in chillies, produced a connective tissue response in Wistar rats (Sirsat and Khanolkar, 1960). However, these observations could not be duplicated in subsequent experimental work (Hamner et al, 1974).

Pindborg and Singh, (1964) observed eosinophils in the connective tissue in this condition and considered that this indicated the presence of an allergic reaction. No aetiological role, however, was found in the work of Wahi et al (1966) who compared the amount and the duration of the consumption of chillies between submucous fibrosis and controls.

1.5.2. Nutritional Deficiencies

Malnutrition is a major problem for the inhabitants of most countries where oral submucous fibrosis is found. Deficiencies of vitamins and iron have been implicated as being of aetiological importance in OSF. In a study of 104 cases of OSF by Wahi et al, (1966) poor nutritional status was present in a significantly greater number of patients than amongst controls. In a study undertaken in Malaysia (Ramanathan, 1981), marked iron and vitamin B complex deficiency was observed in 77% of patients with OSF. Yet despite these observations the actual role played by these factors remains unclear as these micronutrients may be deficient in control compatriot subjects without OSF. Obviously these deficiencies also occur in people who are indigenous to Western countries and who do not have OSF.

A study by McLaughlin et al (1988) implied an important role for carotenes and other vitamins in susceptibility to oral and pharyngeal cancer. Deficiency states of vitamin A causes cellular alteration similar to those induced by chemical carcinogens. Marshall et al (1982) found a lower intake of vitamins A and C from foodstuffs in 425 male patients with oral cancer than in 588 controls with non-neoplastic diseases. This

difference was associated with a slightly increased risk for oral cancer even after controlling for alcohol drinking and tobacco smoking.

No intervention studies have been published, however, of the protective effect of micronutrients on the incidence of OSF

1.5.3. Areca nut (Betel nut) chewing

The strongest evidence regarding the aetiology of OSF associates it with the habit of areca nut chewing (Figure 1.2.). Daftary, (1992) was able to bring together the combined results for a total of 405 cases and show that 77 per cent of the patients used the areca nut in some form. This result was based upon the combined studies undertaken by a number of authors (Lal, 1953, Su, 1954, Desa, 1957, Sharan, 1959, Mani and Singh, 1968, Wahi, Mital, 1970, Akbar, 1976, Shiau and Kwan, 1979, Bhonsle, Murti, 1987)

Figure 1.2. Betel nut is sold combined with spices, tobacco, and slaked lime, which is then formed into a quid by wrapping it in a leaf of the Piper betel vine and sold as "pan"



In a study of 100,000 Indian subjects areca nut chewing was practiced by 52% of the patients with OSF compared to 2.1% amongst the total population (Mehta, et al, 1972).

A 10 year prospective study undertaken on 10,000 individuals in India, Gupta, et al (1980), demonstrated a zero incidence of OSF amongst those who did not chew areca nut. The same study showed that among areca nut chewers there was an incidence of 35 per 100,000 per year. (It should be noted that the non-chewing group included heavy smokers).

Experimental evidence is, however, inconclusive with regard to areca nut extracts and OSF. Canniff et al (1981). and Harvey et al (1986) found that an extract of areca nut stimulated collagen synthesis (in vitro), as well as increasing the cross-linkages between the collagen, in *in vitro* studies

1.5.4. Betel nut, tobacco and oral cancer

The most common form of chewing tobacco in Nepal/India is in the betel quid or 'pan', which is a preparation of betel leaf (*Piper betel*), areca nut¹ (often erroneously called betel nut, the fruit of the areca palm tree), tobacco, lime, and catechu, which is a resinous extract from the matrix of the *Acacia* tree. Frequently various condiments (such as cardamom seeds, aniseed or gambir - an extract from the *Areca catechu* plant) are also included in the quid. The practice of betel-quid chewing is widespread in Asia (Daftary, Murti, 1991), particularly in India, Sri Lanka, and Bangladesh. It is also found in parts of Taiwan, Thailand, the Philippines, Vietnam, Indonesia, and among certain ethnic groups (mainly of Indian origin) in Malaysia, Singapore, and eastern and southern Africa. Betel-quid chewing without tobacco is a habit of antiquity and was widespread in the Orient. Tobacco became a part of the betel quid in the seventeenth century and its inclusion has now become socially acceptable in many societies (Daftary, Murti, 1991).

¹ The pharmacological effects of the areca nut include addiction, euphoria, counter fatigue, excessive salivation, and tremor attributable to the parasympathetic action of arecoline, the major alkaloid of the nut. Another side effect of the nut is its anthelminthic activity.

The dried ripe seeds of *Areca catechu* contain no less than 0.25% of alkaloids calculated as arecoline.

Volunteers who chewed about 12 nuts in a short time experienced decreased heart-rate and blood pressure, and increased sweating due to the parasympathomimetic action of arecoline. - (Jüptner, 1968)

Table 1.3. Relative and attributable risks for oral cancer (Daftary, Murti, 1991)

Habits	Relative risk	Attributable risk (%)
None	1	-
Betel quid without tobacco	1-4	1
Smoking only	3-6	8
Betel quid + tobacco	8-15	30
Betel quid + smoking	4-25	2
Betel quid + tobacco + smoking	20	50
Total		91

Relative risk is defined as the multiplicative factor that defines the increased risk of developing disease if the factor(s) is present compared to the absence of suspected factors where the factor is unity

Attributable risk for a category of risk factors is defined as the proportion (expressed as percentage) of the disease (oral cancer) in the population to the category of the risk factor under consideration. Attributable risks with the same referent category are additive for mutually exclusive categories of risk factors as in above table.(Gupta, 1975)

Source: World Health Organization, 1984

1.5.5. Other aetiological factors

The nature of immunological changes have been described in OSF. Canniff and Harvey et al, (1986) reported an elevated serum IgG level among 30 patients in England, who were of Indian origin. A number of the characteristics of the disease also suggest an auto-immune aetiology :- the female sex bias, the age of onset, alterations in serum immunoglobulins, the incidence of auto antibodies, and the involvement of the DR locus in the genetic predisposition (Canniff and Harvey, 1981, Phatak, 1984, Canniff, Batchelor, 1985, Adhvaryu, Bhatt, 1986, Rajendran, Sugathan, 1986, Borle and Jagtap, 1987). It is possible, however, that the immunological changes are a secondary epiphenomena resulting from OSF, rather than primarily being responsible for the disease.

1.6 Signs and symptoms of oral submucous fibrosis

Although both sexes are affected by OSF, many of the studies show a predominance of females (Rao, 1962, Pindborg, Mehta, 1968, Gupta, Mehta, 1980). Most patients with this condition are in the 20-40 year age group at the time of initial presentation, however the condition demonstrates regional variability in relation to age range (Pindborg, Mehta, 1968, Gupta, Mehta, 1980)

Oral submucous fibrosis seems to affect some areas within the mouth more frequently than others. The retro molar areas and the buccal mucosa are commonly involved, as are the soft palate, palatal fauces, uvula, tongue and labial mucosa. Many observers state that the disease originates from the posterior part of the oral cavity, and migrates forward to involve the anterior structures (Canniff, Harvey, 1986). However, Bhonsle and Murti et al, (1987) demonstrated that there were regional differences in the presentation of the disease, and postulated that these reflected the chewing habits of those who used the areca nut - whether the areca nut juice and quid are spat out or swallowed. In those geographical regions where the juice and quid were generally spat out the patient had a higher involvement of the anterior sites of the mouth compared with those localities where the fashion was to swallow the juices and quid, where the posterior sites were affected.

A common presenting symptom is a 'burning' sensation when eating and a reduced oral opening, associated with slowness and difficulty in eating. Other symptoms include increased salivation, changed gustatory sensation, dryness of mouth, and nasalised voice.

The earliest clinical sign, indicating the presence of OSF, is blanching of the oral mucosa. (Figs 1.5 and 1.6) This blanching imparts a marble-like appearance to the

oral mucosa, and can be localised, diffuse, or in the form of lace-like network.

Pindborg and Bhonsle et al, (1980) suggests that the blanching represents an early form of the disease and the histological features of these regions are consistent with the very early forms of OSF. Indeed Pindborg and Sirsat suggest that the disease process is frequently present for 2-5 years before the patient becomes aware of obvious symptoms (Pindborg and Sirsat, 1966).

Reduced oral opening is associated with stiffening of the oral mucosa in the form of fibrous bands, in the area affected. All studies report the presence of these fibrous bands, either in the buccal mucosa, the posterior part of the palate, or the labial mucosa. Indeed one of the main criteria for the establishment of OSF is the presence of fibrous bands either in the buccal mucosa, the posterior part of the palate or the labial mucosa (Pindborg, 1989). These fibrous bands run vertically in the buccal mucosa and are initially discrete (Figure 1.3. & 1.4), but gradually broaden and coalesce such that the whole of the involved mucosa becomes firm, and now the tissue assumes a leathery consistency. Involvement of the lips is characterised by the presence of circular fibrous bands around the rima oris. In the palate the bands radiate from the median raphe to the anterior faucial pillars. The faucial pillars become short and thick, and frequently the tonsils appear blanched and atrophic (Figure 1.5 & 1.6). When the soft palate is affected its mobility is affected, the voice becomes nasalised, and the uvula is shrunken and often bud-like. (Figure 1.7)

The data gathered personally in Nepal (Cox,) suggests that the reduction in oral opening would appear to be episodic, and related to periods of pain. When the pain lessens the patients are able to open their mouth 'wide', though usually not to the same degree which existed prior to the painful episode.

The tongue can also be affected in OSF. It becomes quite stiff, and protrusion may be limited. Some observers have noted that the tongue may even be devoid of papillae, though such severe involvement has not been noted in Nepal. The floor of the mouth is blanched and leathery and the gingiva is fibrotic, depigmented and devoid of its normal

stippled appearance. The cases seen personally in Nepal (Cox,) demonstrate a marked reduction of both the buccal and lingual vestibular sulci such that there is no unattached alveolar mucosa. Bhonsle and Murti et al, (1981) also describes the occurrence of petechiae in one-fifth of cases - whereas it was observed, personally, in only a single patient in Nepal.

Figure 1.3. & 1.4. Demonstrate the limited oral opening experienced by many with OSF, together with the intra-oral fibrous bands. (A squamous cell carcinoma was present just distal to the fibrous band in this patient.)



Figure 1.5. & 1.6 The disease presents with the earliest signs of blanching in the region of the anterior region of the fauces



Figure 1.7. Uvula is shrunken and is bud-like, the mucosa also has a mosaic pattern



1.7. Histological features present in oral submucous fibrosis

Oral submucous fibrosis shows characteristic histological features consisting of an atrophic epithelium with juxtaepithelial hyalinization (Daftary, 1992).

1.7.1. Thickness of the epithelium

The results of a study undertaken by Pindborg and Mehta et al (1970), showed that 70% of the biopsies had atrophic epithelium in comparison to that of the controls, about 2% were hyperplastic and the remainder were of normal thickness. The atrophic epithelium had in the majority of cases lost its rete ridges, but the remainder appeared normal. (Quantative morphometric studies of OSF and control epithelial thickness were not undertaken to confirm these findings.)

1.7.2. Type of keratinisation

According to Pindborg and Mehta et al, (1970) the buccal mucosa, which is normally non-keratinised, is often altered in OSF with 26% of biopsies exhibiting orthokeratosis and 22% parakeratosis. The remainder were non-keratinised.

1.7.3. Epithelial dysplasia

Slightly less than a quarter of the specimens had features of epithelial dysplasia (deemed present when two or more of the following were present- irregular epithelial stratification, basal cell hyperplasia, increased number of mitotic figures, any abnormal mitoses, increased nuclear-cytoplasmic ratio, loss of polarity of cells, cellular and nuclear pleomorphism, nuclear hyperchromatism and keratinisation of single cells or cell groups in the prickle cell layer) (Pindborg, Mehta, 1970). This result compares with only 7% in a study undertaken in 1966 (Pindborg and Sirsat, 1966)

It should also be noted that the epithelial dysplasia seen in OSF differs from that found in leukoplakia. The main characteristics of leukoplakias in Indian patients were an increased mitotic activity, hyperchromatism, and basal cell hyperplasia (Mehta, Pindborg, 1969), whereas in OSF basal cell hyperplasia was rare, and irregular epithelial stratification, nuclear pleomorphism, and a pronounced intercellular oedema were the predominant features. (Mehta, Pindborg, 1969), (Pindborg, Mehta, 1970).

Pindborg and Mehta et al (1970), also observed an increased number of mitotic figures, nuclear hyperchromatism, and loss of polarity of cells in tissue affected by OSF. Pindborg et al (1970) emphasised the presence of prominent intercellular oedema, especially in the basal cell layers - this was observed in 75% of epithelial dysplasias. In the biopsies which showed no dysplasia, intercellular oedema was found in 52.5% of cases.

Unfortunately, the histological characteristics for the control population were not presented in this study by Pindborg et al (1970).

1.7.4. Other changes

Other changes were also noted in the work of Pindborg and Mehta et al, (1970), In approximately 20% of biopsies there were 'signet-ring' cells in the epithelium, mainly in the basal layer. However, it should be noted that similar changes can result from nuclear shrinkage as a fixation artefact in normal mucosa.

Another abnormality in the epithelium was the marked reduction of melanin pigment in the basal cell layers when compared with that of the controls. With this pigmentary incontinence the melanin accumulated in the upper part of the lamina propria, presumably in macrophages.

Pindborg and Mehta et al (1970) also noted that a number of biopsies demonstrated signs of lichen planus - presence of 'colloid' bodies in the epithelium, a change now thought to be due to apoptotic keratinocytes, a tendency to formation of pointed ridges surrounded by lymphocytes, and a marked lymphocyte infiltration with a band-like distribution in the lamina propria. However on re-assessment it was decided that the lichen planus was superimposed on a co-existent submucous fibrosis.

1.7.5. Connective tissue changes

The original description of the connective tissue changes in OSF was presented by Sirsat and Pindborg, (1967) and appeared under the categories of 'very early', 'early', 'moderately advanced', and 'advanced'. In comparison to the clinically normal tissue (Fig 1.8), the 'very early' changes were characterised by finely fibrillar collagen, marked oedema, large fibroblasts, and blood vessels which are often dilated and congested. The inflammatory exudate consisted mainly of polymorphs and occasional eosinophils. The 'early' stage (Figure 1.9) was marked by the early signs of hyalinization, the collagen bundles were thickened and there were a moderate number of fibroblasts. At this stage the inflammatory cells were mainly lymphocytes,

eosinophils and plasma cells. In the 'moderately advanced'(Figure 1.10.) and 'advanced' stages there was hyalinization of the connective tissue to a variable extent. There was no oedema and the predominant inflammatory cells were lymphocytes, and plasma cells, and the state of the blood vessels ranged from being normal to being constricted or obliterated.

Figure 1.8. Histological presentation of clinically normal gingiva. (stained H & E, x 130)

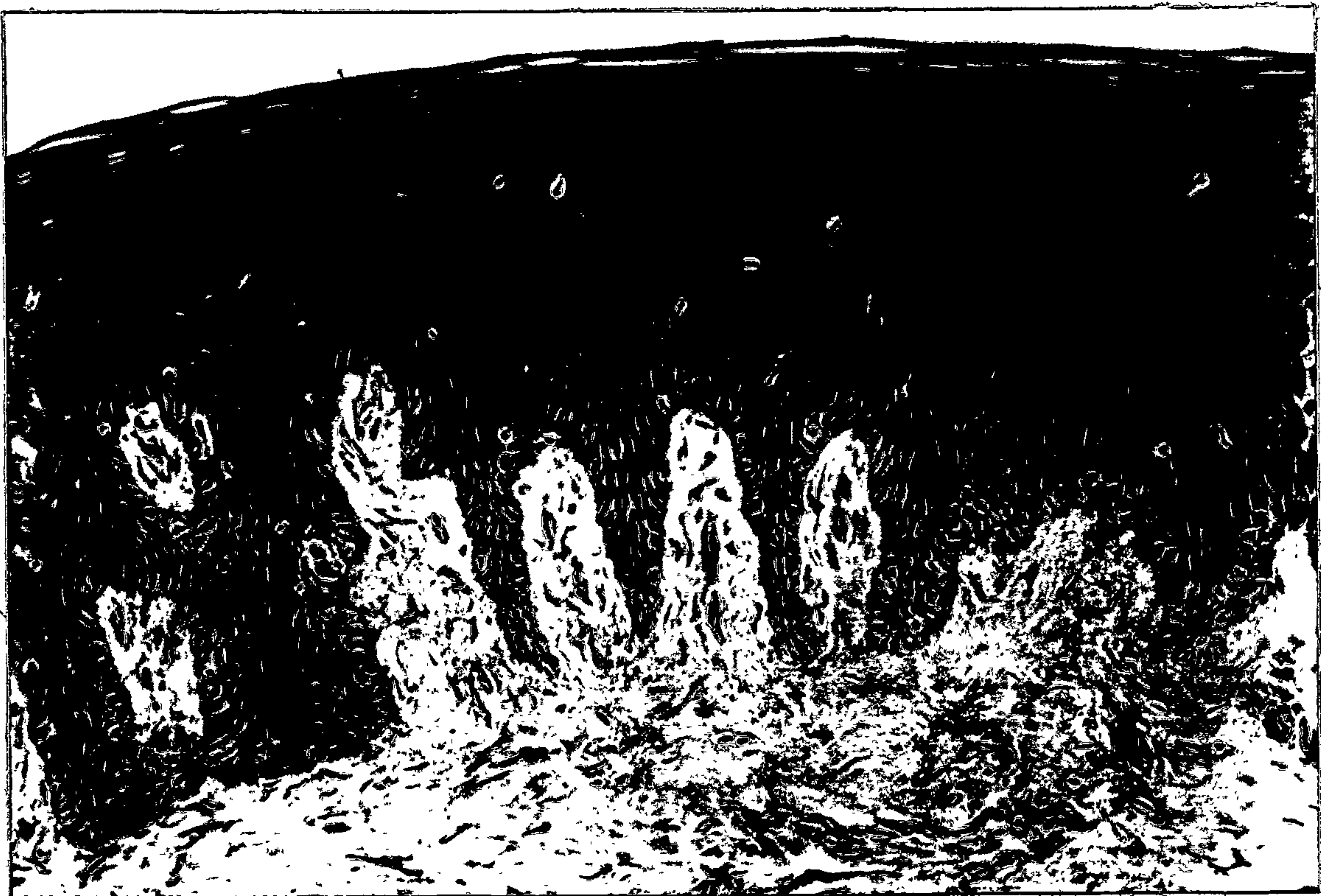


Figure 1.9. 'Early' stage of oral submucous fibrosis demonstrating the early signs of hyalinisation, thickened collagen bundles, moderate number of fibroblasts and an inflammatory exudate consisting mainly of lymphocytes and plasma cells. (stained H & E x 260).

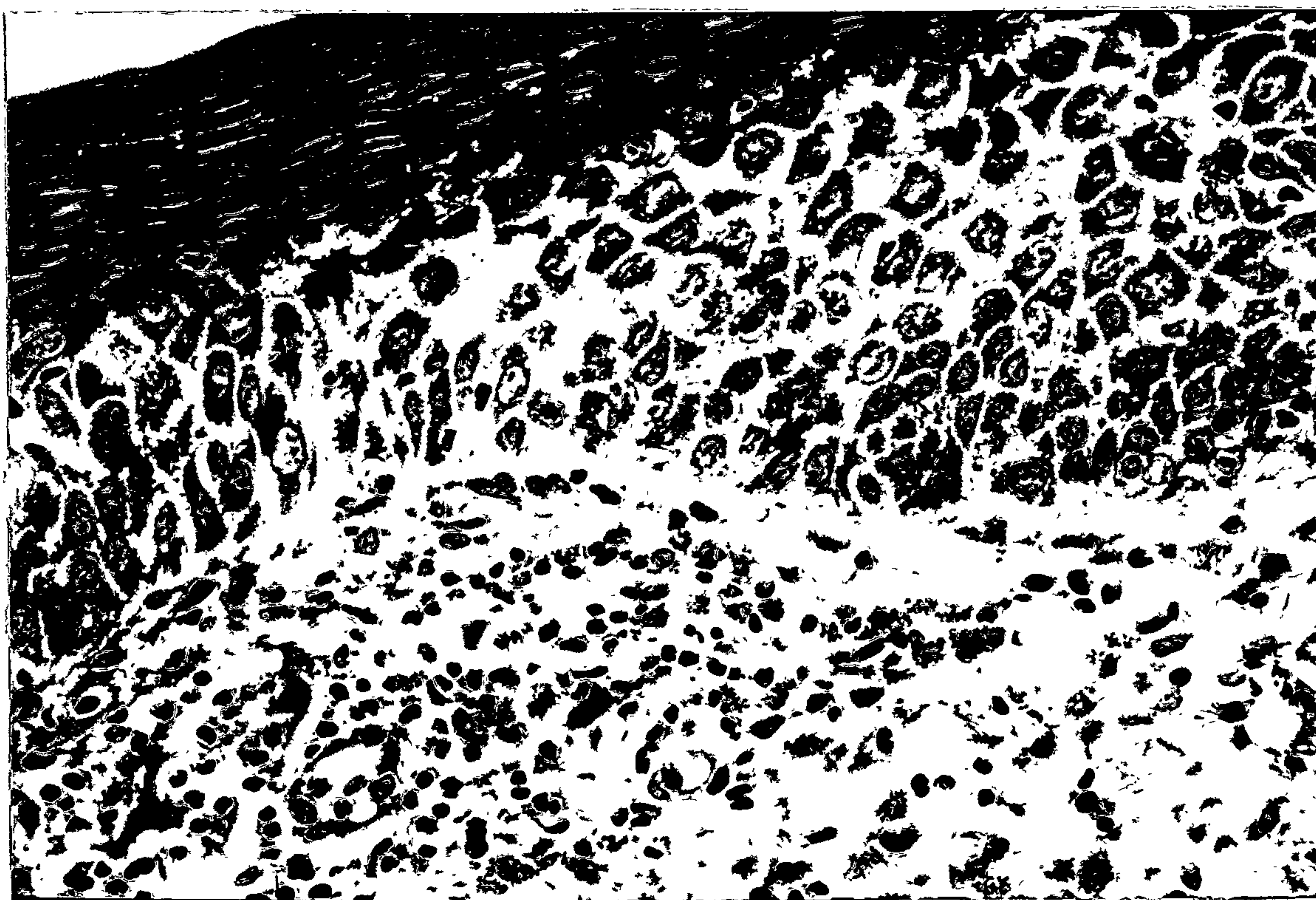


Figure 1.10. 'Moderately advanced' stage of OSF, with little oedema, occasional lymphocyte and plasma cell, and extensive degree of hyalinization of collagen. (stained H & E x 130)



1.7.6. Ultra-structural changes

The collagen in oral submucous fibrosis was reported as normally banded by Sirsat and Khanolkar, (1957). The use of electron microscopy, immunofluorescent microscopy and special staining to detect the presence and distribution of type I and type III collagen allowed van Wyk et al (1990) to conclude that there is an excessive increase of collagen, especially Type I, in OSF, though the fibrils are still morphologically normal.

El-labban and Canniff (1985) reported ultra-structural alterations of muscle fibres in oral submucous fibrosis which they interpreted as degenerative changes. These changes included an amorphous degeneration of the entire cytoplasmic and nuclear structures, while frequently the integrity of the cell membrane was maintained. The authors attributed such changes as being consistent with long standing tissue hypoxia.

1.8. Oral submucous fibrosis and precancer

The precancerous nature of oral submucous fibrosis was originally presented by Paymaster (1956) who noted the presence of squamous cell carcinomas in a third of his patients. However since then there have been a number of figures given as to the incidence of carcinomatous change in patients with OSF (Pindborg, 1965, Pindborg and Zachariah, 1965, Pindborg and Sirsat, 1966).

Table 1.4. Incidence and prevalence of squamous cell carcinoma arising from tissue affected by oral submucous fibrosis.

Reference	Locality	Study group	Prevalence of oral carcinoma (%)	Transformation rate
Paymaster, 1956	Bombay	650	33	NA
Pindborg & Zachariah, 1965	Kerala	100	40	NA
Pindborg and Sirsat, 1966	Lucknow, Bombay & Trivandrum	118	60	NA
Pindborg, Murti, 1984	Ernakulam, Kerala	89	10	3/66 median 8 yrs
Murti, Bhonsle, 1985	Ernakulam, Kerala	66	5.2	4.5 % over 10 yrs / 7.6% over 17 yrs

The criteria in support of the precancerous nature of OSF has been summarised by Pindborg, (1972)

1. Higher prevalence of leukoplakia among OSF patients;
2. High frequency of epithelial dysplasia;
3. Histological diagnosis of carcinoma without the clinical signs of carcinoma;
4. Concurrent finding of OSF among patients with oral cancer;
5. Incidence of oral cancer among patients with OSF.

In a population based prospective study undertaken by Murti and Bhonsle et al (1985) oral carcinoma developed in patients with OSF in 7.6 per cent of cases over a period of 17 years (median 10 years).

The malignant potential and origin of the cancers, being in diverse intraoral locations, in this condition is attributed to the generalised epithelial atrophy present in OSF.

1.9. Management

The aetiology of oral submucous fibrosis is uncertain and therefore the treatment is aimed at ameliorating the discomforting symptoms, reducing the fibrosis and improving oral opening. Basically prevention of OSF is more effective than treatment.

Several medical and surgical approaches have been tried for the management of the condition. Local injections, as well as systemic medication, in an attempt to breakdown the collagen and hyalinized material have been trialed, without satisfactory results (Su, 1954, Desa, 1957, Rao, 1962).

Surgical management to improve the opening of the mouth by merely cutting the fibrous bands may make matters worse. However, split thickness skin grafting following bilateral temporalis myotomy or coronoidectomy seems to be effective (Canniff, Harvey, 1986) as are naso-labial flaps (Kavarana and Bhathena, 1987).

(B) Clinical Study

1.10 Oral submucous fibrosis

Patients who demonstrated the clinical signs of oral submucous fibrosis (see section 1.6.), were included in this study. All patients were resident in the countries of Nepal or India. All the patients from Nepal were seen personally, and had complete clinical notes.

Patients who presented with either the signs and/or symptoms of OSF were recorded, and personal details were taken (see Appendix for copy of 'Record Sheet'). A record was made of any associated abnormality detected clinically and whether the buccal vestibules had reduced sulcus depth. Extra-oral photographs were taken, and where oral opening permitted, intra-oral were also taken.

The study was made up of 20 patients who suffered from OSF, and out of this group full clinical data was available for 13 patients (Table 1.5)

Table 1.5. Biographical data on subjects with oral submucous fibrosis.

Subject	Sex	Age	Oral opening (mm)	Area affected	Habit
1	F	36	22	Generalised	Nil
2	F	53	40	Generalised	Nil
3	M	30	30	Generalised	Nil
4	M	30	27	Generalised	Sup + cig
5	F	25	45	L. retro-molar region	Pan
6	F	22	19	Banding buccal mucosa & lower lip	Sup
7*	M	60	30	Generalised	Cig
8	F	51	40	L buccal mucosa & tongue	Sup
9	F	28	32	Soft palate	Sup
10	F	32	23	Generalised	Sup
11	M	43	62	L buccal mucosa	Cig
12	M	26	18	Generalised	Sup + Tobacco
13	F	55	35	Buccal mucosa & tongue	Pan + Sup

* This subject had a squamous cell carcinoma of the right buccal mucosa.
 Supari is the Hindi word for the betel nut - (areca nut)
 Pan (areca nut , spices & tobacco)
 Cigarettes
 Left
 Chewing tobacco

1.11. Signs and symptoms

The signs and symptoms in this group were quite characteristic and have been described previously. The clinical features in the OSF patients in the study are described and illustrated in section 1.6.

1.12. Limited mouth opening associated with oral submucous fibrosis.

A common feature of subjects with OSF is the intra-oral fibrous banding, with associated reduction in oral opening.

All clinicians dealing with the oral cavity are aware of the difficulty experienced when mouth opening is limited. Many situations can lead to a compromised oral opening and those frequently encountered are trauma, infection and tumours. Although limited oral opening is a frequent feature of clinical practice, very few investigators have actually dealt with the subject of 'normal oral opening'.

Agerberg (1974) determined that the mean maximal vertical opening of the mandible in 20 year old healthy men was 58.6 mm with a range of 44 -77 mm, whereas for women of the same age it was 53.3 mm with a range of 42 -75 mm. Mezitis and Rallis et al (1989) examined a Greek community, ages 18-70 years, and determined that the mean value and range of maximal mouth opening was 52.85 ± 7.41 mm (38.74 to 67.27 mm) for men and 48.34 ± 5.64 mm (36.67 to 60.45 mm) for women. The greatest mean maximal opening was recorded in younger ages (18-30) both in men (56.6 mm) and women (51.04 mm) whereas the lowest was recorded in men aged 51-60 years (47.58 mm) and women 61-70 years (44.45 mm). Maximal opening of the mouth has been described either as the interincisal distance at maximal mouth opening or as the interincisal distance plus the overbite (Wood and Branco, 1979). The Maxillofacial Surgery Clinic in Zurich accepts 36-38 mm incisal distance as the minimal limit for adults (Landtwing, 1978).

Oral submucous fibrosis is clinically characterised by the reduction of inter-incisal distance on maximal opening and the presence of intra-oral fibrous bands and reduction

of oral opening. An objective measurement of the oral opening may be a simple useful screening procedure for particular diseases, such as OSF, in regions where it is known to be prevalent.

The average range of oral opening for people in Asia has not been previously documented. Most studies have been undertaken in populations in Europe and North America, and have utilised the inter-incisal distances. Thus, a preliminary study to ascertain the normal range for inter-incisal distance on maximal opening for the population of Nepal.

1.12.1. Determining 'normal' Oral Opening

Over a six month period, a survey was undertaken at Patan Hospital Kathmandu, to determine the normal range of oral opening as determined by the inter-incisal distance. The survey was conducted on the relatives of patients who attended the paediatric outpatient clinic at the hospital, so as to exclude any natural bias that may be expected amongst patients who are feeling unwell, or who were attending for dental treatment.

1.12.2. Materials and methods

The maximal mouth opening in subjects, of the age range of 18yrs to 70 years, was studied for both sexes from various parts of Nepal. These subjects gave no history of current infection, trauma, or tumour and had no obvious congenital facial anomaly. All subjects were dentate.

The study consisted of 700 subjects (389 males and 311 females). The distance between the incisal edges of the maxillary and mandibular central incisors was measured with the mouth opened maximally using a Vernier calliper. The overbite was not included. Two measurements were made for each subject and the mean value of the two measurements was recorded. Normally the second measurement was greater by an average of 2.5 mm.

If subjects were edentulous, or both central incisors were absent in either the maxilla or mandible, then these subjects were excluded from the survey.

1.12.3. Results

The results of the survey undertaken amongst the relatives and friends of patients attending Patan Hospital, Kathmandu, are presented in Table 1.6.

Table 1.6. The range of mouth opening and mean values of maximal opening in relation to age and sex.

Age (yr)	Mouth opening (mm)					
	Men			Women		
	No.	Range	Mean	No.	Range	Mean
15-19	58	36.0-58.0	49.8	56	38.0-58.0	49.6
20-29	165	34.0-61.0	51.1	101	38.0-64.5	49.0
30-39	89	28.0-64.0	49.9	74	37.0-64.0	49.0
40-49	54	39.0-57.5	47.3	48	37.0-57.0	46.3
50-59	15	37.0-57.0	43.7	25	37.0-57.0	44.3
60-69	8	41.5-58.0	50.6	7	41.5-52.0	48.5
Total	389		46.8	311		47.3

Many factors can influence mouth opening, and there are various groups which may have unique characteristics such as children, old people, patients with maxillofacial injuries, or oral surgery. However the results presented in Table 1.6. and Fig 1.11 demonstrate similarity to the results of studies in Europe and elsewhere.

Figure 1.11. Distribution of number of people demonstrating various inter-incisal distances for maximal oral opening.

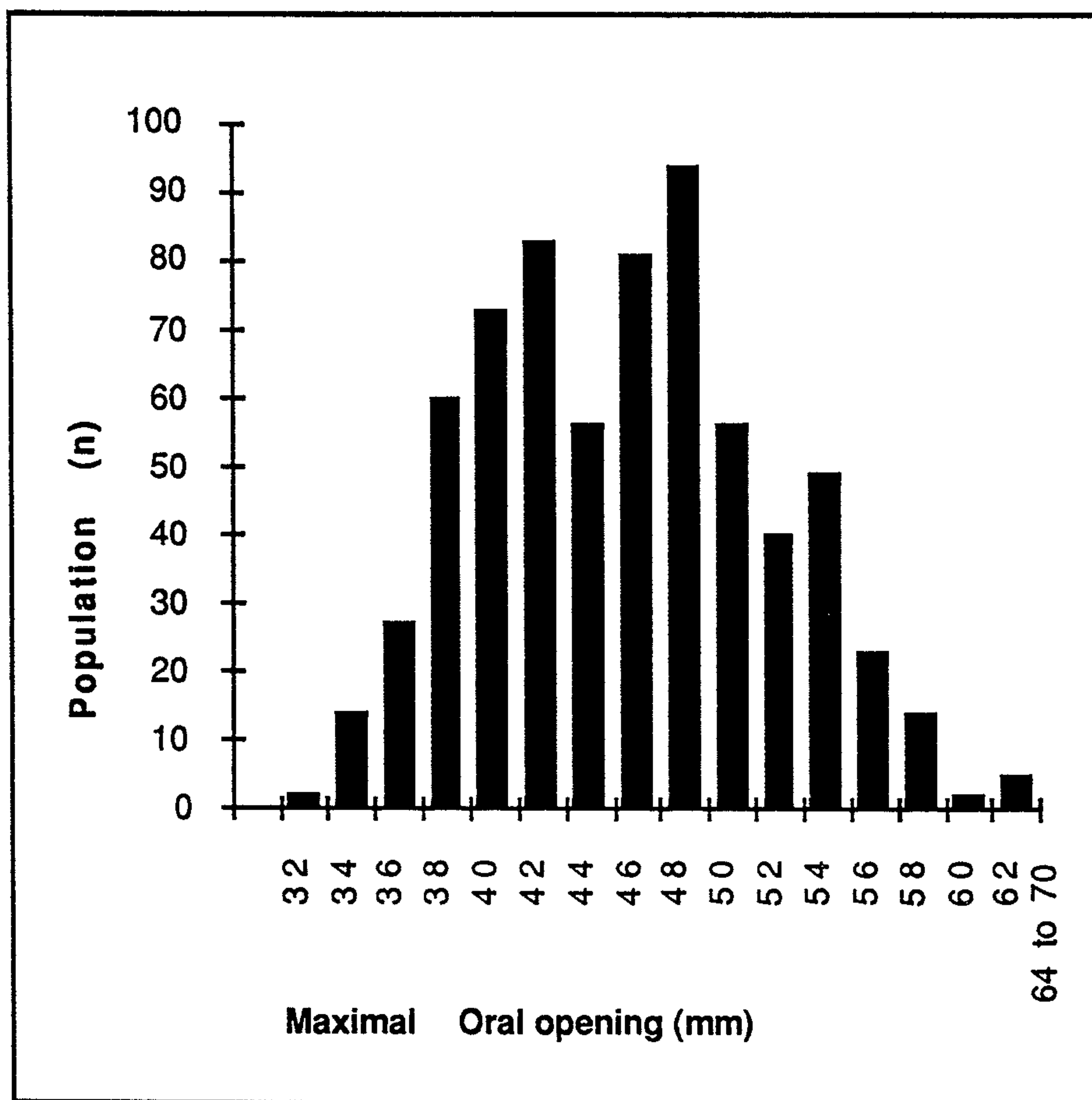


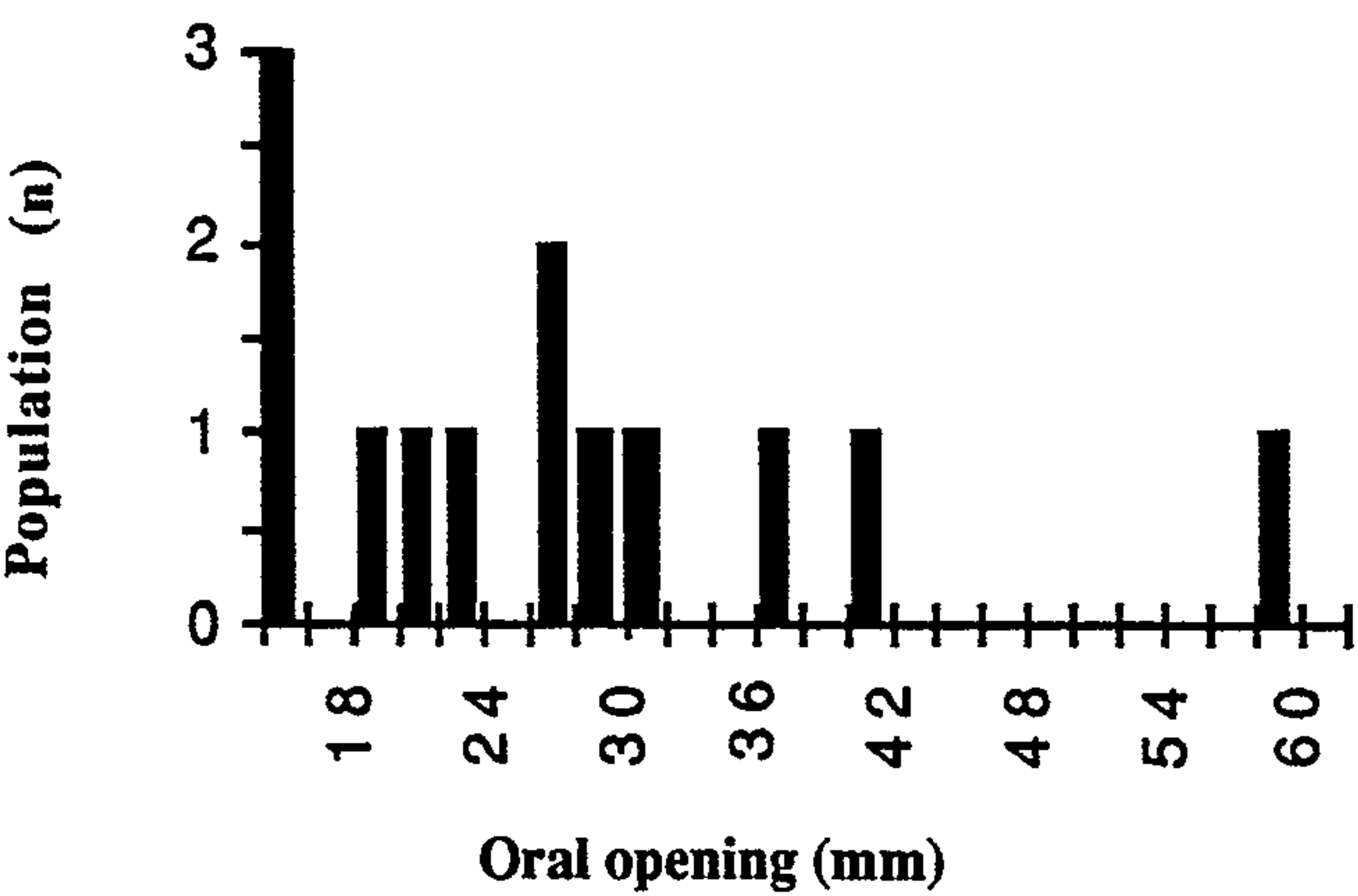
Figure 1.11 demonstrates an unusual 'dip' in the region of 44mm , however consultation with a statistician determined that although this was unusual it was probably due to sampling error, and that the graph should be treated as representing a normal distribution in the population. Therefore the mean opening $\pm$ standard deviation was 47.1 ± 6.7 mm. From this, the mean and normal range of opening (± 2 SD) for the people in Nepal, measured as the inter-incisal distance, was taken as 47.1 (range 33.7 - 60.4 mm) and 98% of the population fell within this range (as determined by the measuring calliper).

Those subjects whose maximal opening was less than 36 mm amounted to 2.2% of the total (15/700) and these were offered followup services in the hospital. Three subjects took up this offer and two were subsequently diagnosed to have OSF, which was confirmed by histology, and the third was diagnosed as having had an early pericoronitis, associated with a lower impacted lower third molar. All three patients were excluded from the survey.

In the light of the above study and the similarity of the data to that determined amongst other populations, the figure of 34-36 mm was taken as the minimal limit for adults in Nepal. This would appear to coincide with the recommendation of the Zurich maxillofacial surgery clinic (Landtwing, 1978)

Figure 1.12. demonstrates the values for maximal oral opening for patients suffering from oral submucous fibrosis. The graph demonstrates the percentage of patients who had a certain oral opening, and all demonstrated the clinical signs of oral submucous fibrosis.

Figure 1.12. Oral opening of patients suffering from oral submucous fibrosis.



Although the sample of OSF patients is small ($n=13$), it is evident that the distribution of oral opening for these patients is quite different to the normal controls.

All measurements were undertaken by auxiliary dental staff who had no particular diagnostic skills, but were trained to carry out the measurements.

The data was analysed to determine whether the maximal oral opening could be used as an objective means of screening a population for oral submucous fibrosis. It was determined that 4 (31%) patients with OSF fell inside the normal range. This meant that the sensitivity of this "test" was quite low at 62%, while the specificity was high at 97%.

All OSF patients with advanced disease had an associated limited oral opening. However, the test was unable to identify those patients with the early stages of the disease.

Comment : The present study would have been improved had the author personally examined all the subjects, to determine the state of the oral cavity, after the oral opening measurements had been undertaken by the staff.

Chapter 2

Epithelial changes in Health and Disease

2.1. Introduction

The overall survival rate for intra-oral cancer (all stages) has not improved significantly in the past 30 years, due probably to an increased proportion of advanced (T3 and T4) cancers (Stell and McCormic, 1985)

Prompt treatment of premalignant oral lesions, has been shown to greatly reduce, or eliminate, the risk of oral carcinoma (Banoczy and Csiba, 1976). However, the assessment of the risk of malignant transformation of oral epithelial lesions by the pathologist has a subjective element and an alternative quantitative method for assessing the malignant potential of oral lesions is needed.

An increased state of cell proliferation is a fundamental change in neoplasia, and can be related prognostically to tumour behaviour (Costa et al, 1981; Meyer et al, 1983), and an increase in mitotic activity is a feature of oral epithelial dysplasia (Kramer et al, 1978).

2.2. Cell Type

The surface epithelium functions as a barrier for many organisms and irritants, and protects the underlying connective tissue. The major cell type in stratified squamous epithelium, making up 95% of the total cell population, is the keratinocyte. It is therefore, the keratinocyte which is placed at risk from a range of agents within the mouth, and the epithelium partially compensates for this risk, by constantly renewing itself and continually desquamating its superficial cells

The keratinocyte moves progressively from attachment to the epidermal basement membrane towards the surface, forming well-defined layers during its transit. The process of terminal differentiation, which is commonly termed keratinisation, is a result of numerous and extensive processes. Specific factors regulate these processes, and involves the transformation of a 'keratinocyte' into a 'corneocyte' (which is not merely a change in keratin expression).

2.3 Kinetics of the epidermis-

2.3.1. Early concepts

It was originally asserted that all cells in the basal layer of epithelium were capable of division and that they were homogeneous with roughly equal cell cycle times. It was also assumed that their removal to a suprabasal position was not only the result of random processes, but was the direct result of an increased basal 'pressure' induced during cell division (Leblond et al, 1964).

2.3.2. Stem cells

The epithelium is a tissue which is capable of permanently renewing the cell population (capable of rapid and continuous cell turnover) (Hall and Watt, 1989). This involves the presence of at least two distinct cell populations: the terminally differentiated cells which have a short lifespan and are replaced through the proliferation of the second subpopulation of cells known as stem cells (Hall and Watt, 1989).

Stem cell populations throughout the body demonstrate three features in common.

First, stem cells have the capacity for unlimited sequential renewal, in the context of the lifespan of the organism. Second, they have the potential for asymmetric division, such that one daughter is itself a stem cell, while the other is committed to undergo terminal differentiation. Third, the differentiation process is irreversible since the daughters of a cell that is committed to terminal differentiation are never stem cells (Hume, 1985, Hall and Watt, 1989)

In epithelial systems one daughter of each stem-cell division will enter the maturation pathway where it will enter a limited number of amplification divisions (probably 3-4) before becoming post-mitotic and eventually being shed or desquamating. The other daughter remains a stem cell. As to which of the two cells is removed for maturation depends on whether this process is stochastic (both cells being equivalent: symmetrical division), or whether this process is a programmed removal of them (the cells not being equivalent: asymmetric division). The latter may result through positional or micro environmental factors acting on the former (Hume, 1985)

2.3.3. Number and site

The epidermis comprises multiple layers, and the deepest layer, which is adherent to a basement membrane, contains most of the stem cells (Hall and Watt, 1989). They are actually present in both the basal and supra-basal layers of the human epidermis-

Penneys et al, (1970) and others (Van Nest et al, 1983, Dover and Watt, 1987) found that 32% of all cells undergoing DNA synthesis were above the basal layer. In order for the epidermis to remain a constant thickness, in normal tissue, the rates of cell production and cell loss must be equal. (The incorporation of tritiated thymidine into cellular DNA has allowed some approximations to be made regarding the cell cycling time for various tissues. It has been shown that in hyperproliferative diseases such as psoriasis the number of cells identified as being in 'cell cycle' will be far greater than that found in normal tissue (Ebling et al, 1992).)

A number of observations suggest that not all the keratinocytes that are capable of dividing are stem cells - perhaps only 10% are stem cells and a further 50% of basal cells that are capable of dividing may constitute a transient amplifying population (progenitor) with the remaining 40% of basal cells presumably being post mitotic and committed to terminal differentiation (Potten and Morris, 1988).

2.3.4. Position of stem cells

Loe et al (1972) demonstrated that mitotic figures occur mainly toward the base of the connective tissue rete ridges in rat gingival epithelium. This has been substantiated in a number of other studies both in rats and humans (Lavker and Sun, 1983, Kellett et al, 1989), and Seddon et al (1992) have postulated that the stem cell lies at the base of the rete ridge.

2.4. Definitions

Cell Populations

The oral mucosa is covered by a stratified squamous epithelium comprised of three types of cells, grouped according to the various functions they perform. There are the progenitor cells or stem cells (basal and parabasal cells) which are the site of cell division; the second group is made up of maturing cells - where cells are undergoing more terminal differentiation (spinous and granular layer): and a superficial group which are mainly cornified squames and make up the superficial keratinised region (either orthokeratotic or parakeratotic). In non-keratinised regions of the mouth (floor of mouth and buccal mucosa) overt and granular cells are absent and the superficial cells are flattened with elongated nuclei (Scully, 1992).

Differentiation

This is a qualitative and relative term used to describe a change in the characteristics of a cell or group of cells. It represents a change in the pattern of gene expression, ie a reprogramming of the genome or a change in the balance of activated and repressed genes (Potten, 1992).

Stem cells

They are the cells ultimately responsible for all cell replacement in the tissue for the life-span of the individual, inspite of physiological or accidental cell loss (Potten and Loeffler, 1990) They may be capable of producing a range of differentiated cell lines and , because of this large division potential, are likely to be the most efficient tissue regenerators (Potten, 1992).

Transit cells

These are relatively short-lived cells produced by a stem cell compartment (Fig. 2.1.). They may, or may not, possess a limited division potential. They are not self-maintaining, being dependent for their replacement on the stem cells (Potten, 1992).

They usually mature and differentiate further as they pass through their life span. Their division capacity would seem to be limited to about 12 cell cycles at most, and in many cases considerably fewer.

The division of transit cells as they mature increases, or amplifies, the cell output from the stem cell compartment and hence these divisions are sometimes referred to as amplifying cell divisions (Fig 2.1). Three transit divisions amplify eightfold and ten divisions 1024-fold. Transit cells are derived from stem cells and may undergo amplifying cell divisions before maturing to become post-mitotic maturing cells incapable of further division.

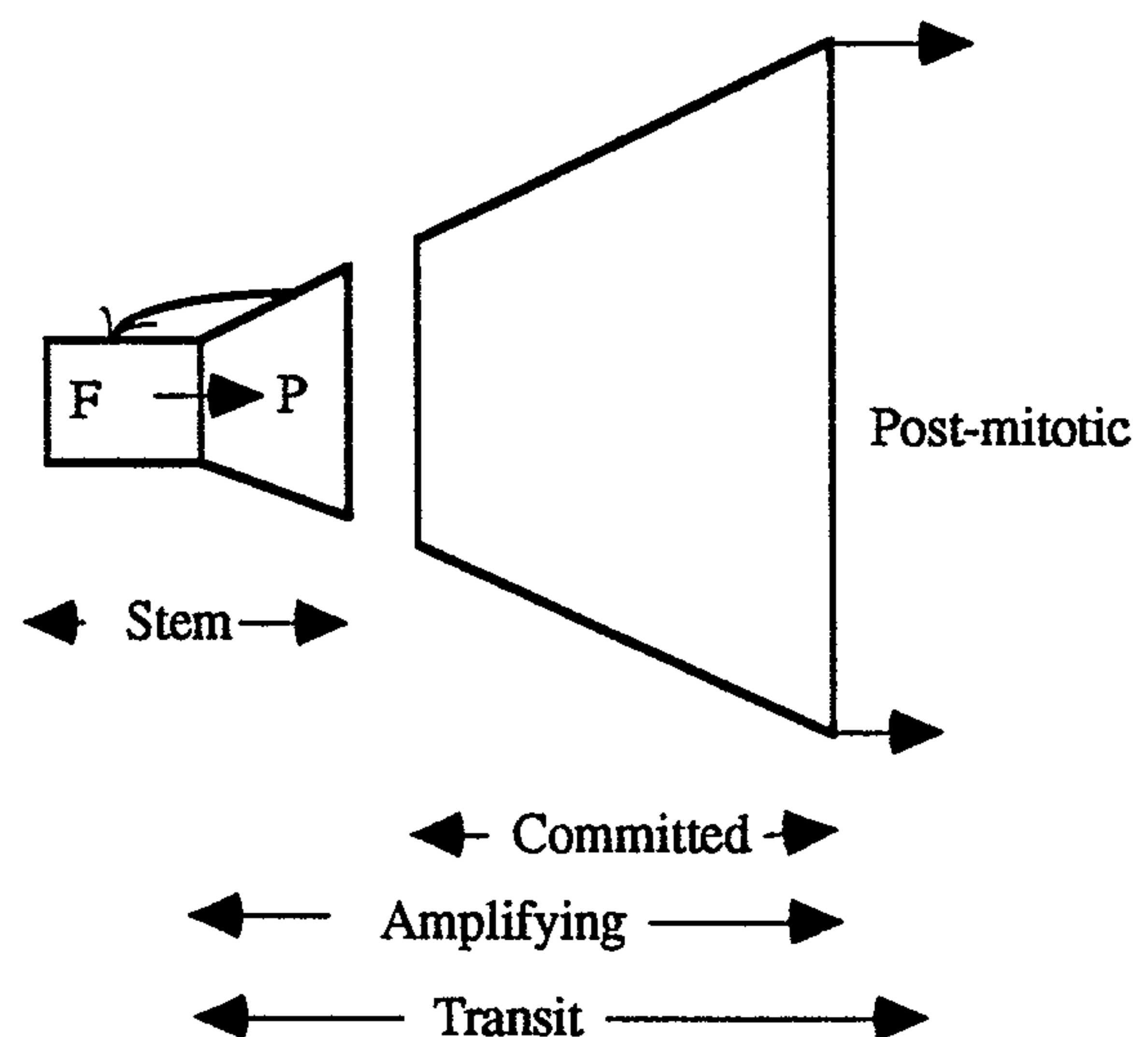
2.5. Epidermal turnover rate

The maintenance of a constant thickness of tissue requires the balance of cell loss against cell production. This is most evident in cases where there is a greatly increased number of cells involved in mitosis, for there is an associated reduction in cell cycle time, and a reduced epidermal turnover time. It is interesting to note that this observation is true over a range of species and pathological conditions.

In most renewing cell populations, the rate of stem cell turnover is lower than for the rest of the proliferative compartment (Potten, 1974). One of the reasons for this slow turnover of the stem cells may lie in their spending prolonged periods of time in a G_0 -like phase of the cell cycle to allow for appropriate "genetic housekeeping" (Lajtha, 1979). This would minimise the risk of errors in DNA sequences being passed to the amplifying cells (and eventually to all the progeny of the stem cells) (Hume, 1985). The experimental data of Chan and Little (1981) indicate that if cells grown to confluence are allowed a 'resting' period, before being treated with a carcinogen, their transformation rate is reduced. Thus it is not only the history of the cell following exposure to carcinogen, but its proliferative history prior to exposure which is important (Hume, 1985).

Figure 2.1. adapted from Hume, 1985 is a schematic representation of the organisation of the stem cell / daughter cell unit. Under normal or steady state conditions the daughter of a functional stem cell (F) undergoes a limited number of amplification divisions before becoming post-mitotic. All suprabasal and basal cells, except for the functional stem cell, are on the transit pathway and are destined to be lost. If this stem cell is killed, there is evidence that the youngest amplifying cells can take up the role of the functional stem cell and even acquire its characteristic features. Hence they can be considered to be potential stem cells (P). This potential is represented by the arrow passing from the F to the P compartments. The older amplifying cells are irreversibly committed to maturation and cannot act as potential stem cells during regeneration.

Further, Hume in 1985 makes the comment that the classical two-stage carcinogenesis experiments in mouse skin



suggests that: (1) initiation is irreversible; and (2) the initiated cell must be long-lived (as a delay in promotion, up to an interval of one year, can still produce neoplasms).

The findings of Fearon and Vogelstein (1990), have been able both to support this observation, and suggest an hypothesis to explain it.

The hierarchical scheme of proliferation shown in figure 2.1. implies that the stem cell, being the only long-term resident in the epithelium, is the important target cell population during initiation.

During tissue regeneration or epidermal diseases which stimulate the cell cycle, it is probable that there are periods when the stem cells have a shorter cycle time. At these times it might not differ from that of their progeny. A consequence would however, be

the diminished time available for 'genetic housekeeping'. Eventually the number of stem cells of "poorer" quality, having spent little time in a G₀-like phase, would increase - possibly to the detriment of the tissue and the organism. One way of removing this threat would be if the probability of the cells being forced on to the maturation pathway were inversely proportional to the time spent in G₀ (Hume, 1985).

2.6. Regulation of epidermal thickness

The rate of production and loss of cells must be controlled to maintain a state of equilibrium. The control mechanism consists of a balance of stimulatory and inhibitory signals.

Some of these control mechanisms have been identified and are in the form of diffusible factors such as cytokines or growth factors. They have different effects on different cell types and the net effect in a complex tissue such as the oral epithelium depends on cellular interactions mediated by complex balances or cascades of *cytokines* and *growth factors*. (Epidermal growth factor has been shown to be able to induce the production of a proliferating cell nuclear antigen (PCNA) (Jaskulski et al 1988), an essential element in the synthesis of DNA. In addition interleukin 2 has also been demonstrated to induce the production of PCNA (Moore et al 1987).

2.7. Growth Fraction (Proliferation state) - a histological marker in health and disease.

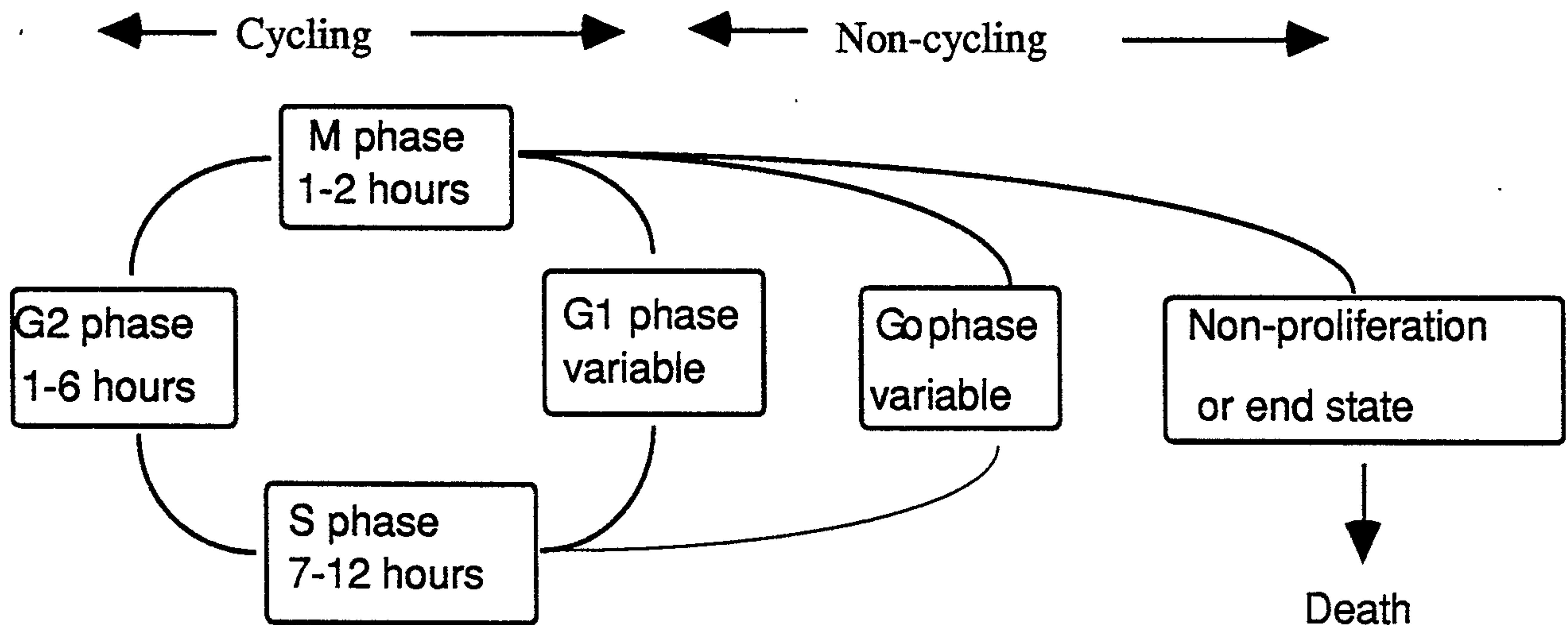
2.7.1. Introduction

One of the frequent changes in the histological appearances of tumours, compared with those of normal tissue, is the increase in the number of mitotic figures. Occasionally bizarre mitotic forms can be observed, though more frequently the tumours have a large number of 'normal' figures which is suggestive of a rapid rate of cell division with an associated increase in tumour size.

2.7.2. Review of the cell cycle

The concept of the cell cycle and its subdivision into a number of phases arose out of the observations of Howard and Pelc in the early 1950's (Howard and Pelc, 1951). The cell cycle is comprised of four main phases designated G₁, S, G₂, and M. The synthesis of DNA and doubling of genetic material take place during the synthetic or S phase, and the phase preceding this is made up of a period of variable length and is known as the gap (G₁) phase because it separates the S phase from the preceding mitosis (M phase). A second gap phase (G₂) follows the S phase and this is marked by apparent inactivity in the cell prior to the next mitosis. "Interphase" comprises successive G₁, S, and G₂ phases and forms the largest part of the cell cycle.

Figure 2.2.: The cell cycle, adapted from Wright (1984). It should be remembered that there may be considerable variation in the duration of phases of the cell cycle within and between tissues, both normal and neoplastic.



In any tissue there are two populations of cells present at any one time, those involved with mitosis and those which can no longer divide. These two types of cells are termed 'cycling' and 'non-cycling' cells. This allows for the concept of the **growth fraction** (proliferative fraction) of any cellular population, which is the ratio of cycling to the total number of cells.

While various cell and animal models demonstrate that cellular proliferation is controlled at a molecular level via a cascade of regulatory genes (Dunphy and Newport, 1988, Lee and Nurse, 1988) (ref 2.6.), the molecular control of mitoses will not be examined any further here. Instead this study has attempted to define and discern the growth fraction of clinically normal oral mucosa.

2.8. Growth Fraction - methods of assessment

Various methods have been devised to allow the determination of the tissue growth fraction at the time of biopsy. This refers to those cells which are actually part of the cycle from G₁ to M phase (Fig2.2), and does not refer to the rate at which the cells enter the cycle, nor how long they remain there. Hence the 'growth fraction' only reflects the state of the tissue at biopsy.

2.8.1 Mitosis

The only phase of the cell cycle which can be recognised by simple morphological examination is the mitotic phase. However there are several potential problems associated with using this phase to assess the growth fraction (Hall and Levison, 1990):-

1. Identification of mitoses can be very difficult.
2. The process of mitosis in inadequately fixed material will go on to completion, while no new mitosis will be initiated, leading to an underestimation of the true numbers of mitosis and apparent regional variation

2.8.2. DNA content markers

Another method of determining the growth fraction of tissue is to take advantage of the changes which occur during the cell cycle. DNA synthesis during the S phase leads to a doubling of the genetic information and the cell becomes temporarily tetraploid. This can be measured in several ways.

Thymidine labelling

Thymidine labelling is considered to be the 'gold standard' for cell kinetic studies. This method requires the viable cells to take up tritiated thymidine and for this to be incorporated into DNA during the S phase of the cell. This is then detected via high resolution autoradiography (Hall and Levison, 1990). This method requires fresh tissue. There are, however, problems in labelling tissue in vitro and in vivo studies in humans carry ethical implications.

Flow Cytometry

Information about the number of cells and their DNA content can be obtained from flow cytometry studies. However, the disadvantages of this technique include the need to disrupt tissues so that the spatial relationship of cellular subpopulations of the cells is lost (Hall and Levison, 1990).

Immunohistochemical Methods

The discovery that there are certain proteins expressed at different phases of the cell cycle has offered an important means of assessing cell proliferation. The advantage of this system is that, using specific antibodies, the immunohistological demonstration of cell cycle related antigens also allows spatial orientation to be demonstrated. Perhaps the best known antibody that recognises proliferating cells is Ki67. This identifies a poorly characterised nuclear antigen associated with the cell cycle, being expressed in all phases except G₀ (Gerdes and Schwab et al, 1983; Gerdes and Lemke, et al 1984). A major limitation to the use of Ki67 has been the need for fresh or snap frozen material as the antigen is very sensitive to fixatives and may deteriorate with time even when stored at -20°C. An alternative marker of

cycling cells, which can be identified in formalin fixed and routinely processed material is the proliferating cell nuclear antigen (PCNA)

An important point to consider is that the **proliferation rate** is made up of two parameters: (1) the growth fraction (assessable by immunochemistry) and (2) the time taken for the cell to complete the cell cycle (not assessable by immunochemistry). Thus the expression of a positive product with immunochemistry only provides information about whether a cell is in cycle or not, it does not tell us about the cell cycle length (Brown and Gatter, 1990). It is for this reason that the results of the immunochemical studies will refer to the growth fraction (or growth index) and not the proliferation rate or growth rate.

2.9. Growth Fraction and Neoplasia

The rate at which tumour cells divide tends to exceed that of normal tissue, and the rate of proliferation of a neoplasm may be significant in relation to its clinical course.

Histopathologists have therefore sought means of determining this as an adjunct to diagnosis. The rate at which a tumour or tissue increases in size is determined by the growth fraction (the number of cells in cycle) and the time taken to complete the cell cycle (Wright, 1984), as well as the rate of cell death (apoptosis), or, in the case of surface tumours, cell loss. A number of authors have demonstrated a strong correlation between the proliferative rate and clinical outcome of tumours (Costa et al 1981, Strang, et al, 1987), including those arising in the head and neck are amongst these (Chauvel, Courdi, 1989). Tubiana and Courdi (1989), in a review of cell proliferation kinetics, concluded that there was a strong association between a high proliferation rate of solid tumours and the degree of tumour aggressiveness.

However, Quinn and Wright (1990) criticise the value these indices are to any quantitative study, stating that the interpretation of the results must be limited to the specific variable being measured. They suggest that the use of indices (such as the number of mitoses or the labelling index) as a measure, is of dubious value for it ignores the problem of the lack of homogeneity in any tumour. It is also questionable whether such measurements actually reflect tumour growth rate. Steel (1977) found no relationship between the thymidine labelling index and the volume doubling time for a range of tumours

Yet it may be stated that the very nature of malignant tumours demand that a number of various indices are used to evaluate their progression and metastatic potential. Malignant tumours tend to be heterogenous in biological, enzymatic immunological, antigenic and ultrastructural aspects (Takahashi et al 1991). Therefore knowledge of the growth

fraction may, in addition to other parameters, contribute to a fuller understanding of the tumour which in turn will allow an informed prediction of the progression and the prognosis of the tumour to be made.

2.10. Pilot study - Immunochemical means of determining the 'growth fraction' in 'clinically normal tissue'

2.10.1. Antibodies used in immunochemistry

Ki-67 antibody identifies a nuclear antigen associated with the cell cycle, being expressed in all phases of the cell cycle, except G₀. Studies have shown a close correlation between Ki-67 immuno-reactivity and thymidine labelling, considered to be the "gold standard" for cell kinetic studies (Hall and Levison, 1990), and therefore Ki-67 is considered a satisfactory marker of cell proliferative states.

Murine monoclonal antibodies recognising the proliferating cell nuclear antigen (PCNA) provides a histological method for reliable detection of proliferating cells in formalin fixed, paraffin embedded material (see section 2.10.3)(Garcia et al, 1989, Takahashi et al, 1991) . Satisfactory detection of the growth fraction depends on establishing the optimal conditions for fixation, antibody concentration, and conditions of incubation. These conditions vary between different tissues and have not been established for oral mucosa. This investigation will determine the optimal conditions for PCNA growth fraction estimation for oral mucosa. The results obtained will be compared with those from Ki-67 antibody labelling, which was used on fresh frozen material (Kamel et al, 1991).

2.10.2. Monoclonal antibody Ki-67

Ki67 is an IgG₁ class monoclonal antibody, raised in Kiel, Germany, in a 67th well of a tissue culture plate, against a crude nuclear fraction of the Hodgkin's disease derived cell line L428.

2.10.2.1 Cell kinetics

The antibody Ki67 recognizes an antigen which is associated with the cell nucleus. As yet the nature and molecular weight of the antigen remains undetermined. Gerdes et al, (1984) have shown that the Ki67 antigen is continuously expressed in all stages of cycling cells, except G₀. The intensity of the staining varies according to the phases of the cell cycle (Verheijen et al, 1989).

It seems also that the expression of Ki67 antigen changes with the nutritional state of the cells. It is known that under conditions of nutrient deprivation tumour cells can be arrested in various phases of the cell cycle. This would be significant in considering the growth fraction of tumours (Verheijen et al, 1989).

In 1983, Gerdes et al (1983) showed that the distribution of Ki67 in normal tissues reflected their known cell kinetics. The same workers (Gerdes et al, 1984) subsequently observed that there was a close correlation between the growth fraction of proliferating cells, as measured by autoradiography using tritiated thymidine incorporation, and the number of cells stained by Ki67. Since then several workers have confirmed this (Schwartz et al 1986, Kamel et al, 1989) and also demonstrated a correlation using

other techniques eg bromodeoxyuridine incorporation (Silvestrini et al 1988), and proliferating cell nuclear antigen (PCNA) expression (Kamel et al, 1991)

Over the past 10 years Ki67 has been used as a prognostic indicator in a large number of tumour tissues including lymphoproliferative diseases (Hall et al, 1988, Cibull et al 1989), connective tissue diseases (Costa et al, 1984), breast diseases (Barnard et al, 1987, Cattoretti et al, 1988), and central nervous system tumours (Patsouris et al 1988).

2.10.2.2. Quantitation

Assessment of growth fractions using immunohistochemical means, is frequently followed by manual counting of cells on tissue sections, and this can be very time consuming as many cells must be counted (Hall et al, 1988). There appears to be no absolute answer as to the number of cells that need to be counted, and in fact the more appropriate question to ask is how many cells need to be counted to be able to distinguish confidently between two values? The concept of the cumulative mean (Underwood, 1987) would appear to be a reasonable approach to the problem. Here the nature of the tissue dictates the number of cells to be counted and when the variation of the cumulative means from a tissue specimen is reduced to 3% then this is the figure recorded. (The 3% was chosen arbitrarily, but it should be of a lower order of magnitude than the differences in the mean values between the groups being compared.)

Heterogenicity within a specimen must also be considered, though this is of particular importance in relation to tumours. It may be important to identify the region of intense growth, and there is evidence that stem cells are present in tumours, and these may represent the predominant biologically and therapeutically relevant population. Thus the assessment of the growth fraction in different subpopulations may provide more meaningful information than crude assays such as flow cytometry which attempt to

include all the cells in the sample. Heterogeneity may extend to there being different kinetic indices in tumour samples from different sites (McFarlane et al, 1986).

2.10.3. PCNA / Cyclin

There are numerous functionally inter-related substances that vary in level or activity during the cell cycle, whose functions (whether structural or regulatory) are to permit the initiation and completion of normal cell division. The proliferating cell nuclear antigen (PCNA) is one such substance. It is a 36 Kd, non-histone, nuclear polypeptide that is associated with cell proliferation. It has been observed that the concentration of PCNA reflects the growth state of normal and transformed tissue (Kurki et al 1988, Galand and Degraf, 1989, Hall et al, 1990, Landberg et al, 1990).

PCNA is a very conserved protein and is present throughout mammals, as well as plant cell and yeasts (Almendral et al, 1987, Matsumoto et al, 1987)

2.10.3.1. Identity of the proliferating cell nuclear antigen (PCNA) and 'cyclin'

The 'proliferating cell nuclear antigen' (PCNA) was originally detected during experiments in which serum from patients with systemic lupus erythematosus failed to show immuno reactivity with resting cells, but rather reacted with nuclei of proliferative cells in normal human tissue (germinal centre cells, cortical thymocytes, spermatogonia of testes) as well as mitogenically stimulated peripheral blood lymphocytes and several

cultured cell lines. Miyachi et al (1978) designated this protein proliferating cell nuclear antigen (PCNA).

'Cyclin' is a nuclear protein, identified by its position in a two dimensional separation of cell proteins, which is preferentially synthesised in the S-phase. This protein was discovered independently by Bravo et al (1981) in a search for cell cycle, phase specific proteins

PCNA and 'cyclin' both have an isoelectric point of 4.9 and MW 36,000, and Mathews et al (1984) demonstrated that PCNA and Cyclin are identical. Since that time PCNA/cyclin has been demonstrated to be an auxiliary protein to DNA polymerase delta (Bravo et al, 1987), and its presence appears to be necessary for DNA replication (Jaskulski et al 1988). The structure of the human PCNA/cyclin gene has been determined, and the protein, a 261-amino acid polypeptide with a high content of aspartic and glutamic acids, has been sequenced.

For the purpose of simplicity the protein shall be referred to simply as proliferating cell nuclear antigen or PCNA

2.10.3.2. Function of PCNA in DNA synthesis

PCNA is an auxiliary protein of DNA polymerase delta (Tan et al, 1986) which appears to be a cell-cycle-related protein, coordinating leading and lagging strand synthesis and rendering the polymerase more processive (Prelich and Stillman, 1988).

2.10.3.3. Character of PCNA during cell cycle

Several investigators have shown that the amount of PCNA protein varies during the cell cycle. In particular the distribution of PCNA increases through the G₁ phase, (immediately prior to DNA synthesis phase) of the cell cycle, peaking at the G₁/S phase interphase, decreasing through G₂, and reaching low levels which are virtually undetectable by immunocytochemical methods in M-phase and quiescent cells. Kurki et al 1988 (1988) using flow cytometry, showed that PCNA starts increasing during G₁, peaks in S phase and declines during the G₂/M-phase.

2.10.3.4. PCNA involvement in DNA repair -expression independent of cell division

There is experimental evidence to support the hypothesis that PCNA expression also increases independently of cell division (Celis and Madsen, 1986, Nishida et al, 1988). Celis et al (1986), in a study of human amnion cells after ultraviolet irradiation, observed a significant increase in PCNA levels in cells not in the S-phase of the cell cycle. As polymerase delta may be involved in DNA synthesis associated with repair (Nishida et al 1988, Toschi and Bravo, 1988), it is possible that PCNA also participates in that process. Therefore, elevated PCNA levels may also indicate a higher activity of DNA repair synthesis.

It now appears that PCNA can exist in the nucleus in three different forms

1. Free in the nucleoplasm
2. Present in the pre-synthetic complex
3. Part of the replicative complex

2.10.3.5. Down regulation of PCNA

It has been shown that PCNA is essential for DNA synthesis, in the cell cycle and DNA repair. PCNA itself seems to be under the regulation of the 'product' of the p53 tumour suppressor gene (Mercer et al, 1991), in addition to that of growth factors and interleukin (2.6).

2.10.3.6. Variation of PCNA available to immunohistochemical staining

Different forms of PCNA exist in resting and in cycling cells (Bravo and Macdonald-Bravo, 1987). a soluble form lost on organic solvent fixation and not involved in replication of DNA, and an insoluble form associated with the sites of ongoing DNA synthesis. Consistent with this hypothesis is the observation that the total concentration of PCNA varies at the most by only 2-3 fold during the cell cycle, but there is a greater fraction of PCNA that is insoluble due to chromatin association in S-phase than in other phases of the cell cycle (Morris and Mathews, 1989).

Two dimensional gel electrophoresis of [35S]methionine-labelled cells show that quiescent cells synthesise PCNA at approximately one-tenth the rate of growing cells. In contrast, the immunoblotting analyses demonstrated that the amount of PCNA present 24-48 hours after growth arrest is 30-40% of that present in growing cells (Bravo and Macdonald-Bravo, 1987).

Synthesis of PCNA in quiescent cells is very low and increases 6-7 fold after serum stimulation (Bravo, 1984, Bravo and Macdonald-Bravo, 1984). It has been

demonstrated that an increase in PCNA synthesis occurs 10-12 hours after stimulation of the cell, shortly before DNA synthesis at a time in which the cells become committed to enter the S-phase (Bravo and Macdonald-Bravo, 1984).

2.10.4. Comparison of the use of Ki-67 and PCNA antigen detection for estimation of growth fraction

This study utilizes the monoclonal antibody Ki-67 and PC-10 as probes to detect the presence of two different nuclear antigens which are both reported as being present during the cell cycle.

Thymidine labelling and cell cytometry have been used as the 'gold standard' for the quantitation of proliferating cells. Estimates of proliferating populations within tissues using the Ki-67 antibody agree with data from the thymidine labelling and cell cytometry methods. The present investigation confirms that PCNA can also be used as a reliable marker of proliferating cells. However, the different antibodies define different subpopulations of cells in the cell cycle, owing to the fact that the antigens present themselves at different stages of the cell cycle. Coltrera et al (1991) undertook to compare the growth fraction as indicated by PCNA and Ki-67. In the four cell lines that were examined the PCNA antibody recorded lower levels of positive cells, and showed a mean deviation of up to $\pm 9\%$ (variation was higher in those lines which had a higher growth fraction)

2.11. Materials & Methods

2.11.1. Oral mucosal tissue

The oral epithelium in 31 specimens of clinically normal mucosa was examined to determine the growth fraction, using an immunohistochemical technique. This tissue came from 17 patients who had attended for some form of oral surgical procedure. The patient's age and sex were noted. They were questioned regarding their habits of tobacco smoking and alcohol consumption. If the patient had presented for treatment of a periodontal condition, then the adjacent tissue was examined together with recording of the indices for the gingival index, plaque index, and the pocket depth using a pressure sensitive periodontal probe. In these cases the tissue which was removed was redundant. There were no keratoses of the gingiva in this group. Each patient was personally examined and apart from the chronic periodontal disease the rest of the oral mucosa was clinically normal.

The following periodontal indices were measured in each of the subjects who presented for periodontal surgery:-

1. Greene and Vermilion plaque index (Greene and Vermillion, 1964),
2. Gingival index (Löe and Silness, 1963) , and
3. Periodontal pocket depth (Silness and Löe, 1964)

The intra-observer error for indexes 2 and 3 was estimated to be 0.4%, and the error for the gingival index was 0.2%.

It should be noted that the periodontal department has a strict protocol, which it observes, for surgical patients and therefore all patients had undergone a course of

scaling, polishing and oral hygiene instruction, commencing at least two weeks prior to surgery.

Table 2.1 Subjects presenting with clinically normal mucosa

Sex	No.	Age (SD)	Biopsy site		Oral Habit*
			Posterior	Anterior	
Male	9	37.1 (11.9)	13	10	6 (67)
Female	8	18.3 (3.9)	9	1	3 (38)

* Oral habits included smoking and /or drinking. Smokers averaged 10 cigarettes per day. Patients attending the periodontal clinic were required to cease their smoking prior to surgical correction of their periodontal condition

The specimens from these patients were collected at the time of surgery, and subdivided into two smaller sections, with one half (approximately) snap frozen in liquid nitrogen and stored at -80°C, and the other half fixed in 10% neutral buffered formalin for varying periods of time (from 6 hours to 9 days), and subsequently processed.

2.11.2. Immunohistochemical method for detection of PCNA and Ki-67 antigen

The PC10-murine IgG₂ anti-PCNA monoclonal antibody (Novocastra Newcastle, UK) was used on 4 µm-thick paraffin sections which were dewaxed, taken through alcohol, and then immersed for 10 minutes in methanol with 0.3% hydrogen peroxide to block endogenous peroxidase activity. They were subsequently taken to phosphate-buffered saline (PBS). The optimal period of incubation and the concentration of the antibody was determined in a chequerboard design series of experiments. The detection system used to visualise the site of antibody binding was a biotin-conjugated goat anti-mouse antibody (Tago) used at a dilution of 1:250, and incubated for an hour at room temperature. This was followed by peroxidase conjugated strepavidin (Tago) used at a dilution of 1:4,000 for an hour at room temperature. Diaminobenzidine was used as a chromagen. Each step was followed by thorough washing in tap water, followed by PBS. The sections were lightly counterstained with haematoxylin.

For Ki67 staining cryostat sections 6µm thick were fixed in acetone at room temperature for 10 minutes. Ki-67 antibody (Dako) was used at a dilution of 1:50 and the sections incubated at room temperature for an hour. The remainder of the staining procedure was similar to that described above.

2.11.3. Estimation of the proportion of PCNA positive basal cells in oral epithelium.

All sections which reacted positively for PCNA were assessed twice, with an interval of 6 weeks between each assessment. A feature of this method was the general heterogeneity of the tissue being examined. It was noted that about 700 cells had to be examined before the cumulative mean began to be consistent. The sections which were examined were usually small measuring 1.5cm in length, on average, and there were at least 4 sections per slide and 3 slides were made from each block.

The actual counting was undertaken using a light microscope with a high power lens, (x 260), and the fields examined were chosen at random. As the basal layer was the only region of interest, the length of the basal layer was divided into lengths equivalent to a single 'high-power' field. These 'fields' were numbered and then identified as either odd or even. Once a section had been randomly selected for counting, then the sequence of whether counting would commence from the one end of the section, and which sequence of odd and even sections would be counted was determined using a table of 1000 random digits. Thus two even or odd integers would result in a field being 'jumped'; and an odd followed by an even (or vice versa) would result in consecutive 'fields' being counted. This procedure would continue until the section had been completed, and repeated with other sections chosen at random until at least 700 basal cells had been counted.

2.11.4. Intra-observer variation

Observer variation was acknowledged as being a source of potential error. It was therefore determined that this variation should be recorded in some objective manner. Therefor all readings were undertaken twice by the same observer, with a minimal time interval of 6 weeks between the two readings

2.12. Results

2.12.1. Period of Fixation

A number of the specimens were set aside for investigation of the result of long term fixation. During the period of the trial 'buffered Millonig's ' formalin was used and the results are demonstrated in Table 2.2. This demonstrates the number of sections retaining immunoreactivity as the period of fixation was increased.

Table 2.2. Frequency of epithelial cell reactivity for PCNA in five specimens of oral mucosa, incubated with PC-10 antibody, according to antibody dilution and period and temperature of fixation in buffered Millonig's formalin.

Fixation time	Room temperature for 1 hr						Overnight at 4°C					
	Concentration of PC-10						Concentration of PC-10					
	1:1000	1:500	1:200	1:100	1:50	1:20	1:1000	1:500	1:200	1:100	1:50	1:20
6 hrs	-	-	±	+	+	+	-	-	-	+	+	+
24 hrs	-	-	±	+	+	+	-	-	+	+	+	+
2 days	-	-	±	+	+	+	-	-	±	+	+	+
3 days	-	-	±	±	+	+	-	-	±	+	+	+
5 days	-	-	-	±	±	±	-	-	+	±	±	±
7 days	-	-	-	±	±	±	-	-	=	±	±	±
9 days	-	-	-	-	-	-	-	-	-	-	-	-

- +

±

-
- All five specimens in the trial were immunoreactive

One or more specimens were immunoreactive

No specimens were immunoreactive

2.12.2. Antibody dilution studies

Several trials were undertaken to determine the optimum dilution of primary antibody PC-10 used for PCNA localisation, using tissue from clinically normal oral mucosa as the substrate. This is the dilution level at which all the maximum tissue sections demonstrated immunoreactivity, with minimal background staining. All the trials had a known positive and negative control included (Table 2.2).

Similar trials were carried out for the primary antibody Ki-67 and the following protocol was observed for the remainder of the study :-

Ki-67 1:50 dilution, incubated overnight at 4°C.

PC-10 1:100 dilution, incubated for an hour at room temperature

2.12.3. The patterns of immunolocalisation of PCNA and Ki-67 antigens in oral epithelium from clinically normal mucosa.

The distribution of Ki67 antigen was nuclear and nucleolar (Fig 2.3). PCNA immunostaining was similar to that of the Ki67, though there was no cytoplasmic staining. Relative to the Ki-67, there was less homogeneity in the staining pattern of the PCNA (Figure 2.4.), though both did express a range of staining patterns. Staining intensity was variable, more markedly with the PCNA immunostaining.

Figure 2.3. Localisation of Ki-67 antigen as detected by Ki-67 antibody in clinically normal tissue. (Nuclear staining only was considered as positive, though there is cytoplasmic staining in the supra basal region.)(x 260)

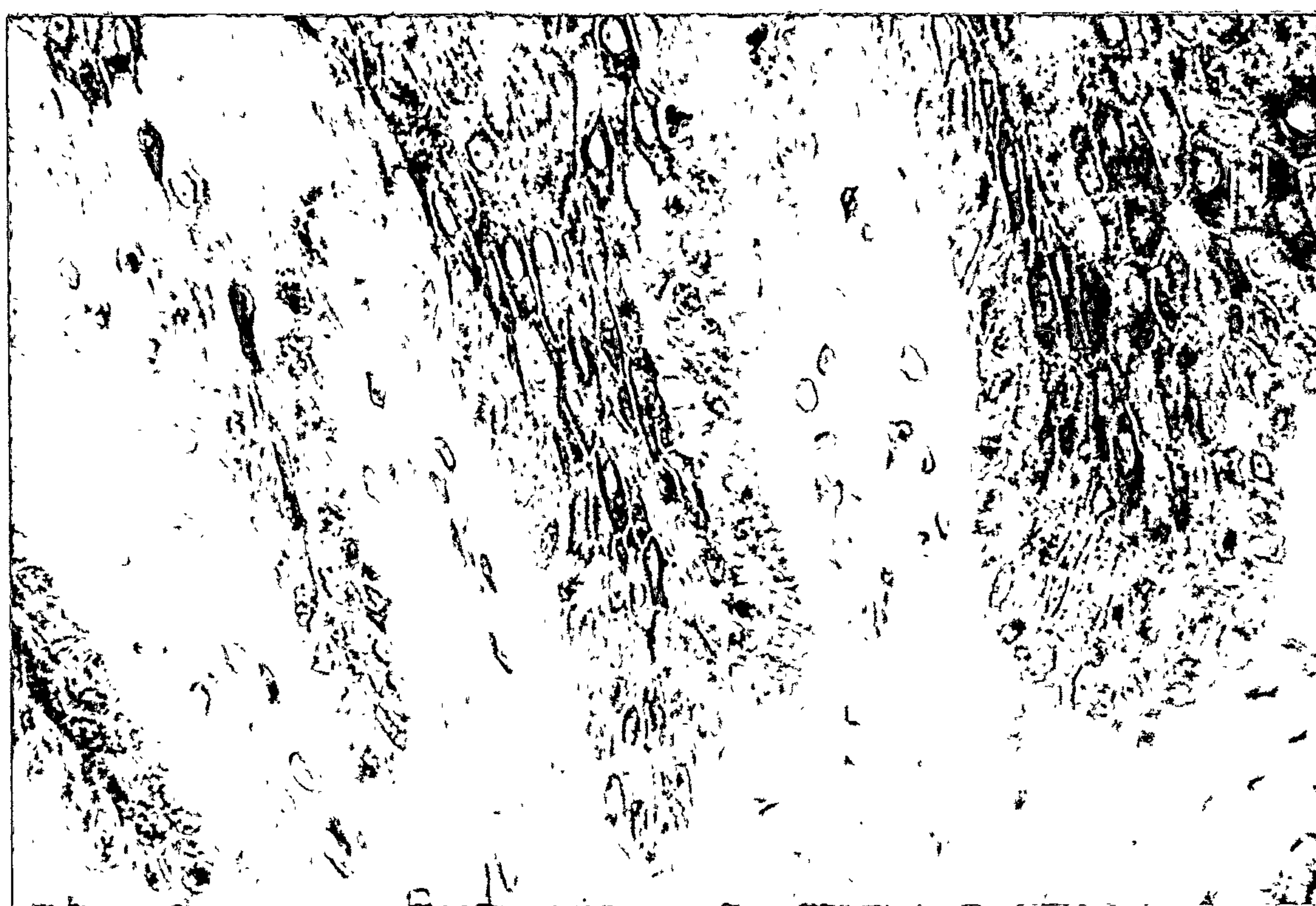
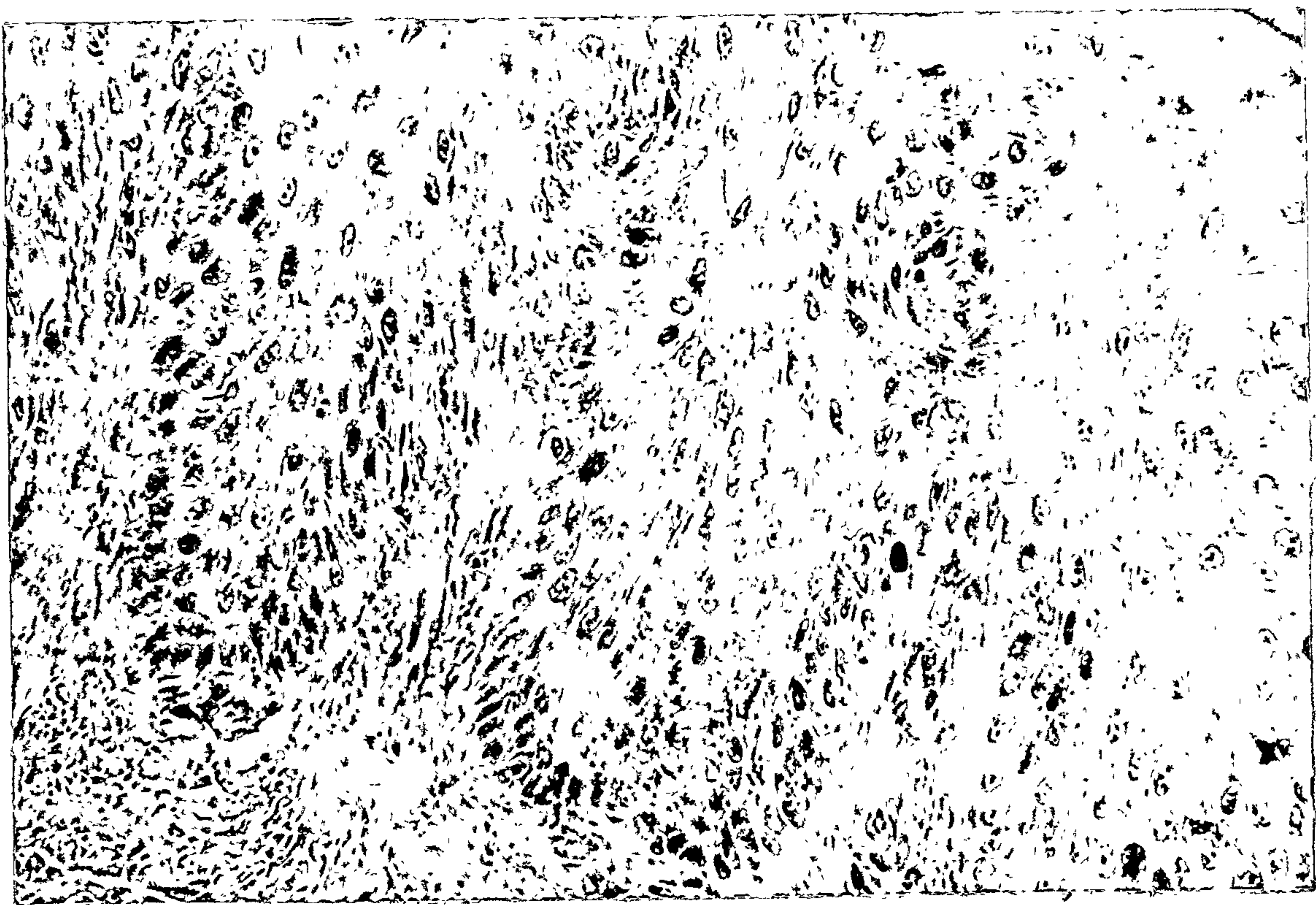


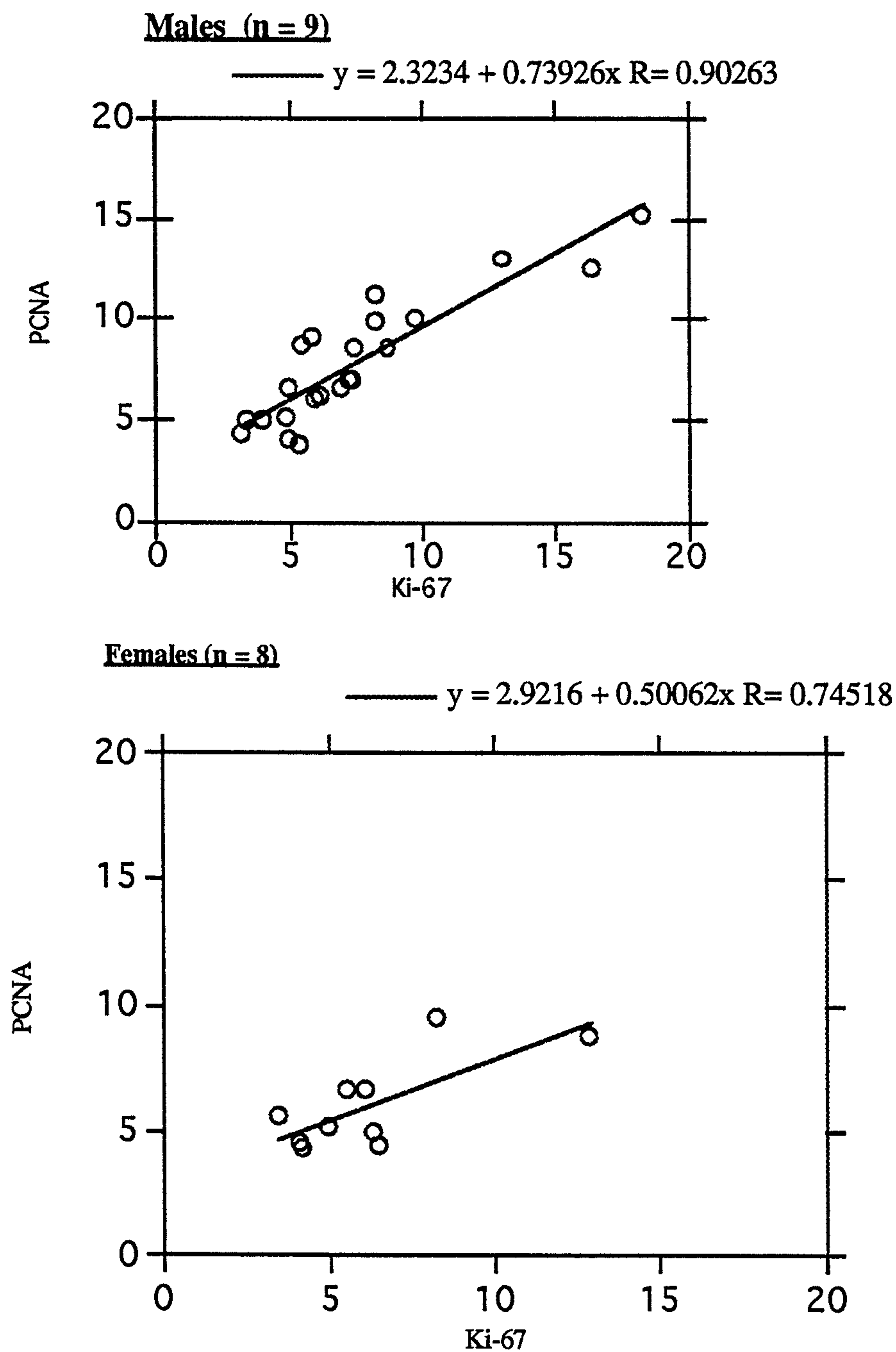
Figure 2.4. Localisation of PCNA antigen as detected by PC-10 antibody in clinically normal tissue. (Brown nuclear stain was considered positive)(x 260)



2.12.4. Correlation between growth fractions of basal epithelial cells in clinical normal oral mucosa determined by PCNA and Ki-67 antigen detection.

There was a significant correlation between the growth fraction determined by the immunohistochemical visualisation of PCNA and Ki-67 antigens respectively in cycling cells, for males, females (Fig 2.5.)and for the combined sexes (Fig. 2.6.)

Figure 2.5. Correlation of the growth fraction (proportion of positive cells %) for the basal layer of oral epithelium of clinically normal mucosa, recorded by the two antibodies Ki-67 and PC-10 for the same biopsies (n = 17, and biopsy specimens totalled 31, with a number of specimens being obtained from several subjects).



The mean and standard deviation for Ki67 and PCNA are reported in Table 2.4, and the difference in the growth fraction for the smoking and non-smoking population is also presented.

Figure 2.6.Correlation of growth fractions for PC-10 and Ki-67 in basal epithelial cells in clinically normal tissue for the same biopsy.(male and female subjects, n = 17)

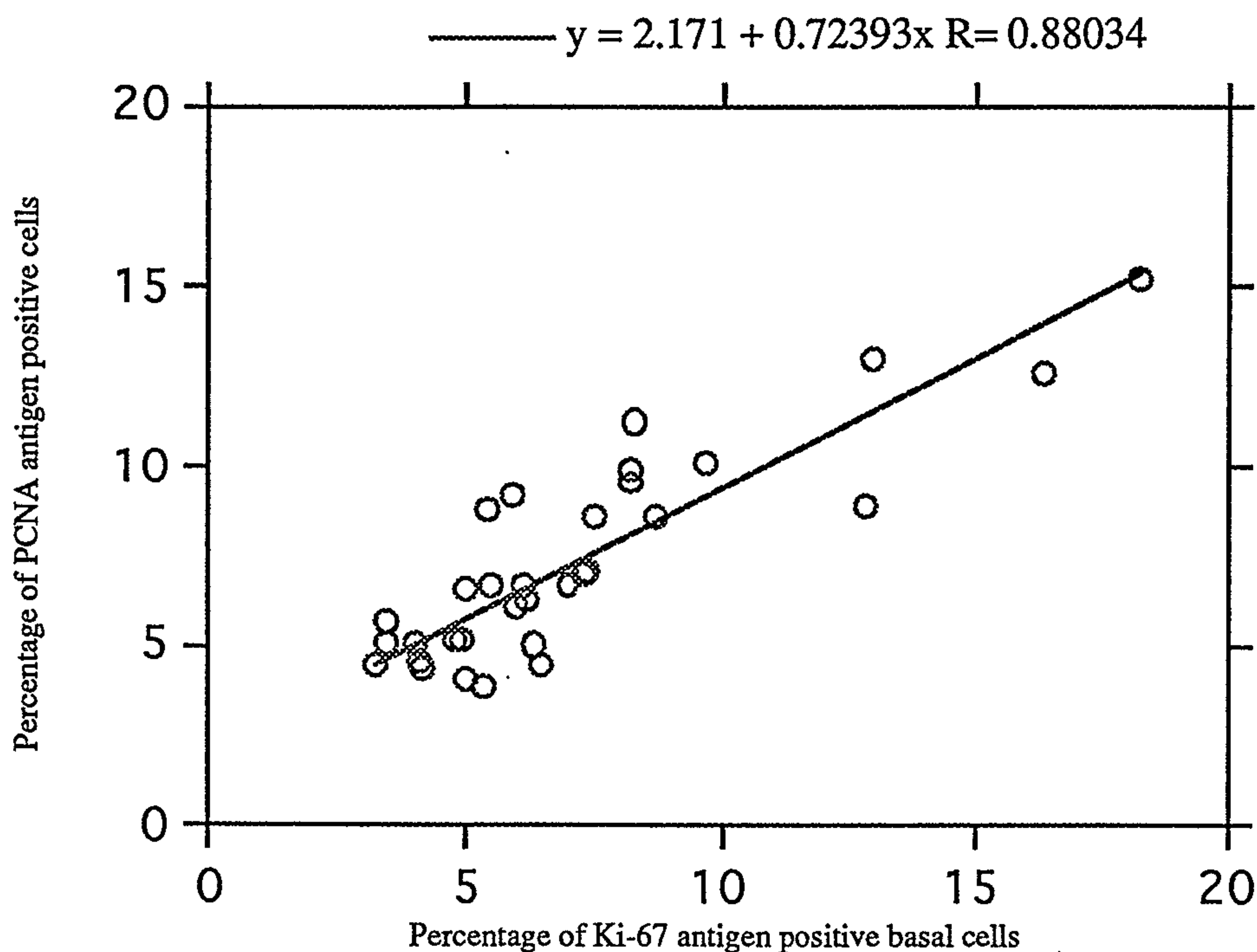


Table 2.4. Growth fraction as determined by Ki-67 and PC-10, according to the presence of an oral habit

		No of subjects	No. of biopsies	Mean (SD)	Median
Ki67	All subjects	17	33	7.3 (3.8)	6.1
	Smoking	9	23	8.6 (3.9)	7.4
	Non-smoking	3	10	4.8 (1.8)	5.0
PCNA	All subjects	17	33	7.6 (3.6)	6.8
	Smoking	9	23	8.8 (3.8)	7.6
	Non-smoking	8	10	5.2 (1.8)	5.2

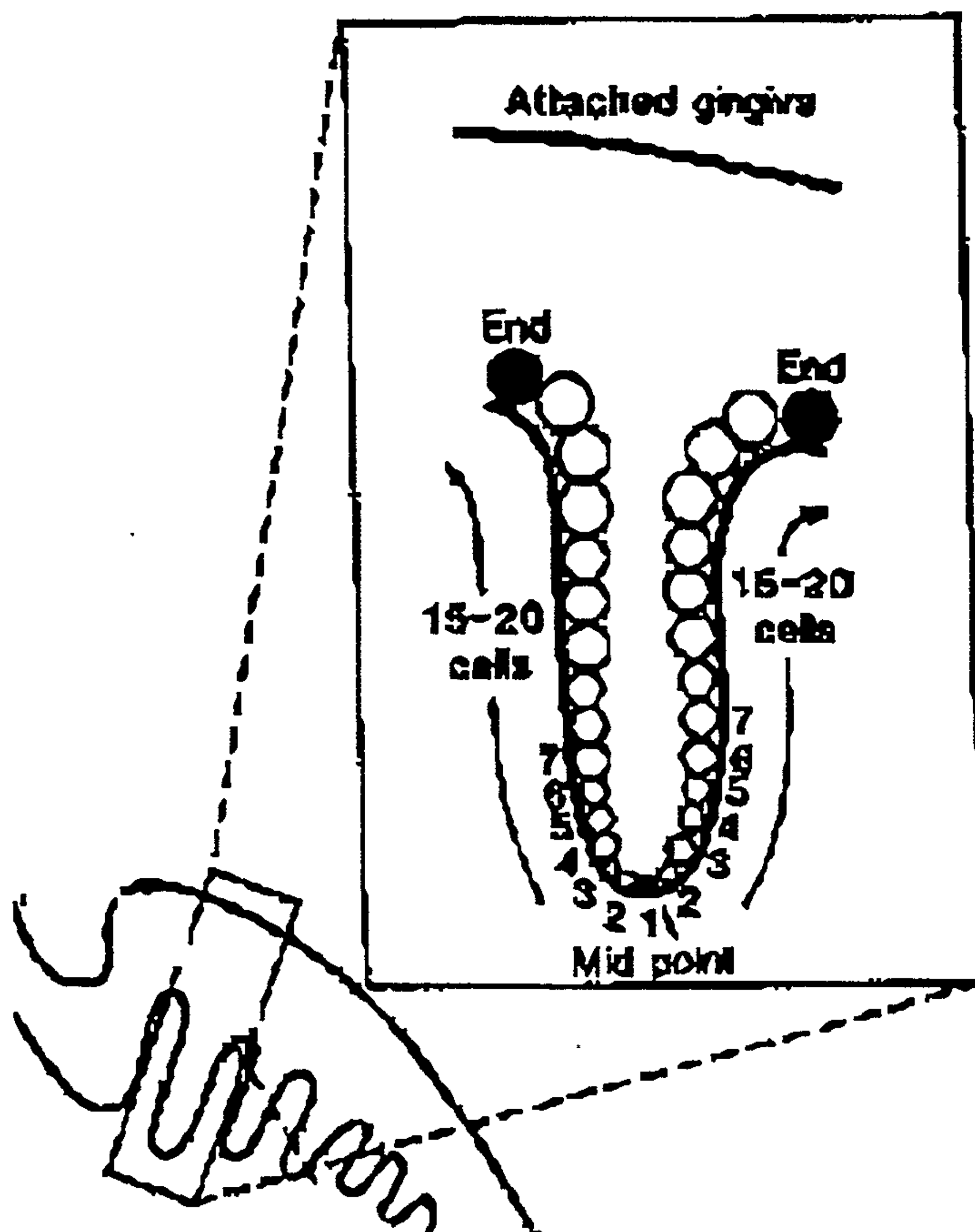
Combined correlation between PC-10 and Ki 67 shows a good correlation coefficient ($r = 0.88$) for the total subject population when the basal layer is examined. (Figure 2.6.). Only minor variations occurred when the subjects are placed in groups according to smoking habits.

There was a significant difference in the growth fraction of the basal epithelial cells between the subjects who smoked and those who had never smoked ($p < 0.05$)

2.12.5. Distribution of cells positive for cell cycle associated antigens relative to the anatomical structure of oral epithelium.

The position of cells along the basal layer were recorded topographically, such that each cell was numbered according to its relation to the cell most distant from the epithelial surface, on the rete ridges. Figure 2.7. demonstrates this system. (This diagram illustrates the system of numbering of cell position, and does not represent the total number of cells found in the human oral epithelium).

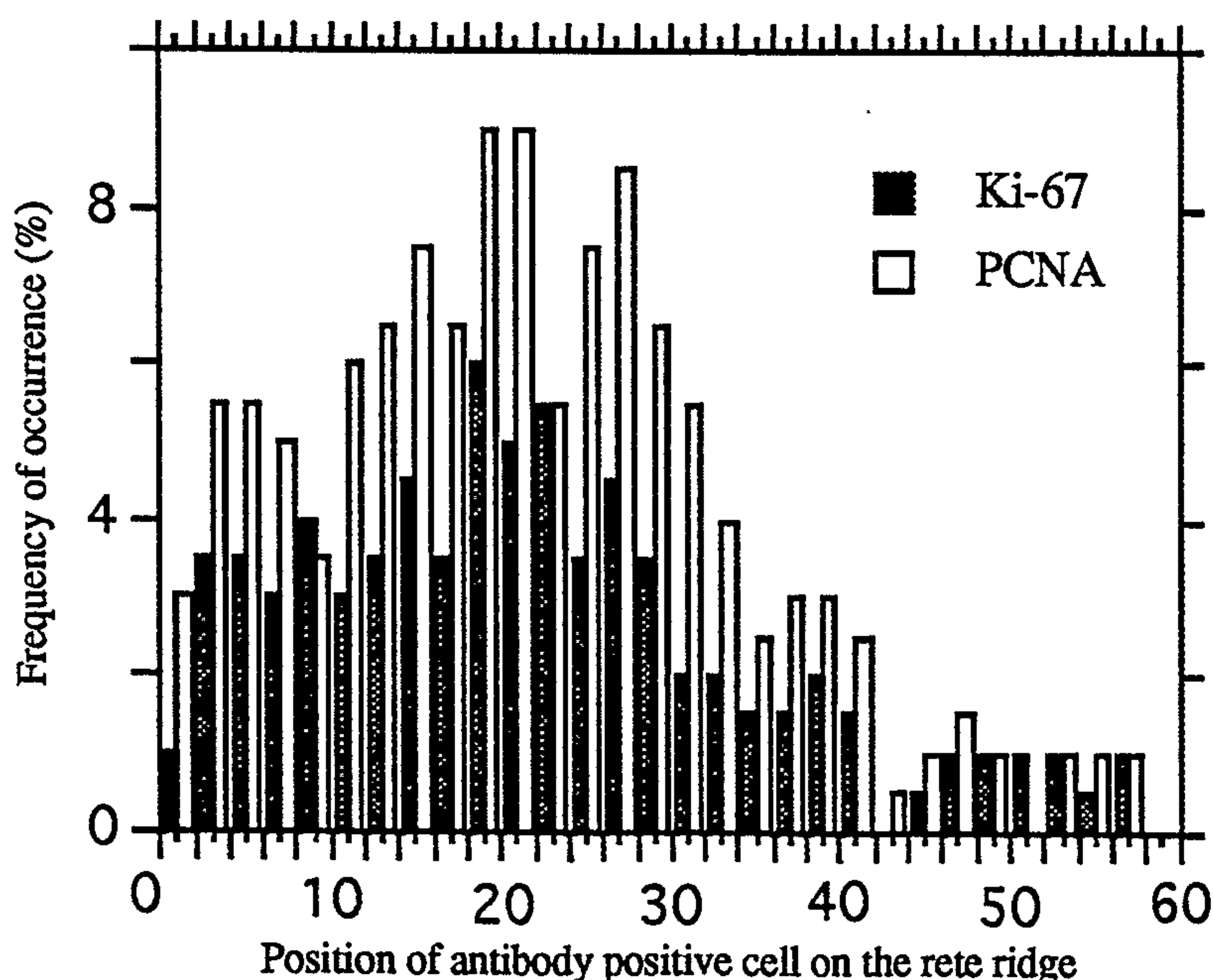
Figure 2.7.Standardisation process for identifying the position of cells on the rete pegs of the gingival epithelium.



The results as shown in Figure 2.8. demonstrate that although Ki 67 and PC-10 identify different proteins, present during the cell cycle, there is sufficient similarity in the basal layer, for the results to be comparable. This figure demonstrates the frequency (per hundred rete ridges) of immunopositive cells occurring at the various sites along the rete peg. The diagram is a cumulative graph obtained from counting at least 300 rete ridges- 300 in the Ki67 studies and 400 in the PC10 studies. The rete ridges examined were made up of at least 20 cells from the mid point, from each tissue block (rete ridges of at least 20 cells were chosen to reduce the incidence of obliquely sectioned rete ridges being included in the study).

Most of the biopsy specimens were taken from gingival areas, and thus this data reflects the situation for the gingiva specifically.

Figure 2.8. Growth fraction by detection of Ki-67 and PC-10 antigens in the basal layer according to cell position in the rete ridges of oral epithelium. (Tissue biopsies from the gingiva).



Numbers on the x-axis reflect the position of the cell on the rete ridge according to Fig 2.6

There was a degree of variation for the specific cell positions, and this was understood to be due to the relatively small number sections examined. Therefore, Figure 2.8 attempts to smooth out these variations in frequency with which any one position was observed to have a positive cell, by each bar in the histogram representing two cell positions

Figure 2.9.Frequency of occurrence of positive Ki-67 cells for each cell position on the rete ridge.

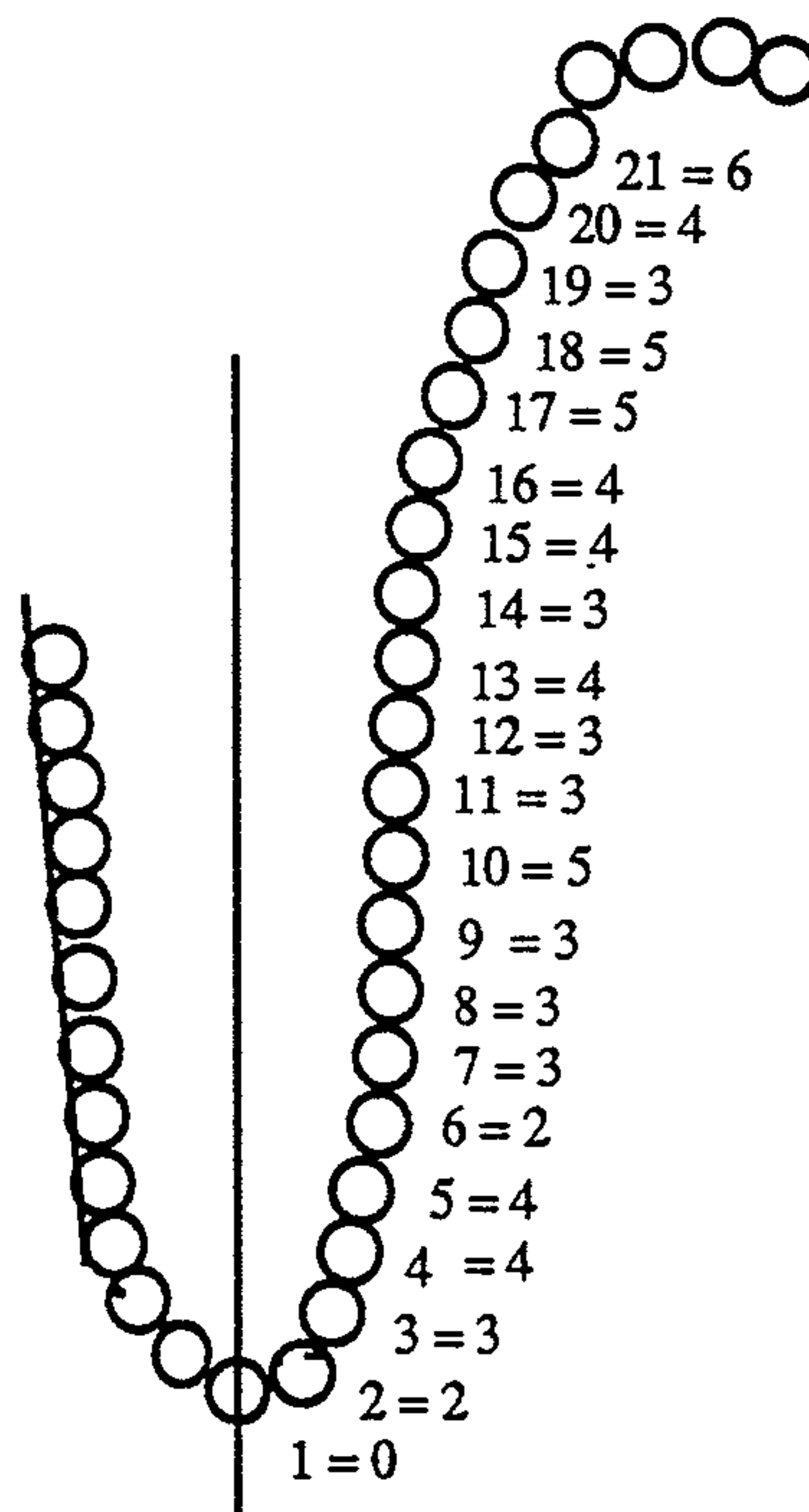


Figure 2.9 represents the frequency of the first 20 basal cells in the rete ridge presenting as positive to Ki-67. Some of the rete ridges were made up of up to 58 cells (without demonstrating any of the signs of an oblique section). However the majority of the rete ridges in the sections examined were made up of approximately 20 basal cells.

Figure 2.9. demonstrates that position 1 was never observed to have a cell positive for the Ki-67, while the other cells on the basal layer all demonstrated a degree of variability in the frequency of positive staining.

2.12.6. Correlation with periodontal indices

The results for the growth fraction were examined alongside the periodontal indices, to identify any trends. Although there was a general trend for the growth fraction to increase as the median value for the pocket depth increased (from a mean value of 7.8-9.3 with SD of ± 3.6 for pocket depths of 4-12 mm) the results were not significant

The other periodontal indices could not be analysed due to insufficient patients being present in the full range of the indices. This was due to the protocol observed in the periodontal department for surgical patients which would allow surgery if the mouth was in a poor state of health.

2.12.7. Growth fraction and Tobacco smoking

Tobacco smoking was a common habit among the subjects surveyed, being practiced by 76% . It was noted that there was a higher growth rate in the gingival tissue, amongst tobacco smokers compared with that of non-smokers - demonstrated in Table 2.6., though the difference in regard to distribution is not statistically significant.

Table 2.6. Variations in the growth fraction of basal epithelial cells in clinically normal oral mucosa according to tobacco smoking and site of the mouth.

Site	No.	Mean growth fraction (SD)	
		Smoking	Non-smoking
Posterior	22	7.4 (2.2)	4.9 (1.0)
Anterior	11	9.4 (4.0)	5.6 (0.6)

2.12.6. Intra-observer variation

Minimal variation in the determined mean values of growth fraction was obtained using the method of the 'cumulative mean'(Underwood, 1987).. The mean and standard deviation for intra-observer error for the number of immunopositive cells recorded as a percentage after 700 cells were counted, was 2.5% (SD 1.1)

2.12.9. Methodological problems with antibodies to PCNA.

A number of problems were encountered with the immunochemical techniques. Most of these were due to the lack of previous experience. The main problems encountered were as follows:-

1. Only partial staining of the specimen occurred during the early days of the laboratory procedure. This was attributed to the lack of adequate mixing of the antibodies when placed on the specimen, and was corrected when a dropper bottle was used.
2. An 'out-dated' batch of PAb Ki-67 was substituted without my knowledge for the recently purchased antibody in the immunohistochemistry laboratory, in the interests of economy. There followed three weeks of systematic analysis of each step of the procedure, with the final conclusion being that the primary antibody had deteriorated.

2.13. Discussion

2.13.1 Growth fraction in 'normal' tissue

The study showed that PCNA immunoreactivity was confined to the nuclei, and not to the cytoplasm of proliferating cells, and this is consistent with the nuclear origin of PCNA. The growth fraction as recorded by the proportion of cells showing a positive reaction with the primary antibodies Ki-67 and PCNA lay within a range of 2-18 cells per 100 cells (2-18%). The mean number of positive cells (PCNA - mean = 7.6%, SD = 3.6; Ki-67 - mean = 7.3%, SD = 3.8) was very similar for both of the cell cycle associated antigens visualised, and correlates well with data from other sources (Tsuji et al (1992) demonstrated a mean of 9.8% with a SD of 4.9 for clinically normal oral mucosa.

The studies of the effect of fixation time on the presentation of the PCNA protein indicated that its immunoreactivity is highly sensitive to fixation time, with the recommended time being 24-48 hours. Longer periods of fixation presumably caused significant antigen denaturation.

It was decided to count only those antigen-positive cells situated in the basal layer of the epithelium. PCNA is expressed both in the G₁/S phase of actively cycling cells (ref 2.10.3.3.), and during DNA repair (ref 2.10.3.4.). PCNA-staining might predominantly reflect proliferating cells engaged in DNA synthesis rather than repair at this anatomical level in the epithelium but further work would be necessary to dissociate these two cell functions. The same approach was used for the Ki-67 staining for reasons of consistency.

Basally situated cycling cells as determined by PCNA and Ki-67 antigen detection (Fig. 2.6.) were shown to be most frequently located near position 18 - 20 on the rete ridge, which frequently represented the junction of the rete ridges with the overlying epithelium, rather than at their bases (which was the region determined by Seddon et al

(1992) for the tongue epithelium of the mouse). Although at any one position on the rete ridge the number of cells PCNA positive cells were consistently higher than those for Ki-67 the general distribution of the cells (and mode of the distribution) was similar Fig 2.6..

A good correlation existed between the Ki67 and the PC-10 as markers of the growth fraction of the oral mucosa (correlation coefficient $r = 0.88$). This correlation does not appear to have been demonstrated previously in oral mucosa.

Together these results support the use of PAb PC-10 as a reliable and convenient indicator of the number of basal epithelial cells which are in the cell cycle at any particular point in time, provided only the basal layer of cells is considered. The major advantage of the monoclonal antibody PAb PC-10 is that it can be used on routinely processed biopsy material, and therefore particularly valuable in retrospective studies of archival paraffin embedded studies, as was used in the subsequent investigation of oral submucous fibrosis.

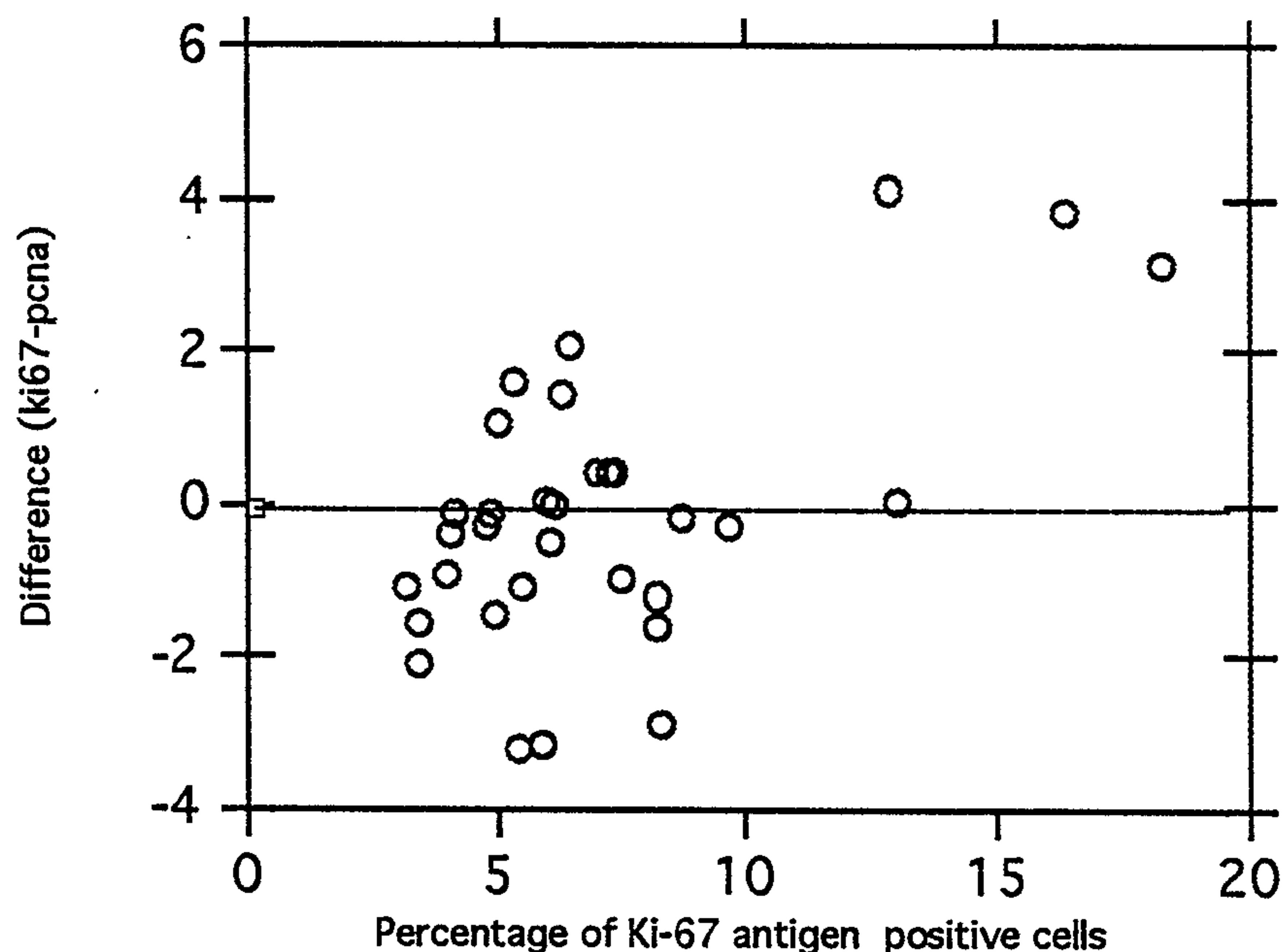
There are a number of reports in the literature to suggest that PCNA is in fact a poor indicator of the growth fraction in neoplasia, showing no correlation with other cell markers (Leonardi et al, 1992). Although there is a linear correlation between Ki67 and PCNA immunoreactivity in nodal lymphoma and between S phase fraction and PCNA staining in gastric lymphoma and now in this study, recent observations of gastric cancer, and of haemangiopericytomas have shown that the correlation between S phase fraction and PCNA immunoreactivity is very poor (Leonardi et al, 1992)..

The lack of correlation between PCNA and other markers of 'growth fraction' could be attributed to several factors. PCNA appears to be present both in G₁ and S phase of the cell cycle; it is also known that the half-life of PCNA protein is about 20 hours (Bravo and Macdonald-Bravo, 1987), which may permit its immunohistochemical detection in cells which have recently left the cell cycle. It is possible that the half-life of PCNA

remains constant and is independent of the rate of proliferation. In addition PCNA can be involved in DNA repair as well as cell proliferation.

Although from Figure 2.10 it might be supposed that at the lower levels of growth fraction there is good agreement between the two methods for deriving growth fractions this was not obtained at values exceeding 10%, concurring with other comparable studies (Landberg and Roos, 1991). When the variation is expressed as a proportion of the absolute value, the reduced agreement appears less significant (Fig 2.10). This correlates with the observation of Allegranza et al (1991) that the number of immunopositive cells are different for Ki-67 and PCNA, with the latter usually being higher.

Figure 2.10. Difference in the growth fraction as determined by PC-10 and Ki-67 plotted against the value determined by Ki-67



This data is significant in that for the range of clinically normal tissue the variation between Ki-67 and PCNA is ± 4 . However, it would be unwise to suggest, on the strength of this data, that the same would apply for the tissue from patients with carcinomas.

Chapter 3

Proliferating cell nuclear antigen (PCNA) in malignant and premalignant lesions of epithelial origin in the oral cavity: an immunohistochemical study.

3.1. Introduction

Malignant tumours are frequently characterised by heterogeneity (Langdon et al, 1977) in biological, enzymatic, immunological, and ultra-structural aspects. This emphasises the need to have various indices which are able to evaluate tumour progression and metastatic potential. Of these the growth fraction of the tissue may provide valuable information concerning cellular growth kinetics, tumour progression - including metastatic potential and prognosis.

Immunological methods of assessing cell proliferation have the advantage of being able to relate, *in situ*, cell proliferation to the pathological features. In this study the monoclonal antibody, PC10, which recognises the proliferating cell nuclear antigen (PCNA), will be used to assess the growth fraction and pattern of cell proliferation and its correlation with dysplasia and tumour grade, in pre-malignant lesions and malignant tumours of epithelial origin, respectively.

Giglio et al (1992) states that high levels of PCNA correlate with more advanced disease stages, as well as with shorter doubling times for chronic lymphoid leukaemic cells. It therefore may be a very useful variable in assessing subjects, with OSF, who are particularly at risk of developing an oral carcinoma.

Although a wide variety of malignant neoplasms can arise from the oral tissues, the vast majority of these are squamous cell carcinomas of the oral tissue (90% world wide) (Daftary et al, 1991).

3.2. Materials and methods

This study involved a total of 44 cases of oral squamous cell carcinoma (International classification of Diseases groups 141, and 143-145) (World Health Organization, 1977) 20 cases of oral submucous fibrosis, 6 cases of fibroepithelial hyperplasia, 5 cases of oral lichen planus, and 5 cases of oral keratosis. All cases were confirmed histopathologically.

3.2.1. Subjects

The biopsy material used in this study was obtained from four main subject groups:-

3.2.1.1. Oral submucous fibrosis

Patients who demonstrated the clinical signs of oral submucous fibrosis (see section 1.6.), who were resident in the countries of Nepal or India. All the patients from Nepal were seen personally, and had complete clinical notes.

All patients who presented with either the signs and/or symptoms of OSF were recorded, and personal details were taken (see Appendix for copy of 'Record Sheet'). A record was made of any associated abnormality detected clinically and whether the buccal vestibules had reduced sulcus depth. Extra-oral photographs were taken, and where oral opening permitted, intra-oral were also taken.

Patient profile is presented in Table 1.5.

3.2.1.2. Control subjects without submucous fibrosis

a/ Subjects who attended the Westmead Hospital for surgical treatment of inflammatory periodontal disease, or other minor oral surgical procedures. In these cases the tissue which was removed was redundant. There were no keratoses of the gingiva in this group. Each patient was personally examined and apart from the chronic periodontal disease the rest of the oral mucosa was clinically normal (Table 3.1). The patients had undergone a course of scaling, polishing and oral hygiene instruction, commencing at least two weeks prior to surgery.

b/ Nepali and Indian subjects presenting for routine removal of impacted "wisdom" teeth who required the removal of redundant tissue adjacent to the surgical site. Apart from the chronic inflammation of the operculum associated with partially erupted teeth, there were no keratoses or other abnormalities of the gingiva or rest of the oral mucosa. These patients were not seen personally.

c/ Archival material from patients, who attended Westmead Hospital, with the following conditions:-

- i/ Oral lichen planus
- ii./Oral keratosis
- iii/ Fibroepithelial polyp

These groups were not seen personally.

d/Summary data regarding tumour patients is given in Table 3.3.

e/The total number participating in the study, together with their age and tobacco habits is presented in Table 3.4.

Table 3.1. Details of control subjects

	Subject	Sex	Age	Oral Habit
Clinically normal mucosa	1	M	24	Smoking
	2	F	22	Smoking
	3	F	18	Smoking
	4	F	23	Smoking
	5	F	26	Non smoking
	6	M	41	Smoking
	7	F	12	Non smoking
	8	F	24	Non smoking
	9	F	24	Smoking
	10	F	28	Smoking
	11	F	19	Non smoking
	12	M	56	Smoking
	13	M	68	Smoking
	14	M	40	Non-smoking
	15	M	54	Smoking
Keratosi	16	F	64	Smoking
	17	F	39	Smoking
	18	F	56	Smoking
	19	M	74	N/A
	20	F	41	N/A
	21	F	63	Non smoking
Lichen Planus	22	M	42	Non smoking
	23	F	33	Smoking
	24	F	54	Non smoking
	25	M	54	Smoking

3.2.1.3. Positive control patients with oral cancer

In this study archival material was retrieved from the files of the Anatomical Pathology Department, Institute of Clinical Pathology and Medical Research, by reviewing the histopathology reports of Westmead Hospital, NSW, Australia, during the period 1989-1990. All cases of oral squamous cell carcinoma were noted, and the microscopic sections were examined, and the presence of carcinoma was confirmed.

This material was used as the positive control for the immunohistochemical studies.

Clinical data regarding the age, sex, and oral habit as well as the site of the biopsy was documented for each patient, (Tables 3.2 and 3.3) and the information recorded (layout of the record from in appendix B). Those patients who suffered from OSF were additionally assessed for their 'oral opening" (Table 1.5.).

Summary data regarding tumour patients is given in Table 3.3.

The total number participating in the study, together with their age and tobacco habits is presented in Table 3.4.

Table 3.2. Patients with oral squamous cell carcinoma

Patient	Sex	Age	Site	TNM	Smoking	Drinking
1	M	72	1	T3,N1,M0,S-III	20	20
2	M	73	2	T1,N0,M0,S-I	NS-30yr	20
3	M	53	2	T1,N0,M0,S-I	10	20
4	M	55	3	T2,N0,M0,S-II	40	100
5	M	59	4	T2,N0,M0,S-II	20	120
6	F	56	2	T2,N0,M0,S-II	NS-6yr (10)	40
7	M	67	2	T3,N0,M0,S-III	10	156
8	M	53	3	T4,N2b,M0,S-IV	40	80
9	F	64	1	T4,N2b,M0,S-IV	20	60
10	M	60	4	T1,N0,M0,S-I	50	Nil
11	F	73	2	T4,N2a,M0,S-IV	NS-4yr (10)	30
12	F	53	3	-	NS-3yr (20)	20
13	M	70	2	T3,N0,M0,S-III	NS-9yr (50)	30
14	M	67	5	T3,N2b,M0,S-IV	20	5
15	M	71	4	-	NS	Nil
16	F	53	2	T1,N0,M0,S-I	20	Nil
17	F	85	3	T2,N0,M0,S-II	NS	Nil
18	F	66	4	T2,N0,M0,S-II	10	10
19	M	66	6	T4,N1,M0,S-IV	NS-10yr,(10)	150
20	M	87	1	-	20	60
21	F	72	3	T2,N1,M0,S-III	20	140
22	M	74	6	-	NS-7yr (20)	Nil
23	M	66	1	T3,N0,M0,S-III	60	60
24	M	46	4	T4,N2,M0,S-IV	20	150
25	F	76	6	T2,N0,M0,S-II	20	5
26	M	63	2	T2,N0,M0 S-II	20	80
27	M	56	6	-	NS-25 yr, (5 for 10 yr)	5
28	F	88	6	T2,N0,M0,S-II	5	Nil
29	M	63	1	T2,N2,M0,S-IV	25	120
30	M	60	3	T3,N2,M0,S-IV	20	80
31	M	63	3	T3,N0,M0,S-III	40	30
32	M	51	3	T3,N0,M0,S-III	20	100
33	M	34	6	T4,N2,M1,S-IV	20	240
34	F	69	5	T3,N2b,M0,S-III	NS	5
35	F	94	4	-	-	-
36	M	71	2	T3,N0,M0,S-III	20	80
37	F	56	1	T2,N0,M0,S-II	20	Nil
38	M	44	6	T4,N0,M0,S-IV	20	40
39	F	73	1	T1,N0,M0,S-I	NS 11YR,(10)	5
40	M	59	4	-	80	140
41	F	63	3	T2,N0,M0,S-II	NS-9yr (50)	60
42	F	68	5	T2,N0,M0,S-II	NS	Nil
43	F	69	6	T2,N3a,M0,S-IV	40	150
44	M	55	1	-	-	-

TNM* T represents tumour size, where; T₁ = < 2cm in greatest diameter; T₂ = 2-4 cm, T₃ = 4-6 cm and T₄ > 4cm and deeply penetrating underlying structures.

N₀ = no clinically detectable nodes;

N₁ = enlarged ipsilateral lymph nodes present ≤ 3 cm

N₂ = ipsilateral single > 3-6 cm or ipsilateral multiple ≤ 6 cm or bilateral or contralateral ≤ 6 cm;

N₃ = any palpable node > 6 cm.

M₀ and M₁ = absent or detected distant (blood borne) metastases.

Site: 1 = retro-molar trigone region; 2 = tongue; 3 = palate; 4 = floor of mouth; 5 = alveolar mucosa; 6 = buccal mucosa.

Smoking = cigarettes per day; NS = non smoker; () = the number of cigarettes previously consumed.

Drinking = Alcohol (gm) per day.

(* The UICC (1988) TNM staging system (Harris, 1991))

Table 3.3. Patients with oral squamous cell carcinoma

		Male	Female
Number		27	17
Age (yrs)	-median	63	69
	-Standard deviation	10.5	12.9
Tumour size at time of presentation (%)			
	Tx	0	2 (5)
	T1	3 (7)	2 (5)
	T2	4 (9)	10 (23)
	T3	9 (20)	1 (2)
	T4	5 (11)	2 (5)
	Unknown	6 (14)	1 (2)
Non-smokers (%)		4 (9)	4 (9)
Primary cancer sites			
	Retro-molar trigone	5	3
	Tongue	6	3
	Buccal	5	4
	Floor of mouth	4	1
	Palate	6	4
	Alveolar mucosa	1	2

Table 3.4. Summary of participants in the study

		No.	Age	Tobacco
			Mean (SD)	Habit (%)
Clinically normal	Westmead, Australia	15	30.6 (15.1)	31*
	Nepal/India subjects	14	Not known	Not known-
Keratoses		6	52.6 (11.9)	50
Lichen planus		4	43.5 (8.6)	50
Fibroepithelial polyp		6	23 (12.9)	20
Carcinoma		44	64.9 (8.1)	77#
OSF		23	38.1 (8.1)	71

* excludes subjects who ceased smoking for more than 10 years.
5 subjects had never smoked, while 2 had ceased smoking more than 10 years previously

The tissues used in this study were fixed in a solution of formal-saline for a period of 24 hours, dehydrated and embedded in paraffin . Serial sections were cut at 4 μm, and

the deparaffinized sections were stained with haematoxylin and eosin for routine histopathological examination. The immunohistochemical procedure for PCNA staining was carried out in the manner described in section 2.11.4. The dilution of the primary antibody (PAb PC-10) was 1:100, this being the dilution which provided the greatest intensity of nuclear staining with minimal or no background staining. This dilution was also that at which, under the above conditions of fixation, time and temperature of incubation, the antigen was disclosed in each of the normal mucosal specimens used in the preliminary study

3.2.2. Epithelial dysplasia

All epithelial sections, apart from the carcinoma specimens, were examined for the extent of epithelial dysplasia. The histological grading method recommended by Smith and Pindborg, (1969) was used. This grading system examined 14 characteristics of cells and two of the tissue, and assigned a numerical value to the severity of the various forms of cellular atypia. This system also sought to standardise the histological assessment through the use of references, in the form of photographic plates. The results for cellular atypia are recorded in terms of the total value of the severity score over the number of dysplasia categories

The term epithelial dysplasia is being used to refer to the changes in the epithelium as a whole which are associated with an increased risk of malignancy. The term cellular atypia is used to denote those changes in the individual cells eg. at the edges of wounds or intensely inflamed hyperplastic states, which are not associated, necessarily, with malignant potential.

3.2.3. Oral epithelial malignancy score

The examination of the invasive nature of the tumour and prognosis was undertaken using Bryne's modification of Anneroth's index (Bryne et al, 1989). This modification recommends that all features examined are registered in the most anaplastic area of the most invasive site of the tumour. A high malignancy score indicates a poorly differentiated tumour. The extent of PCNA (PC10) positivity was evaluated as described previously (2.5.2.1.). All cells which demonstrated immunoreactivity were counted as positive regardless of the staining intensity. The significance of PCNA positivity and tumour grades, and atypia was assessed.

3.3. Results

The growth fraction, as determined by PAb PC-10, of the oral mucosa from normal controls, fibro epithelial hyperplasia, carcinoma and OSF are given in Table 3.5

Table 3.5. Percentage of PCNA-positive cells (growth fraction) in the basal epithelial layer, as appropriate for clinically normal tissue, carcinomas and oral submucous fibrosis.

		No.	% of PCNA positive nuclei Mean (SD)
Clinically normal	Westmead	29	7.6 (3.6)
	Nepal/India	13	6.1 (6.0)
Keratosis		6	5.6 (2.5)
Lichen Planus		4	9.5 (4.2)
Fibroepithelial hyperplasia		10	
Surface epithelium			6.8 (2.5)
Epithelium deep in the corium			23.3 (19.7)
Carcinoma		52	39.4 (22.4)*
OSF		23	31.8 (21.9)

* Positive cells in neoplastic tissue

Squamous epithelium from clinically normal oral mucosa showed positive nuclear PCNA immunoreactivity in the basal cells with a mean of 7.6% of the cells. Most cases of malignant and premalignant tissue showed positive nuclear staining of varying intensity. The pattern of staining in the nucleoplasm was normally granular.

No staining of mitotic figures was observed. The tissue presenting with features of oral keratosis and lichen planus showed positive nuclei mainly in the basal and lower

spinous layers, with a few in the upper spinous layer. However, only positive cells in the basal epithelial layer were counted for this comparison.

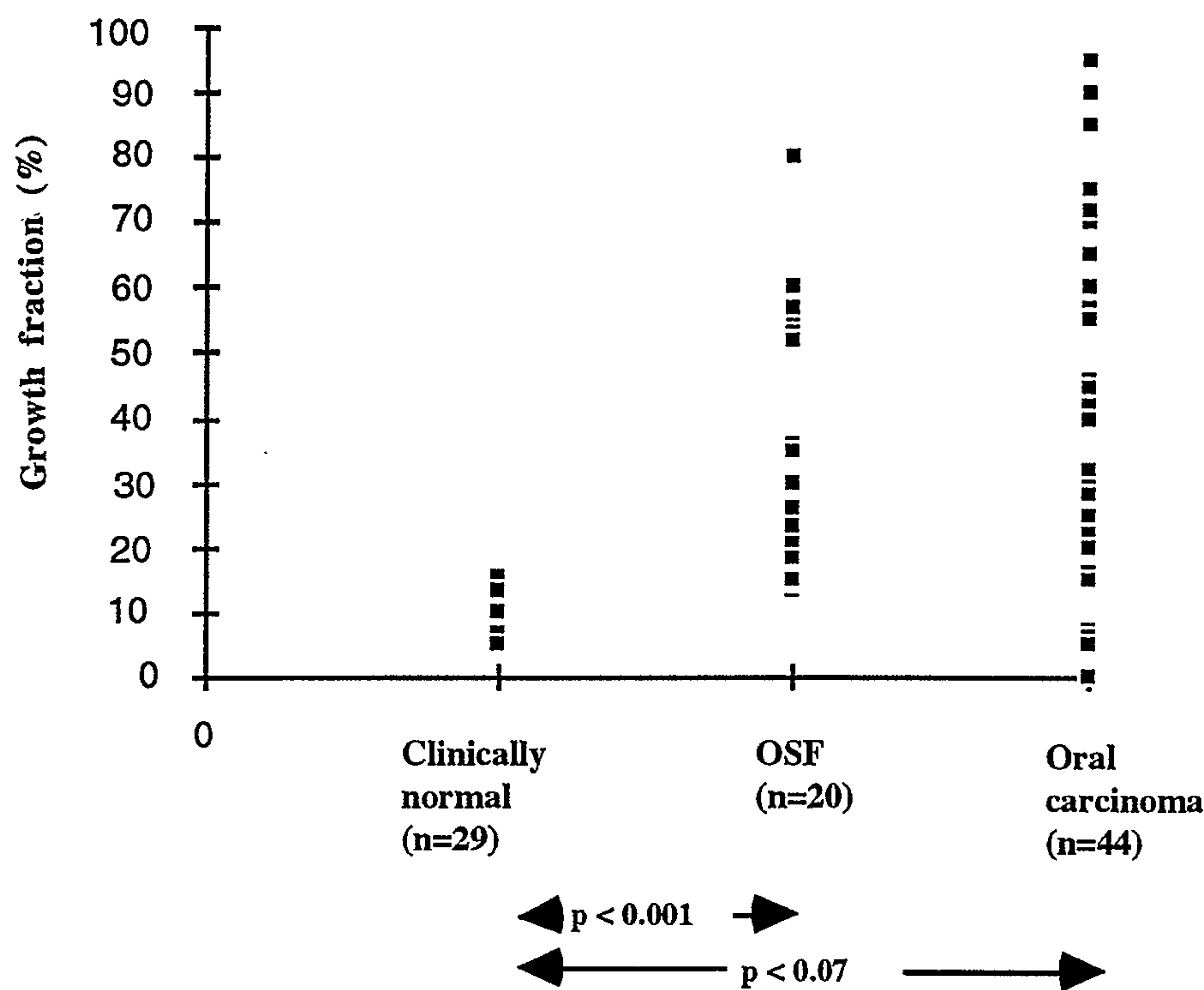
The superficial epithelium of the fibro epithelial polyp had a similar PCNA value as that for normal control mucosa specimens. However there were strands of epithelium which ramified deep through the connective tissue of the corium and this was a frequent characteristic of the fibroepithelial polyps 66% (4/6) with the two remaining biopsies demonstrating negative immunoreactivity to PAb PC-10. The strands of epithelium found deep in the corium exhibited a higher growth fraction (23.3%), as determined by PCNA, compared with that of the surface layer, but the number of specimens was too low for any meaningful statistical analysis.

There was a significant difference between the growth fraction, as detected by PCNA testing of the basal epithelial layer, in clinically normal mucosa and that of both the oral submucous fibrosis and the malignant tissue (Table 3.5.). The positive nuclei in the OSF were confined to the basal layer, and only occasionally was the lower spinous layer involved.

The positive nuclei in the squamous cell carcinomas were frequently found throughout the surface epithelium as well as in the invading cells. The regions of actively invading epithelial cells demonstrated immunoreactivity to a varying degree, and occasionally failed to demonstrate any product..

There were 6 cases which failed to show any immunoreactivity for PCNA, 4 of these were poorly differentiated carcinomas, the fifth was moderately well differentiated, and the last was well differentiated. It is for this reason that Figure 3.1 demonstrates a zero growth fraction for some of the tumours in the oral carcinoma column.

Figure 3.1. Growth fraction (%), as determined by PAb (PC-10), in normal mucosa , oral submucous fibrosis and oral squamous cell carcinoma.



3.3.1. Oral carcinoma

The squamous cell carcinomas frequently showed intense nuclear staining in the invasive region whereas the surface tissue would stain poorly for PCNA.

Table 3.6. Correlation of immunohistochemical staining for PCNA with tumour cell differentiation as recorded on the histopathology reports.

Differentiation	No	% PCNA-positive nuclei		% Total positive mean (SD)
		1-20	>20	
Well differentiated	7	2	5	43.2 (30.2)
Moderately differentiated	18	4	14	38.8 (20.6)
Poorly differentiated	8	4	4	37.4 (21.3)

3.3.2. Analysis of growth fraction against survival

A univariate analysis was undertaken for the growth fraction against survival. The growth fraction was initially used as a continuous variable where the risk of dying, associated with an increasing growth fraction was 0.97 ($p = 0.28$).

The failure to gain any significant result was thought to be possibly associated with an absence of appropriate grouping of the cases. The cases were then grouped into four according to the respective growth fraction:- group 1 = 0-4%; group 2 = 5-19%; group 3 = 20-39%; and group 4 = 40 + % (following the precedent set by Bodner et al. 1992). (Group 2 had a growth fraction similar to the normal controls). The result of this analysis was that the lowest growth fraction as recorded by PCNA, had the poorest prognosis, with a risk of dying for patients in the other groups, compared to group 1 being 0.71 though this was not significant ($p < 0.2$),

Table 3.7.Correlation of PCNA value and the Bryne score for oral squamous cell carcinomas.

Bryne score	No.	PC-10 positivity
≤ 10	24	28.7 (17.4)
> 10	21	41.0 (19.5)
	3	Negative

Table 3.7 demonstrates a trend between the growth fraction, determined by PCNA, for the tumours based upon the two groups as determined by their Bryne score. These results did not prove to be significant ($p < 0.08$)

3.3.3. Relationship between the growth fraction and epithelial dysplasia score with the stage of OSF

The clinically normal mucosa and OSF biopsy material were assessed for epithelial dysplasia, using the Smith and Pindborg (1969) epithelial dysplasia score. These scores were then compared (Table 3.8) with the histological stage of the OSF, determined by the scheme proposed by Sirsat and Pindborg (1967)

Table 3.8.Correlation of stage of oral submucous fibrosis and the degree of dysplasia ("atypia" - Smith and Pindborg, 1969)

Degree of "atypia"	No.	Submucous fibrosis staging				% Total
						Mean (SD)
		Very Early	Early	Moderately	Advanced	
Advanced						
Mild	0	0	0	0	0	-
Moderate	22	1	4	8	9	30.5 (16.4)
Severe	9	0	3	2	4	33.8 (21.7)
% Total Mean(SD)		-	25.4 (10.8)	35.9 (17.9)	32.6 (27.8)	

None of the OSF biopsy specimens were classified as exhibiting mild atypia, and most had moderate atypia. There was no significant correlation between the dysplasia index of Smith and Pindborg (1969) and the staging of the disease.

Table 3.9. The growth fraction and epithelial dysplasia score for the different grades of oral submucous fibrosis as defined by Sirsat and Pindborg (1967).

Stage	No.	Growth Fraction	Epithelial	Number of categories
		Mean (SD)	dysplasia.	of dysplasia
			Mean	Mean
Very Early	1	18	4	2
Early	7	25.4 (10.8)	8	4
Moderately advanced	10	35.9 (17.9)	10	5
Advanced	5	32.6 (27.8)	11	6
Normal	29	7.6 (3.6)	1	1

An attempt was made to ascertain if the growth fraction, as determined by PCNA correlated with the dysplasia index as presented by Smith and Pindborg. Table 3.9 shows that the dysplasia index changes with the stage of OSF but not with the growth fraction. (standard deviation for the median of the dysplasias was between 0.8 and 6.2, demonstrating the lack of significance of these results).

The growth fraction in moderately advanced and advanced stages of OSF was significantly higher ($p < 0.001$) than in normal mucosa.

3.4. Discussion

The use of immunohistochemical methods can potentially simplify and expand the clinical analysis of proliferating cells, as it preserves tissue architecture and cell to cell relationship and viable tumour cell areas may be evaluated and separated from necrotic areas with a simultaneous evaluation of tumour cell kinetics and histology. This is the reverse of the situation of flow cytometry, where the tissue is lost to further followup. Radiolabelling with tritiated thymidine also has a number of drawbacks, including the requirements of fresh, viable tissues for thymidine incorporation, and the uncertainties inherent in *in vitro* labelling techniques. *In vivo* labelling with radioisotopes can usually only be used in the experimental animal model, due to ethical considerations in man

3.4.1. Growth Fraction

Recently a number of papers have presented PCNA as being an excellent marker for cells in S-phase in breast carcinoma (Dierendock et al, 1991), and lymphomas (Kamel et al, 1991). Indeed Tsuji et al (1992) in a study of oral squamous cell carcinomas were able to demonstrate that PCNA positivity correlated well with the differentiation and atypia of the tumour cells - this correlation was lost in poorly differentiated tumours. The Tsuji et al (1992) study showed immunopositive cells only in the basal layer in normal tissue, and in more diverse regions in premalignant and malignant lesions. The present study was able to demonstrate a relationship between the growth fraction and the Bryne index, which is a combination of the dysplastic and invasive characteristics of the tumour and the hosts response. The tumours with a Bryne index of ≤ 10 had a growth fraction of 28.7% (SD = 17.4) while those with a score of >10 had a growth fraction of 41.0% (SD = 19.5) (Table 3.7). This was a significant difference ($p <$

0.03). It was also remarkable that the specimens which failed to stain positive for PCNA were from the poorly differentiated group of oral carcinomas. However there was no significant difference between the growth fractions of the three differential histological grades of oral cancer studied: well, moderately and poorly differentiated (as recorded on the pathology report of the hospital). This might indicate that a semi-quantitative system for grading, like the Bryne index, correlates better with the proliferative activity than the previous conventional grading system for oral squamous cell carcinoma, which concentrates solely on tumour differentiation (not a systematic numerical system). However, the present study did not go on to compare the Bryne index and the conventional grading system (well, moderately and poorly differentiated) in their correlation with patient survival .

The growth fraction of the OSF tissue is 31.8% (SD 21.9) of basal cells which is significantly different to that of the clinically normal ($p < .001$) and keratoses ($p < .001$), though not so when compared with the oral carcinomas ($p < 0.1$). (Significantly there is one case of oral submucous fibrosis which has a squamous cell carcinoma present in the same section - Fig 1.3 & 4)

Another feature of OSF is that although there is a high growth fraction the epithelium is frequently normal or atrophic in regards to thickness. This feature would suggest that either the cells are caught in G_1/S phase, or perhaps there is an increased rate of loss of the epithelial cells via rapid shedding of the superficial cells or through apoptosis. (A number of the sections demonstrate civatt bodies, sometimes in association with lichen planus - a finding also reported by Pindborg and Mehta et al, (1970)

3.4.2. Negative immunoreactivity

Negative reactivity was observed in 6.8 % (3/44) of the cases of carcinoma. The literature also presents variable results even with the use of monoclonal antibodies, for the fraction of PCNA positive cells (Kurki et al 1988, Garcia et al, 1989). At the present time, from this study and other reports, the PCNA index does not appear to reliably correlate with the conventional WHO-type histological grading of oral squamous cell carcinoma.

The results from the present study show that the growth fraction of basal epithelium in clinically normal tissue is 7.6% (SD 3.6) of basal cells, compared with the results of Tsuji et al (1992) where the growth fraction was determined to be 9.8% (SD 4.9). The growth fraction of the squamous cell carcinomas in the present study -39.4 % (SD 22.4) is of the same order as the result of 43.8 % (SD 6.0) obtained by Tsuji et al. However, the standard deviation in the present study differs widely from that of TSuji et al . The possible reasons for this may be the wide degree of heterogeneity in the tumours, and the variation in staining of different tumours (despite the positive and negative controls being standardised throughout the study). This in turn might result from differing duration of fixation of the tissue specimens, and the type of fixative used. Table 2.2 demonstrated the loss of immunological recognition for the PCNA protein with increasing fixation time, for clinically normal oral mucosa. Although the table was specifically for clinically normal tissue, it is reasonable to assume that the same would apply to tumour tissue - that the PCNA negative tissue is possibly an artefact of the fixation and processing procedures. As was noted (2.12.1.) the buffered formal saline is an additional complication as the hospital has been experimenting with different media for the past 18 months in an attempt to improve their immunohistochemical results. This was unknown to the candidate until late in the study. To examine whether this is a significant factor the various fixation media should

be analysed against PCNA antigenicity and time of fixation. The study of Tsuji et al attempted to standardise the tissue fixation and preservation.

3.4.3. Tobacco Habit

Amongst the normal controls 31% (11/36) had a tobacco habit, while amongst those patients with oral squamous cell carcinoma 76% (32/42) used tobacco in some form or other. This is a similar proportion to that found in the study of Field et al (1991)

The growth fraction of patients with oral submucous fibrosis failed to demonstrate a significant difference between those patients with, and those without, an oral habit - only three patients had no habit, and of the patients who had a habit none of them smoked without also using the areca nut - a factor also noted by Daftary et al (1991).

Chapter 4

The 'tumour suppressor gene" - p53: A possible marker for high risk carcinoma and premalignant states.

4.1. Introduction

In head and neck cancer treatment the control of the tumour and survival remain disappointing despite the advances in surgery and radiation therapy. A number of determinants have been advocated as good prognostic indicators, and the clinical staging is perhaps the major one (Schuller et al, 1980, Kramer et al, 1986, Hahn et al, 1987). However this remains a 'late stage' prognostic indicator, and a multi-disciplinary approach has been advocated (Ervin et al, 1985).

Some studies have analysed the pattern of failure of tumour treatment, either considering a specific tumour site (Bradfield and Scruggs, 1983, van den Bogaert, et al, 1983, Bataini et al, 1985) or head and neck tumours in general (Kramer et al, 1986). These have generally shown that recurrence at the primary site is the main cause of failure in tumour treatment.

Bryne et al (1989) was able to demonstrate the value of histopathological grading of oral squamous cell carcinoma in the buccal and maxillary alveolar mucosa. It may be possible to enhance Bryne's malignancy grading by using an immunohistochemical approach to probe for the markers associated with cell proliferation (growth fraction) which might in turn be related to tumour behaviour.

4.2. Control of Cell Proliferation

The part played by the tumour suppressor gene, p53.

Studies undertaken by Mercer et al (1990) demonstrate that the presence of wild type (wt) human p53 protein limits the proliferation of cells in culture (hamster cells transformed by simian virus 40), whereas mutant forms of p53 had no anti-proliferative effect on these cells. This observation led to the postulate that expression of p53 protein correlates with inhibition of cell cycle progression and that cell cycle inhibition is accompanied by selective down-regulation of PCNA mRNA, and protein expression

A number of authors (Baker et al, 1990, Mercer et al, 1990) have provided experimental evidence showing that human p53 protein has a growth suppressing activity which is abrogated in mutated forms of the protein. However, the work by Mercer et al (1991) links this activity with the observed changes in the expression of the proliferative cell nuclear antigen (PCNA). The down regulation of the PCNA expression is not a consequence of the inhibition of DNA synthesis *per se*, because PCNA mRNA is not down-regulated when DNA replication is inhibited by drugs which hold the cell in S phase (aphidicolin, or hydroxyurea). The conclusion drawn by

Mercer et al (1991) is that down-regulation of PCNA expression may be a direct consequence of the expression of wt p53 protein.

While the study by Mercer et al (1991) provides the evidence that expression of wt-p53 protein is associated with the down-regulation of another cellular growth-regulated gene, the molecular mechanism by which p53 exerts its control over gene expression is presently unknown. There are many steps and components involved in the progression of cells through the G1 and S phases, and it is more than likely that expression of other cellular genes could also be modulated by wt-p53. It is uncertain whether the effect of p-53 on PCNA is exerted indirectly of other cellular suppressor genes(Kern et al, 1991) or by direct action (Braithwaite, 1987, Gannon and Lane, 1987)).

In the OSF oral mucosal biopsies from Nepal, the proportion of actively cycling epithelial cells, as detected by PCNA, was significantly higher in comparison to that of normal mucosa from control subjects both from Australia and from Asia (Table 3.5.). It therefore seems not unreasonable to suggest that these results arise from a loss of control of the normal cell proliferation rates. It was pertinent in the present study, therefore, to look for any correlation between the growth fraction and p53 expression in OSF and to compared this with that of clinically normal oral mucosa and that of oral squamous cell carcinomas.

4.3. Theories of carcinogenesis

4.3.1. Background

The Knudson hypothesis (1985) states that any tumour requires two events and that these events involve the same gene whether the tumours are of an hereditary or non-hereditary form. In reality any cancer arising from a single 'step' must be rare, because age specific incidences of virtually all carcinomas suggest at least two steps in their origin. An autonomously oncogenic mutation in the germ line, stem cells, would almost certainly be lethal, because every target cell would be malignant. Any non-lethal constitutional abnormalities of oncogenes would necessarily be less severe and probably require another event to produce a cancer (Knudson, 1985). It has also been shown that even in the absence of oncogenic mutations two steps are necessary for the *in vitro* transformation of normal cells by DNA transfected from human tumours.

The somatic mutation hypothesis of Knudson implies that there are genetic loci with mutations that lead to cancer (Knudson, 1983). Thus any attempt to understand precancer and the process of carcinogenesis would necessarily be concerned with discovering these genes and understanding their behaviour in health and disease (Knudson, 1985). There are two broad classes of such genes identified to date: they consist of the oncogenes (tumour inducing genes - originally discovered through the study of critical genes in the acutely transforming retroviruses); and the second class is the anti-oncogenes or tumour suppressor genes (identified through the study of hereditary tumours) (Knudson, 1983 a & b).

4.3.2. Oncogenes

The theory of a multi-step process in the formation of a tumour has long been considered, at least as early as the 1950's (Foulds, 1958). However, it has only been recently possible for the molecular basis of this statement to be examined, with the process of initiation and progression of human tumours (Bishop, 1987, Weinberg, 1989)

The normal, non neoplastic cell, has two basic controlling influences to stimulate growth: the external stimuli for growth, in the form of 'growth factors' [growth factors may act on adjacent cells (paracrine effect), or on the cell producing the factor (autocrine)], and the internal - signals coded by proto-oncogenes, which are genes present in normal cells which influence growth, proliferation and differentiation. The mechanism of action is via the production of various protein products that form an intra-cellular communication network. The protein products of proto-oncogenes influence growth at one or more steps in the growth signalling pathway.

The main feature in cancers is that the normal cell control seems to be absent. The common mechanism for cancer initiation appears to be through damage to the DNA resulting in uncontrolled cell proliferation, for cancer cells do not seem to have the same growth control restraints that affect normal cells.

4.3.3. Proto-oncogenes and tumourigenesis

Some agents which initiate carcinomas, such as viruses, affect proto-oncogenes by stealing genes, by inducing translocations, or by inserting genes, near to or within proto-oncogenes and thus influencing their activity. It is the transformed proto-oncogenes, (producing abnormal products, with loss of normal function), which are termed 'oncogenes'. Oncogenes can thus lead to the uncontrolled proliferation of malignant neoplasia.

Oncogene activation is not an uncommon event, yet only rarely does a malignancy result. Thus other changes must also occur. Oncogenes may be involved in the different stages of neoplasia, some in tumour formation or initiation, others in promotion, progression or metastasis. The same oncogene is not necessarily involved in individual tumours and indeed most tumours result from changes inflicted by a number of oncogenes, frequently four or five (Fearon and Vogelstein, 1990).

4.3.4. Suppressor genes

Experimental work has shown that if cultured transformed cells are hybridised with normal cells then the malignant characteristics of the cell line are inhibited (Mercer et al, 1991, Yin et al, 1992) This suggests that the normal cells contain a gene which suppresses cancer development, and that this gene is dominant. These tumour suppressor genes (also known as anti-oncogenes, or recessive cancer susceptibility genes) have now been identified on at least three chromosomes - 11, 13, and 17. Whether these genes act by regulating oncogenes or by possibly producing proteins that antagonise the effects of onco-proteins, is unknown. However, a mutation or

deletion of these tumour suppressor genes may result in a defective suppressor protein, and consequently a loss of tumour suppression.

4.4. Human cancer and p53

Mutations in the p53 gene are turning out to be the most commonly observed genetic alteration in human cancers (Cattoretti et al, 1988, Rodrigues et al, 1990, Hollstein et al, 1991, Levine et al, 1991). The p53 gene appears to function as a tumour suppressor gene - negatively regulating the cell cycle and requiring loss of function mutations for tumour formation. In addition, expression of cDNA or genomic clones of wild-type p53 suppresses the transformation of cells in culture by other oncogenes (Finlay et al, 1989), the growth of transformed cells in culture (Martinez et al 1991), and the tumorigenic potential of cells in animals (Chen et al, 1990). Finally, deletions or mutations of wild-type p53 alleles occur in several animal (Mowat et al, 1985) and human (Baker and al, 1989) tumours.

4.5. The tumour suppressor gene p53

The p53 gene produces a protein, which is a 375-amino-acid, 53-kilodalton nuclear phosphoprotein. The normal p53 protein has a very short half-life (6-20 minutes)(Lane and Benchimol, 1990, Field et al 1991).

The human gene lies on chromosome 17p (Soussi et al, 1990)

4.5.1. Null allele of the p53 gene

Donehower demonstrated that the loss of p53 function is sufficient to predispose animals genetically to spontaneous tumour development, and that a p53 oncogenic point mutation is not required as an initiating factor (Donehower et al, 1992).

Recent studies of the p53 null allele in thymocytes demonstrated that these cells are extraordinarily resistant to the induction of cell death following irradiation, when compared to the normal thymocyte (Clarke et al 1993, Lowe et al 1993). These studies focused on the role of p53, via the process of apoptosis, in the control of abnormal cell proliferation.

4.5.1.1. Apoptosis and the p53 null allele

Apoptosis was first described in 1970's (Kerr et al, 1972) and is the process of an apparent programmed cell death. This process can be induced by normal developmental control processes, but can also be induced by toxic insults, especially by any agents that damage DNA.

High levels of p53 can be induced by DNA damage and this observation has led to the concept that p53 might be part of a damage-control pathway rather than the normal pathways that are present in normal development (Kastan et al, 1991).

The studies of Lowe et al (1993), and Clark et al (1993) demonstrate that the effect, on the cell, of radiation is dependent on the number of p53 genes which are present : cells from heterozygous mice which contain only one copy of the p53 gene are slightly more resistant to radiation induced apoptosis than are homozygous normal mice with two copies (which are highly sensitive), while the homozygous null mice with no active

copies are highly resistant. If this information is applied to our understanding of tumour progression, it implies that cells, in which one p53 allele has been somatically mutated, may have a survival advantage in the presence of genotoxic agents over those that have two intact copies of the gene. This in turn could make them more likely to survive a second mutation at the p53 locus (frequent in cancer cells) or any other locus.

The mechanism by which p53 induces apoptosis is unclear. It may activate transcription of apoptosis inducing genes (Kastan et al, 1992), alternatively, p53 may interact more directly with the DNA repair mechanism, perhaps increasing its fidelity, but delaying repair so that lesion-induced apoptotic signals are sustained (Branch et al, 1993)

This inter-relation of p53 and apoptosis would appear to be a significant defence mechanism to protect the organism from the propagation of cells that have sustained mutation following genotoxic insult.

4.5.2. Mutation in p53

Mutations in p53 genes, usually in the form of simple base substitutions, are the commonest cancer-related change at the gene level, and they show several characteristics. Most occur within a restricted region of the gene and are concentrated within four highly conserved domains - residues 117-142, 171-181, 234-258, and 270-286 (Levine et al 1991). Their frequency and distribution vary for different cancers in different tissues, and they can be categorised as distinctive "mutational spectra" according to the types of mutation and their location. Different mutational spectra appear to reflect different aetiological agents (Carter, 1992).

The work of Nigro et al (1989) highlighted three important features:

- a) most tumours with allelic deletions for one p53 allele contain point mutations of the genome which resulted in amino acid substitutions (thus the mutations were significant, resulting in an altered protein);
- b) such mutations are not confined to tumours with allelic deletion, but also occur in at least some tumours that have retained both parental 17p alleles; and
- c) p53 mutations are clustered in four 'hot-spots' which exactly coincide with the four most highly conserved regions of the gene. These results suggest that p53 mutations play a role in the development of many common human malignancies (Nigro et al, 1989).

Nigro et al (1989) further presents the hypothesis that mutations in the p53 gene accumulate during the process of tumourigenesis, and through a dominant negative effect (Sturzbecher et al, 1988, Baker et al, 1989, Eliyahu et al, 1989, Finlay et al 1989), cause tumour progression. The dominant negative effect may be mediated by binding of the mutant p53 product to the wild-type product, creating an inactive oligomeric complex which is much more stable than the wild type p53. Due to the fact that wild-type products remain in the cell, however, a further loss of growth control can result when the wild-type allele is deleted, leaving the cell with only a mutant allele (Nigro et al, 1989). Indeed, it has been shown that the loss of a chromosome 17p allele (tumour suppressor gene, p53) is significantly associated with tumour progression in the human host (Kern et al, 1989).