QUANTITATIVE MORPHOLOGICAL ASPECTS OF EARLY EXPERIMENTAL ORAL CARCINOGENESIS

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"The best laid schemes o'mice and men

Gang aft a-gley."

Robert Burns

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PREFACE

The experimental work described in this thesis was undertaken in the departments of Pathology at Glasgow Royal Infirmary, Glasgow Dental Hospital and the department of Medical Oncology at Glasgow University from October 1974 to August 1977. In the initial stages of the work the author was a Nuffied Foundation Fellow in Pathology and later a lecturer in the department of Oral Medicine and Pathology of the University of Glasgow.

The work described was undertaken individually by the author or by technical staff under his direct supervision. Those parts of the thesis which are repetitions of previously published work are indicated in the appropriate sections.

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CHAPTER ONE

ORAL CARCINOGENESIS IN MAN AND ANIMALS

1.1 INTRODUCTION

In most parts of the world malignant neoplasms of the oral cavity are rare. In Great Britain and the United States oral cancer accounts for about 2-3 per cent of all malignant tumours (Binnie 1975) but there are areas of India where over 40 per cent of all malignancies are oral (Pindborg 1965a). Over 90 per cent of oral malignant neoplasms are squamous cell carcinomas (Waldron 1970), the remainder being mainly salivary gland carcinomas, soft and hard tissue sarcomas and melanomas.

Although oral cancer does not form a large proportion of total malignant tumours in Western civilisations, its importance lies in the very high morbidity and mortality with which it is associated. Despite the readily visualised and accessible nature of the oral tissues many patients present initially with the disease in an advanced stage when treatment is often disfiguring and the long-term prognosis poor.

At present the roles of the various aetiological factors thought to be associated with oral carcinogenesis are not clear and frequently several factors appear to be involved. Until such time as the disease can be prevented by recognition and elimination of the appropriate aetiological agents the most important prognostic factors

are early diagnosis and treatment.

A proportion of cases of intraoral squamous cell carcinomas appear to be preceded by or co-exist with other distinctive epithelial lesions and these lesions are thought to be precursor or premalignant (MacDonald 1975). Recognition of the disease at this stage may allow adequate treatment and offer the best chance of permanent cure under the present state of knowledge.

1.2 HUMAN ORAL PREMALIGNANCY

A premalignant lesion is one which is associated with a greater risk of malignant change than corresponding normal tissue. Premalignant conditions are distinguished by some authorities as generalised disturbances or diseases which predispose to the development of a neoplasm in a particular site. The latter term does not necessarily imply the existence of an observable change in the site before malignant transformation (Smith 1976). Both these terms need to be distinguished from tumour progression which is the development of a malignant neoplasm from its benign counterpart (Butler and Jones 1976).

The reasons for believing a given type of lesion to be premalignant may be several. Firstly, if followed over a sufficiently long period the lesion may be seen to undergo malignant transformation. The lesion may be found frequently in association with a particular type of neoplasm and histopathological examination may reveal cytological features similar to those seen in frank carcinomas but without

evidence of invasion. A number of lesions of the oral mucosa have been suspected as premalignant and the more frequent of these will be considered in detail.

Considerable confusion has arisen over the terminology used to describe the various white patches which can arise in the mouth, in particular the precise meaning of the term "leukoplakia", the oral lesion most commonly alleged to have a premalignant potential (Sprague 1963). In an attempt to clarify this problem the World Health Organisation accepted a definition of leukoplakia as "a raised, white patch of oral mucosa measuring 5mm or more which cannot be attributed to any other diagnosable disease" (Mehta et al 1971). Many authors, however, tended to disregard the somewhat arbitary size stipulated in this definition. In the latest communication from the WHO Collaborating Centre for Oral Precancerous Lesions (1978) the definition was simplified to "a white patch or plaque that cannot be characterised clinically or pathologically as any other disease". Thus diagnosis of leukoplakia is essentially clinical and derived by exclusion and the term has no specific histological implications. Lucas (1978) has pointed out that this definition will be refined in the future as distinctive entities are recognised and eliminated from the general group.

Epidemiological studies of the prevalence of leukoplakia in the general population have been few. In Hungary, the reported incidence of leukoplakia varied from 0.6 per cent

to 3.6 per cent (Bruszt 1962; Bánóczy, Radnai and Reményi 1969) and in India from 0.2 per cent to 4.9 per cent depending on the district (Mehta, Pindborg and Hamner 1971).

The reported incidence of malignant change in leukoplakia shows wide variation in different sites and population groups and has tended to show a marked decline in the last few decades. The older literature gave an average of 30 per cent of cases of leukoplakia eventually developing carcinomas (Shafer and Waldron 1961). More recent studies using larger numbers of patients and less highly selected groups give an incidence of malignant transformation in the region of 3 to 6 per cent (Einhorn and Wersäll 1967; Pindborg, Barmes and Roed-Petersen 1968; Silverman and Rozen 1968; Bánóczy and Sugár 1972). The period prevalence probably gives a more accurate indication of the risk of malignant transformation. For example, Einhorn and Wersäll (1967) noted that oral cancer developed in 2.4 per cent of patients with leukoplakia after 10 years, but this had risen to 4 per cent after 20 years. The reasons for the decline in the incidence of malignant transformation are not wholly clear but may be related to improved diagnosis and awareness of intraoral white patches, more adequately controlled epidemiological studies, the reduced incidence of syphilitic leukoplakia and the general decline in the incidence and morbidity of oral cancer.

The commonest clinical presentation of leukoplakia is

a homogeneous white plaque. Pindborg et al (1963) drew attention to a rarer variant which consisted of white plaques intermingled with erythematous areas which was called speckled leukoplakia. Some authors also recognise a verrucous variant (Bánóczy and Sugár 1972). The major significance of the different clinical types of leukoplakia is that speckled leukoplakia and to a lesser extent verrucous leukoplakia, are more likely to undergo malignant transformation than the homogeneous type (Pindborg et al 1963; Bánóczy and Sugár 1972).

Some studies have attempted to compare the incidence of leukoplakia in particular sites with the incidence of malignancy in those sites. In many cases there has been no significant correlation. For example, Bánóczy and Sugár (1972) found that while 62.8 per cent of cases of leukoplakia involved the commissures and buccal mucosa, these sites accounted for only 19.4 per cent of oral carcinomas, whereas on the tongue, in which 38.8 per cent of oral carcinomas arose, leukoplakia was seen in only 8.5 per cent of cases.

It has been recognised recently that homogeneous leukoplakia of the floor of the mouth and ventral lingual mucosa behaves rather differently from that elsewhere (Kramer, El-Labban and Lee 1978). At one time lesions of this type were thought to be developmental in origin and were called oral epithelial naevi (Cooke 1956). However, Pindborg, Roed-Petersen and Renstrup (1972) thought that leukoplakias in the floor of the mouth were not developmental

in origin and could indeed be premalignant, a view repeated and reinforced by the retrospective study of Kramer et al (1978) which showed that 24.1 per cent of patients with such lesions developed oral carcinomas. Kramer and his co-workers felt the lesion was distinctive enough to justify its inclusion in a separate category of leukoplakia and called it "sublingual keratosis".

Syphilitic leukoplakia is a manifestation of tertiary syphilis and is secondary to chronic interstitial glossitis. Until fairly recently this was a common cause of leukoplakia (Hobaek 1946) and was frequently associated with malignant transformation and carcinomas in the anterior two thirds of the tongue (Wynder et al 1957; Cawson 1969; Bánóczy and Sugár 1972). Smith (1973) has pointed out the possible influence of medicaments such as arsenicals and heavy metals used in the treatment of syphilis and the subsequent development of oral carcinoma.

Jepsen and Winther (1965) in an investigation of oral keratotic lesions frequently found hyphal forms of Candida albicans, especially in cases of speckled leukoplakia. Cawson (1966) and Renstrup (1970) also found candidal infection could be associated with leukoplakia, especially of the speckled variety. Bánóczy and Sugár (1972) found a 61 per cent incidence of Candida in cases of speckled leukoplakia and in addition noted that 65 per cent of cases of leukoplakia which had undergone malignant transformation contained Candida. Whether candidal infection is a causal factor in oral leukoplakia or merely a secondary invader

of altered epithelium has been a question of some controversy. However, Cawson (1969; 1976) has argued strongly for an aetiological role of Candida in proliferative mucosal lesions. He pointed out that leukoplakia is commonly seen in children with mucocutaneous candidosis but is rare in normal youngsters. He thought the fact that Candidal hyphae are intracellular parasites indicated a means by which the behaviour of epithelial cells could be altered (Cawson and Rajasingham 1972). In addition proliferative plaques of candidosis can be produced experimentally on the chick chorioallantoic membrane (Cawson 1973). The precise relationship between candidal infection and malignant transformation of leukoplakia is not certain, but Cawson (1969) found that six out of ten patients with chronic hyperplastic candidosis eventually developed carcinoma. Candidal involvement appears to be a relatively common feature of speckled leukoplakia, which, as described previously, has a greater propensity for malignant transformation than homogeneous leukoplakia.

Leukokeratosis nicotina palati (stomatitis nicotina) is a specific type of leukoplakia which affects the palate and presents as white patches with red umbilicated centres which are the dilated orifices of the minor salivary gland ducts (Thoma 1941). It is seen most frequently in pipe smokers and reverse cigarette and cigar smokers. In Western civilisations the condition does not appear to have a significant premalignant potential but in parts of India

there appears to be a close correlation between reverse smoking, leukokeratosis nicotina palati and carcinoma of the palate (Ramulu et al 1973).

Although white patches or plaques in the mouth are the lesions usually associated with premalignant potential it is becoming increasingly apparent that red lesions are more dangerous (Cawson 1975). Such lesions are called erythroplakia, or more rarely erythroplasia, and have been defined as well-demarcated red, often fiery red, patches which cannot be attributed to other causes (Mehta, Pindborg and Hamner 1971). The importance of erythroplakia is emphasized by the study of Mashberg, Morrissey and Garfinkel (1973) of asymptomatic, early squamous cell carcinomas. They found that over 90 per cent of such lesions had an erythroplakic component whereas less than 7 per cent were solely white. Erythroplakia is uncommon. For example, in the survey of 50,000 Indian villagers by Mehta, Pindborg and Hamner (1971) only 9 cases were recorded, most on the buccal mucosa. Some cases are flecked with white and are called speckled erythroplakia (Shear 1972) but lesions of this type may be indistinguishable from speckled leukoplakia. The most significant feature of erythroplakic lesions is the large proportion of cases which show histological features of epithelial dysplasia which may range from mild to severe, and not infrequently squamous carcinoma is present at the time of biopsy (Shear 1972).

Oral submucous fibrosis is a disease seen almost

exclusively in Indians and is characterised by dense fibrosis of the corium, especially in the buccal mucosa and soft palate. The related muscles undergo atrophy, as does the overlying epithelium and in many cases there is hyperkeratosis and consequent leukoplakia (Pindborg 1965b; Pindborg, Mehta and Daftary 1970). The recent epidemiological data has been reviewed by Pindborg (1972) who concluded that there was a significant association between oral submucous fibrosis and carcinoma of the mouth and that submucous fibrosis was a premalignant condition.

Sideropaenic dysphagia (Kelly-Paterson syndrome; Plummer-Vinson syndrome) which comprises dysphagia, anaemia and glossitis is associated with an increased incidence of post-cricoid carcinoma and also oral carcinoma (Wynder and Fryer 1958). The syndrome has a high incidence in Sweden and is thought to account for the relatively high incidence of oropharyngeal tumours in Swedish women (Watts 1961).

The evidence for and against an association between lichen planus and oral cancer is discussed in detail by MacDonald (1975). He believes that the available evidence points to such an association and Kövesi and Bánóczy (1973) have recorded that malignancy may supervene in erosive or atrophic lichen planus. There is little evidence to support associations between chronic ulcers and fissures of oral mucosa (Lucas 1976) or squamous papillomas (McCarthy and Shklar 1964; Shklar 1965a) and

the development of oral carcinomas.

1.3 EXPERIMENTAL CHEMICAL CARCINOGENESIS

1.3.1 Historical Introduction to Experimental Chemical Carcinogenesis

Sir Percival Pott (1775) noticed that the high incidence of cancer of the scrotum seen in chimney sweeps appeared to be related to prolonged exposure to soot and introduced the concept of an association between environmental factors and carcinogenesis. However, in the following 140 years attempts to verify this concept experimentally were singularly unsuccessful. Most early investigators used crude tar oil as a carcinogen, but their experiments failed because they either used unsusceptible animals like the rat (Hanau 1889) and dog (Cazin 1894) or did not apply the carcinogen for sufficiently long periods (Haga 1913). Bayon (1912) reported abundant epithelial proliferation but no invasion four weeks after injecting tar into a rabbit's ear. The first chemically-induced invasive neoplasms were produced by Yamagiwa and Ichikawa in 1914 (Yamagiwa and Ichikawa 1918) by regularly painting the ears of rabbits with coal tar. This success stimulated a large volume of work on experimental tar carcinogenesis which was reviewed at length by Woglom (1926). In the 1920's Kennaway and his co-workers synthesised the first carcinogenic hydrocarbon 1,2,5,6 di-benzanthracene in pure form and later Cook, Hewett and Hieger (1933) isolated the powerfully carcinogenic

hydrocarbon 3,4 benzpyrene from coal tar. Numerous pure chemical carcinogens have since become available and have been used to study carcinogenesis in a wide variety of sites and animals.

1.3.2 Historical Introduction to Experimental Oral Chemical Carcinogenesis

Early attempts to produce experimental malignant oral tumours were largely unsuccessful, the oral mucosa being considerably more resistant to the actions of chemical carcinogens than skin. Bonne (1925) noticed that some mice whose skin had been painted with coal tar developed papillomas in their mouth and stomach, probably due to tar which had been ingested when the animals licked themselves. Later, Bonne (1927) reported three squamous cell carcinomas developing in the palates of 50 mice which had been treated with coal tar for over a year. Leukoplakia was described in the palates of rabbits which had been repeatedly exposed to cigarette smoke for prolonged periods (Roffo 1930). Oyama (1935) succeeded in producing lingual carcinomas in 2 out of 16 rabbits by injecting coal tar into the tongue. Levy (1948) however, failed to produce any neoplastic or preneoplastic changes in the labial gingiva of mice painted with 20 methyl-cholanthrene for up to 16 weeks and Kreshover (1952) found murine labial mucosa to be resistant to repeated applications of tobacco smoke.

The essential combination of a susceptible tissue and a potent carcinogen was discovered by Salley (1954) when he

showed that local applications of one of several polycyclic hydrocarbons could induce squamous cell carcinomas in the thin lining mucosa of the hamster cheek pouch. Since this discovery malignant neoplasms have been produced in a variety of animals in several intraoral sites and the experimental models described in the literature will be considered in detail.

1.3.3 Experimental Chemical Carcinogenesis in the Hamster Cheek Pouch

The anatomical and physiological features of the hamster cheek pouch were described by Keyes and Dale (1944). The first documented attempt to produce tumours in this site was by Wantland (1954) who sprayed and painted 20 methylcholanthrene, 1, 2, 5, 6 di-benzanthracene and 2 acetyl-aminofluorine into the hamster cheek pouch for up to six weeks. The only change detected was epithelial hyperplasia.

The first successful attempt to produce tumours in the pouch was by Salley (1954). He investigated the effects of the powerfully carcinogenic polycyclic hydrocarbons 9, 10 dimethyl -1, 2-benzanthracene (DMBA), 20 methyl-cholanthrene (20 MC) and 3, 4 benzpyrene (3, 4 BP) dissolved in either acetone or benzene. Each of these carcinogens was painted onto the pouch thrice weekly for 16 weeks and the animals were observed at intervals for an additional nine weeks. During the first two weeks there was an inflammatory phase with necrosis and sloughing of the distal part of the pouch. This was followed by healing

and shrinkage of the pouch. The mucosa subsequently passed through four histologically recognisable stages; hyperplasia, papilloma, carcinoma-in-situ and invasive squamous cell carcinoma, with or without metastases. DMBA in acetone was the most effective carcinogen and the first tumours appeared after seven weeks of painting. Although 20 MC and 3, 4 BP were effective carcinogens the latent period was long (16-25 weeks) and the tumour yield was low.

In a later communication Salley (1957a) described the early changes occurring in carcinogen-treated cheek pouch in more detail. White patches resembling human oral leukoplakia developed after eight or nine applications of carcinogen. Histologically the pouch wall passed through four distinct stages before the appearance of overt neoplasms, these being inflammation, degeneration, regeneration and hyperplasia.

Attempts to standardise the experimental techniques used for hamster cheek pouch carcinogenesis were made by Morris (1961). He demonstrated that the pouch epithelium of old hamsters was more resistant to the action of DMBA than that of young animals. From the standpoint of ease of manipulation and tumour production five weeks appeared to be the optimum age for commencing studies on experimental oral carcinogenesis. A 0.5 per cent solution of DMBA in acetone produced the maximum tumour yield with minimum latent period and no loss of animals. A 1.5 per cent solution, however, was associated with high

morbidity and mortality and a 0.1 per cent solution produced tumours only after a prolonged latent period. A 0.05 per cent solution was not effective in producing tumours. A shorter latent period was required for tumour production when animals were painted three times per week as opposed to twice weekly, but the animals sex and caging conditions did not affect the tumour yield.

Morris and Reiskin (1966) investigated the response of cheek pouch mucosa to varying lengths of carcinogen exposure to determine whether a critical duration of exposure to the carcinogen was necessary for tumour induction. They found that painting the pouch three times a week for four weeks resulted in tumours developing in all animals, but that painting for less than three weeks failed to produce any tumours. This finding implied that the critical change responsible for subsequent malignant transformation had occurred following an exposure of three to four weeks. At this stage no gross or histological changes indicating that tumour formation was destined to occur could be detected.

MacDonald (1978) discussed how the response to carcinogen could be more effectively localised. Animals painted with a 0.25 per cent solution of DMBA had significantly fewer tumours developing outside the experimental area than hamsters painted with a 0.5 per cent solution of carcinogen. There was, however, no significant difference in the yield of tumours within the experimental areas between the groups painted with different concentrations

of carcinogen.

Although Salley (1954) reported frequent cervical lymph node metastases in his original paper, he did not mention metastases in later papers and indeed most subsequent workers have failed to demonstrate metastases despite thorough searches of regional lymph nodes and internal organs. Rwomushana, Polliack and Levij (1970) reported one case of cervical lymph node metastasis out of 562 carcinogen-treated hamsters. Recently, however, Craig (1977) has shown that cervical lymph node metastases can be produced by extending the duration of the tumour-bearing period.

DMBA has been dissolved in a variety of solvents which have been found to influence the latent period, morbidity, mortality, distribution and yield of tumours. Salley (1954) found that acetone was less toxic and was associated with a lower mortality and shorter latent period than benzene. Heavy mineral oil solvent reduced the latent period for tumour production from seven weeks (as seen with DMBA in acetone) to four and a half weeks (Salley 1955; 1957a). This reduction in latent period was probably due to increased tissue penetration of carcinogen rather than a true co-carcinogenic action (Berenblum and Shubik 1947; Salaman and Roe 1964). As Stormby and Wallenius (1964) claimed that mineral oil was prone to cause digestive tract disturbances, some workers have preferred to use liquid paraffin (Smith 1968; Franklin 1977). Attempts to localise and prolong the carcinogenic effect have been made by

incorporating DMBA into the adhesive vehicle Orabase (Renstrup, Smulow and Glickman 1962). Dimethyl sulphoxide (DMSO), a solvent which readily penetrates skin, has been shown to reduce significantly the period for tumour production (Dachi, Sanders and Urie 1967; Shteyer and Lalonde 1974) and increase the tumour yield (Elzay 1967). Some workers however, have produced contradictory results using DMSO solvent. Siegel and Shklar (1969) and Shklar, Turbiner and Siegel (1969) for example, found that animals treated with DMBA in DMSO developed fewer neoplasms than animals treated with DMBA dissolved in heavy mineral oil.

Relatively few investigators have studied electron microscopical changes in carcinogen-treated cheek pouch. Listgarten, Albright and Goldhaber (1963) demonstrated a marked widening of intercellular spaces within two days of DMBA application but also observed similar changes when a non-carcinogenic irritant was used. Carcinoma cells showed clumping of tonofibrils at the cytoplasmic periphery. Bulbous epithelial cell pseudopodia which project through the lamina densa have been described in preneoplastic lesions of the cheek pouch (Woods and Smith 1969a; 1970; MacDonald 1973; McKinney and Singh 1977). These pseudopodia, however, do not appear to be specific to carcinogen-treated mucosa, being produced also by applications of 4-hydroxyanisole, (Woods and Smith 1969b) a chemical of no known carcinogenic activity. The pseudopodia produced by 4-hydroxyanisole, however, regress soon after

applications of the chemical are stopped, whereas those produced by DMBA persist following cessation of painting.

Changes in enzyme activity during cheek pouch carcinogenesis have been described. Alteration in the activity of several hydrolases and dehydrogenases were demonstrated by Mori et al (1962) in experimentally produced carcinomas. They found a marked increase in alkaline phosphatase activity in the stratum spinosum, especially in relationship to inflamed areas. They speculated that the increased enzyme activity was a result of inflammatory and regenerative changes in the carcinogen-damaged tissues. A progressive increase in the alkaline phosphatase activity in the basal and prickle cell layers of carcinogen-treated cheek pouch was found by Luthra, Bharadwaj and Wahi (1969). Smith (1972) also showed increased acid phosphatase activity in premalignant cheek pouch lesions. This activity occurred as fine droplets in the basal cells and similar droplets were seen in human oral leukoplakia. Smith (1972) suggested that these changes were due to alterations in lysosomes. A further important observation in this experiment was that in some instances the changes described preceded the development of epithelial atypia. Shklar (1965b) found that lactic dehydrogenase activity was markedly increased and succinic dehydrogenase activity decreased in carcinomas as compared with normal pouch. This observation suggested an alteration from aerobic to anaerobic respiration during carcinogenesis.

Cell cycle characteristics have been studied in the cheek pouch model. Reiskin and Berry (1968) and Thilagaratnam and Main (1972) showed that in DMBA-induced cheek-pouch carcinogenesis the cell cycle time is shorter than normal and the cell proliferation rate is higher. Thilagaratnam and Main (1972) found that the reduction in cell cycle time was progressive with advancing stages of carcinogenesis. All phases of the cell cycle were reduced, especially G1 which was shortened by about 95 per cent.

Berenblum (1954) discussed the concept that carcinogenesis is made up of at least two stages. In the initiating stage cells are irreversibly converted into tumour cells but these may remain latent or dormant. Subsequent treatment of such cells by an agent, which itself has no carcinogenic properties, causes them to undergo progressive growth. This process is called promotion. In addition, some agents augment the action of dilute carcinogens when applied simultaneously and are called co-carcinogens (Berenblum 1970a).

The hamster cheek pouch model has been used to examine a variety of carcinogenic, co-carcinogenic and promoting agents thought to be associated with human oral carcinogenesis. In addition, the influence of several systemic factors on the formation of oral neoplasms have been investigated. Although much of this work is empirical some interesting observations have been made.

In the experimental situation croton oil has been shown to exert a co-carcinogenic action with DMBA

in cheek-pouch carcinogenesis in older hamsters by Silberman and Shklar (1963). However, in young animals this combination resulted in a retardation of carcinogenesis which Silberman and Shklar (1963) thought was due to the extensive inflammatory reaction which resulted from croton oil applications to young animals in some way inhibiting the direct carcinogenic action of DMBA.

Agents thought to be of consequence in human oral carcinogenesis have also been investigated in the cheek pouch model. Alcohol has been found to act locally with DMBA as a co-carcinogen and as a promoting agent, reducing the latent period for tumour development and producing more aggressive neoplasms (Elzay 1966). Henefer (1966) studied the influence of 30 per cent ethanol as the animals sole source of fluid on DMBA-induced cheek-pouch carcinogenesis. Although he found no significant difference in tumour latency or incidence when compared with water-receiving controls, the small number of tumour-bearing animals in his series would preclude any definitive statement about the influence of alcohol consumption on oral carcinogenesis. Dachi (1962) showed that Tween 60 (polyoxyethylene sorbitan monostearate), a powerful tumour promoter in the skin, acted co-carcinogenically with DMBA in the cheek pouch. Tween 80 (polyoxyethylene sorbitan monooleate) however, appeared to have no influence on DMBA-induced cheek-pouch carcinogenesis (Sabes, Chaudhry and Gorlin 1959). Many of these experiments on co-carcinogenesis were less than ideal as most used the optimum tumour-producing concentration

of DMBA (0.5 per cent) which would tend to mask any weak co-carcinogenic action of the agents being examined.

The cheek pouch model has been used to examine the effects of whole cigarette smoke and cigarette smoke condensates (Tabah et al 1957; Kreshover and Salley 1957; Moore and Miller 1958; Kendrick 1964). All these studies yielded negative results but Elzay (1969) reported that whole cigarette smoke acted as a promoting agent but not as a co-carcinogen in DMBA-induced cheek-pouch carcinogenesis and was a more potent promoter than alcohol. Snuff and chewing tobacco (Peacock and Brawley 1959) and various ingredients of betel quid (Dunham and Herrold 1962) have also failed to produce neoplastic changes in the pouch. Dunham, Muir and Hamner (1966) reported that repeated applications of calcium hydroxide to the pouch produced inflammatory hyperplasia with occasional areas of epithelial atypia. Chang (1966) found that while separate applications of extracts of betel nut or slaked lime to the pouch produced hyperplastic and hyperkeratotic lesions, when these agents were applied together a few papillomas resulted.

Chronic mechanical irritation is another of the many factors implicated in the aetiology of human oral malignancy (Hobaek 1946; Watanabe 1970) and the effects of such irritation on the development of carcinomas have been studied in the cheek pouch. Renstrup, Smulow and Glickman (1962) showed that chronic mechanical irritation from a stainless steel wire ligated around a molar tooth and projecting into

the pouch, did not produce any tumours. However, this irritation decreased the latent period for carcinogenesis when used in combination with DMBA applications. Shteyer and Lalonde (1974) using a similar experimental model to that of Renstrup, Smulow and Glickman (1962) failed to demonstrate any enhancing effect of chronic mechanical irritation. Shklar (1968) showed that manipulation and incision of carcinogen-treated cheek pouch did not influence tumour yield, latency or spread.

The effect of the nutritional status of the hamster on cheek-pouch carcinogenesis has received little attention. A higher incidence of tumours in vitamin A-deficient animals was reported by Rowe and Gorlin (1959). These workers also showed that hamsters on a restricted calorie diet had a lower incidence of tumours than those fed ad libitum or on a vitamin A-deficient diet. In contrast, local applications of vitamin A palmitate had a potentiating effect on DMBA-induced cheek-pouch carcinogenesis when the vitamin was applied simultaneously with the carcinogen (Levij and Polliack 1968) before DMBA treatment (Levij, Rwomushana and Polliack 1969) or following DMBA treatment (Polliack and Levij 1969). Salley, Eshleman and Morgan (1962) showed that the latent period for tumour development in chronically thiamine-deficient hamsters treated with DMBA was significantly shorter than that for the control group. Dietary zinc excess was found to cause inhibition of cheek-pouch carcinogenesis by Poswillo and Cohen (1971) but Edwards (1976) failed to

show a distinct inhibitory effect of zinc on this form of carcinogenesis.

The influence of a number of hormones on hamster cheek-pouch carcinogenesis has been studied. Applications of cortisone prior to DMBA painting were shown to increase the incidence of tumours in the pouch (Sabes, Chaudhry and Gorlin 1963). Shklar (1967) and Siegel and Shklar (1969) found that topically applied cortisone and triamcinolone inhibited DMBA cheek-pouch carcinogenesis. Conflicting results have also been reported on the action of systemically administered steroids on cheek-pouch carcinogenesis. Thus, Shklar (1967) reported more rapid tumour development in cortisone treated animals and the development of larger and more deeply invasive neoplasms. Smith (1967), however, found no evidence that systemically administered cortisone produced earlier invasion or more aggressive tumours. The effects of cortisone may be due to its influence on lymphocytes and the immune response. Indeed, immunosuppression by antilymphocyte serum (Woods 1969; Giunta and Shklar 1971) greatly enhanced tumour induction in the cheek pouch model. Similarly, more rapid and more anaplastic tumours developed after systemically administered antimetabolic drugs like methotrexate (Shklar, Cataldo and Fitzgerald 1966). Stimulation of the immune system by BCG delays chemical carcinogenesis in the cheek pouch model (Giunta, Reif and Shklar 1974). A similar effect can be produced by systemically administered levamisole (Eisenberg and Shklar 1977), an agent thought to enhance cell-mediated immunity

(Churchill and David 1973). A higher incidence of tumours has been reported in DMBA-treated cheek pouches of castrated hamsters receiving systemic oestrogens by Polliack, Charuzy and Levij (1969). They suggested that this enhancement resulted from either an oestrogen-induced increase in permeability of cell membranes or a co-carcinogenic effect between DMBA and the mitogenic action of oestrogen.

1.3.4 Models of Intraoral Carcinogenesis in Extra-Pouch Sites

The oral mucosa proper of the hamster is considerably more resistant to the action of chemical carcinogens than the cheek pouch. In his initial experiments on cheek-pouch carcinogenesis Salley (1954) recorded carcinomas occasionally developing in the palate, buccal mucosa, tongue, oesophagus and stomach, presumably as a result of overflow of carcinogen into the oral cavity. In a later study, Salley and Kreshover (1959) found that thrice weekly painting of the palates of hamsters with DMBA resulted in carcinomas developing in 54 per cent of the animals after 16 weeks. When a similar technique was applied to hamster gingiva only 10 per cent of the experimental animals developed carcinomas (Al-Ani and Shklar 1966) but several papillomas and numerous areas of dysplasia were present. Mesrobian and Shklar (1969) applied powdered DMBA to hamster gingiva once weekly and held the carcinogen in place by cyanoacrylate tissue adhesive. All the experimental animals developed invasive squamous cell

carcinomas by 20 weeks. Dachi (1967) induced lingual carcinomas in 4 out of 15 hamsters by applying DMBA in DMSO. However, larger and more anaplastic tumours developed in adjacent skin and oral mucosa and these lesions were frequently responsible for the animal's premature death. A more successful model of lingual carcinoma in the hamster was developed by Fujita et al (1973a; 1973b). They applied DMBA to the tongue after first scratching and ulcerating the area with a root-canal barbed broach. There were regional variations in the susceptibility of the lingual mucosa to the carcinogen but when the lateral border was treated in this manner, all the experimental animals developed invasive neoplasms between 13 and 25 weeks.

The production of oral tumours in mice has proved to be difficult. Van Prohaska,Brunschwig and Wilson (1939) reported one carcinoma developing in the oral mucosa of mice which had been fed with 20 MC in olive oil over a period of six months. No tumours were produced by painting mouse gingiva with 20 MC for up to 16 weeks (Levy 1948) but when 20 MC was injected under the dorsal mucosa of their tongues, malignant epithelial tumours developed (Levy 1958). Goldhaber (1957) found that when the salivary glands were removed from mice 20 MC could induce carcinomas in the labial mucosa. Carcinomas of murine buccal mucosa were produced by Protzel, Giardina and Albano (1964) using 3, 4 BP as the carcinogen. When the carcinogen was applied to animals in which severe liver damage had been induced the frequency of tumours was increased and the latent period decreased.

Labial carcinomas were produced in nearly half the experimental animals when mice were painted with the water-soluble carcinogen 4-nitroquinoline N-oxide (4 NQO) for prolonged periods (Fujino, Chino and Imai 1965).

Initial attempts to induce oral tumours in rats also met with limited success. Wallenius (1966) showed that only about 30 per cent of experimental animals developed oral squamous cell carcinomas after painting with DMBA for 16 months. When a similar method of carcinogen application was used in desalivated rats, malignant tumours developed in all animals by 11 months. Attempts to produce lingual neoplasms in the rat were unsuccessful even when DMBA was bonded to the tongue with cyanoacrylate tissue adhesive (Giunta and Shklar 1972). When the water-soluble carcinogen 4 NQO was used, however, palatal carcinomas were produced in all experimental animals by seven months (Wallenius and Lekholm 1973a). In addition 75 per cent of animals developed carcinomas on the dorsum of the tongue and 20 per cent showed carcinomas of gingiva or stomach. The Japanese workers Yamamura et al (1975) attempted to prolong the action of chemical carcinogens by implanting them into surgically-created caecal pouches in rat lip. The carcinogens investigated were DMBA, crystalline 20 MC and crystalline N-methyl-n'-nitro-nitrosoguanidine. These carcinogens induced a variety of neoplasms including squamous cell papillomas, carcinomas, neurofibromas, lymphangiomas, haemangiomas and haemangiosarcomas. This wide variety of neoplasms, whilst being interesting as an observation,

would make the model system of limited value in a study of squamous cell carcinoma.

Primates appear to be very resistant to the actions of known chemical carcinogens in most sites (Kent 1960) and there is no satisfactory simian model of oral carcinogenesis. One monkey developed a carcinoma of the tongue after a radioactive source was implanted into the maxillary antrum (Melnikov 1963). Cohen and Smith (1967) painted monkey oral mucosa bi-weekly with DMBA for nearly four years without finding any changes indicative of transformation to malignancy. When tobacco paste was inserted into surgically-created pouches in monkey cheek there were minimal histological changes apart from "ballooning" of epithelial cells (Cohen and Smith 1967), a feature previously described in human oral epithelium following exposure to snuff (Pindborg and Renstrup 1963), chewing tobacco (Zegarelli, Everett and Kutscher 1961) and components of betel quid (Pindborg, Srivastava and Gupta 1964). However, when the application of tobacco to these cheek pouches was repeated at intervals for up to three years, epithelial atypia developed (Cohen, Poswillo and Woods 1971). Hamner (1972) showed that moderate to severe epithelial atypia could be induced in protein-deficient baboons by implanting betel quid with tobacco into surgically-created buccal mucosal pouches.

1.3.5 Resistance of the Oral Mucosa to Chemical Carcinogens

The oral mucosa, apart from the hamster cheek pouch, is much more resistant to the action of chemical carcinogens than skin (Levy 1948; Levy, Gorlin and Gottsegen 1950; Kreshover 1952; Kolas 1955). The most frequent explanations for the differences in susceptibility are the influence of saliva in the mouth and the presence of 'portals of entry' in the skin formed by sebaceous glands and hair follicles.

Kolas (1955) attempted to demonstrate an anti-carcinogenic action of saliva by applying saliva to the ears of mice before painting with 20 MC. He found no protective effects. Further attempts to show a protective action of saliva by extirpating the major salivary glands failed to have any effect in either mice (Goldhaber, Weisberger and Sognnaes 1956) or hamsters (Kreshover and Salley 1957). However, the degree of desalivation achieved in these experiments was only partial as the minor salivary glands were left intact. Wallenius has undertaken a number of careful experiments to determine the role of saliva in carcinogenesis. Stormby and Wallenius (1964) found that hamsters with reduced salivation following surgical removal of the major salivary glands developed more neoplastic lesions after intraoral carcinogen applications than the controls, but the difference was not statistically significant. In 1966 Wallenius showed that when the cheek skin of rats was transplanted into the buccal

mucosa and then painted with carcinogen no changes were found in the transplanted skin after 11 months whereas in its normal situation tumours developed within 6 months. Wallenius (1966) then applied carcinogen to rats' oral mucosa after surgical removal of the major salivary glands and repeated injections of an anti-sialogogue. Xerostomic animals developed buccal carcinomas after 11 months of carcinogen application, whereas none of the normal controls and only a third of animals with reduced salivation developed tumours. Wallenius believed the protective action of saliva was due to the formation of a moist mucous barrier rather than a diluting action as proposed by Kreshover and Salley (1957). Wallenius and Lekholm (1973a) produced high yields of malignant tumours in rat palate and tongue using the water-soluble carcinogen 4 NQO in less than 7 months in animals with intact salivation. They attempted to explain the increased efficiency of 4NQO over DMBA in producing oral tumours in the rat by suggesting that the salivary layer was protective against DMBA which is fat-soluble but not against the water-soluble 4NQO. This supposition appeared to be verified by in-vitro experiments (Wallenius and Lekholm 1973b).

Although there is a good deal of evidence that saliva exerts some protective influence in oral carcinogenesis the importance of portals of entry is much less clear. There is little doubt that adnexal structures play an important role in chemical carcinogenesis in skin.

Lacassagne and Latarjet (1946) painted 20 MC onto areas of mouse skin in which appendages were absent due to scarring or UV light irradiation and found these areas to be resistant to tumour induction. Suntzeff, Carruthers and Cowdry (1947) also failed to induce neoplasms in the skin of newborn mice, which have immature sebaceous glands and hair follicles. The skin appendages could be important in aiding retention of the carcinogen. This is supported by Simpson and Cramer (1943) who showed by using fluorescence microscopy that 20 MC accumulated in sebaceous glands after a single application of carcinogen.

Despite the postulated importance of portals of entry in cutaneous carcinogenesis their role in intraoral carcinogenesis is far from clear. Levy (1958) found that when 20 MC was injected into the submucosa of mouse tongue there was a marked increase in the frequency of tumours developing in the overlying epithelium when compared with superficial painting of the carcinogen. This observation appeared to support a role of portals of entry in intraoral carcinogenesis. However, the oral tissue most susceptible to the action of chemical carcinogens is the hamster cheek pouch which contains no appendages (Salley 1961a). Studies utilizing the fluorescent properties of some polycyclic hydrocarbons (Salley 1961b) and ^{14}C labelled DMBA (Meskin and Woolfrey 1964) have shown that in the cheek pouch carcinogens pass through apparently intact epithelium and are retained in the lamina propria. In the palate Salley (1961b) showed

that carcinogen could be detected in the lamina propria before the minor mucous glands. Goldhaber (1957) suggested that ulceration of mucosa allowed penetration of carcinogen, but this was not supported by the work of Morris (1961) who showed that very low concentrations of DMBA could produce neoplasms in hamster cheek pouch without any macroscopical evidence of ulceration. However, Homburger (1969) has suggested that microscopical defects are formed in the mucosa during mastication and these cannot be ruled out as potential portals of entry for chemical carcinogens. Listgarten, Albright and Goldhaber (1963) considered that intercellular oedema induced by carcinogen painting could facilitate penetration of the carcinogen.

CHAPTER TWO

METHODS OF ANALYSIS OF EPITHELIAL MORPHOLOGY IN NORMAL AND DISEASED MUCOSA

2.1 COMPARTMENTS IN CELL RENEWAL SYSTEMS

Tissues have been classified into three types according to the degree to which they preserve their ability to divide in postnatal life (Bizzozero 1894). Labile cells, such as epidermis, alimentary, respiratory and urinary tract epithelium normally continue to multiply throughout life. Stable cells such as the parenchymal cells of liver, pancreas, thyroid gland and many other organs normally cease to divide when somatic growth ceases. These cells, however, retain their mitotic ability during adult life and may divide in response to tissue injury. On the other hand permanent cells, of which neurones are the classical example, have no capacity to multiply from shortly after birth.

A slightly different classification of cells is into so-called populations (Leblond 1964). A population is a group of cells of similar morphology and presumably function. Such populations can be sub-divided into static, expanding or renewing populations.

A static cell population shows no mitotic or DNA synthetic activity. Expanding cell populations show DNA synthetic activity and mitoses and this accounts for the increase in the size of organs or tissues during somatic

growth. The renewing cell population shows evidence of plentiful DNA synthesis and cell division but this is balanced by a correspondingly high rate of cell loss.

Quastler and Sherman (1959) and Quastler (1960) introduced the concept that a renewing cell population can be divided into compartments in which the constituent cells have distinctive morphology, function and sometimes location. The reproducing cells involved in new cell production are known as progenitor cells and these cells as a group are called the progenitor cell compartment. A proportion of these cells leave the progenitor compartment to form a compartment of mature, functional cells, while some cells remain in the progenitor cell compartment and act as stem cells.

In non-keratinized oral epithelium it is possible to identify cell populations which correspond to the progenitor and maturation compartments which were originally described in mouse intestinal epithelium (Quastler and Sherman 1959; Quastler 1960). See Fig 2.1.

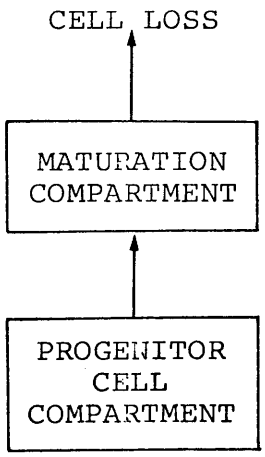


Fig 2.1. Two compartment cell renewal system.

This type of cell renewal system in the steady state has certain characteristics. Cells are born only in the progenitor cell compartment whereas cell death occurs almost exclusively in the maturation compartment. In addition, once having entered the functional compartment cells almost never return to the progenitor cell compartment (dedifferentiate). In some types of oral epithelium there is a keratinized layer and this is sometimes regarded as a third, cell-free compartment.

In stratified squamous epithelium the basal cell layer consists of cells in contact with the basement membrane. These and suprabasal cells which are morphologically similar to the basal cells are included in the progenitor cell compartment. The maturation or functional compartment consists of cells in the stratum spinosum and stratum granulosum. These cells produce keratin, die and then become part of the keratinized compartment before they are finally desquamated.

Cell division occurs in the basal cell layer or, in some locations, in more superficial cell layers. Identification of the progenitor cell compartment is based mainly on the morphology and location of the cells, but in some instances the distinction between maturation compartment cells and progenitor compartment cells may be difficult. The presence of mitoses, and labelled cells in autoradiographs may help delineate the distribution of progenitor cells. Suprabasal dividing populations of cells

are not seen in relatively thin epithelium such as rat oesophagus (Leblond, Greulich and Pereira 1964), murine epidermis (Iversen, Bjerknes and Devik 1968) or hamster cheek pouch (Thilagaratnam 1969). However, in thicker epithelium or epithelium with a rapid turnover suprabasal dividing cells are frequently seen. Examples include hamster palate (Thilagaratnam 1969) and ventral lingual mucosa (MacDonald 1973) and rat palate and ventral lingual mucosa (Sharav and Massler 1967). When attempting to estimate the size of such a suprabasal dividing cell population serial sections should be examined in order to exclude those mitoses which, although appearing to be suprabasal in one section are found to have a basal location when adjacent sections are examined (MacDonald 1971). In most normal stratified squamous epithelium cell division is restricted to the three cell layers closest to the basement membrane (Löe, Karring and Hara 1972). Suprabasal dividing cells appear to have the same morphological features as basal cells (Leblond, Greulich and Pereira 1964). However, the morphologically defined progenitor cell compartment in stratified squamous epithelium is not homogeneous and may contain subpopulations of non-proliferating keratinocytes as well as non-keratinocytes such as melanocytes, Langerhans cells and Merkel cells. These subpopulations however, probably form only a small proportion of the total number of cells in this situation (Hamilton and Potten 1972). In addition, progenitor

cells may be grouped into functional units. Potten (1974) has shown that in mouse epidermis basal cells are organised into "epidermal proliferative units" in which there appears to be a central stem cell surrounded by approximately ten basal cells. Cell division is restricted to the basal layer but the central stem cell appears to be cycling at a different rate from the surrounding basal cells.

Division of stratified squamous epithelium into cell compartments as described in the preceding paragraphs has the advantage over division into the more usual histological strata in that compartment analysis can be used to relate morphological distribution to cell production and turnover. Morphometric analysis of cell compartments using stereology has been undertaken by MacDonald (1973) in normal and carcinogen-treated hamster oral mucosa. Work by Warnakulasuriya (1976) using this method suggests that there could be diurnal variations in the sizes of cell compartments of human buccal oral epithelium.

2.2 HISTOLOGICAL ASSESSMENT OF PREMALIGNANCY

The histological assessment of potentially premalignant lesions of the mouth is generally based on subjective criteria. It is known that some oral lesions have cytological features normally associated with squamous cell carcinomas but show no histological evidence of invasion. These lesions are regarded as showing

histological evidence of premalignancy. The individual cellular changes are referred to as atypia and the general disturbance in the epithelial architecture is called dysplasia (WHO 1978). The use of the older term dyskeratosis is now restricted to abnormal keratinization only. The histological features which comprise epithelial atypia have been described by Smith and Pindborg (1969) and the histological and cytological features which they considered to be important in the diagnosis of epithelial atypia were:

1. "Drop-shaped" rete ridges
2. Irregular epithelial stratification
3. Keratinization of cells below the keratinized layer
4. Basal cell hyperplasia
5. Loss of intercellular adherence
6. Loss of polarity of basal cells
7. Nuclear hyperchromatism
8. Increased nucleo-cytoplasmic ratio
9. Anisocytosis and anisonucleosis
10. Pleomorphic cells and nuclei
11. Increased mitotic activity
12. Mitoses at abnormally superficial sites
13. Presence of bizarre mitoses

The value of epithelial atypia in predicting future malignant transformation has not been fully elucidated. However, Mehta et al (1969) found 59.1 per cent of cases of speckled leukoplakia showed epithelial atypia whereas only

8.4 per cent of cases of homogeneous leukoplakia showed atypia. This suggests that epithelial atypia is much more common in those types of leukoplakia which have the highest premalignant potential. Similarly, Pindborg, Mehta and Daftary (1970) found atypia in 22.6 per cent of cases of submucous fibrosis. Mincer, Coleman and Hopkins (1972) described one of the few longitudinal studies of patients with epithelial atypia. This study showed that in about 11 per cent of cases the lesions reduced in size or disappeared without surgical intervention. 35 per cent of cases, however, recurred following surgery and 11.1 per cent became frankly malignant.

The histological features associated with epithelial atypia are not found exclusively in premalignant lesions, and Berenblum (1970b) postulated that many of the so-called features of premalignancy are reactive and not involved in the neoplastic process. This view is supported by MacDonald and Rennie (1975) who found evidence of atypia in denture-induced hyperplasia, a lesion not generally considered to have any premalignant potential.

Several studies have attempted to evaluate epithelial atypia as an indicator of premalignancy and to assess the relative importance of the various histological features previously described. Smith and Pindborg (1969) graded the histological features of epithelial atypia by using photographic standards and giving weighted scores to each feature. This work has confirmed a fairly high level of variation between individual observers and indeed

between the same observer at different times (Smith 1976). The system is now under examination by the World Health Organisation International Reference Centre for Oral Precancerous Conditions. MacDonald (1973) applied this technique to lesions induced in the hamster pouch by DMBA. He found that those premalignant lesions which proceeded to malignancy had significantly higher atypia scores than lesions which did not undergo malignant transformation. Kramer et al (1970) and Kramer, El-Labban and Sonkodi (1974) attempted to assess the relative importance of the histological features of premalignant lesions by using an objective, computer-aided discriminant analysis. They compared 39 histological features in cases of leukoplakia which were known to have progressed to malignancy with cases of leukoplakia which had not developed malignancy. The histological features found most useful in distinguishing progressive premalignant lesions were abnormal mitoses in the spinous or basal layers, disturbed polarity of epithelial cells, nuclear hyperchromatism, Russell bodies in the lamina propria, enlarged nucleoli in the spinous layer, epithelial cell pleomorphism and intraepithelial keratinization.

Recent evidence by Kramer, El-Labban and Lee (1978) suggests that site may be an important indication of likely future tendancy to malignant transformation. In high risk sites such as the floor of the mouth and the ventral surface of the tongue it is recommended that greater attention should be given to even the lesser

degrees of atypia (WHO 1978).

Although epithelial atypia appears to be the best single guide to the likely behaviour of premalignant oral lesions one of the major management problems in such cases is that foci of atypia may be very small and this makes the selection of the site for biopsy critical. Also, Cawson (1975) has highlighted another major difficulty in the diagnosis of premalignant lesions ie. "the subjective nature of the evaluation of the cellular changes and the virtual impossibility, under routine conditions, of quantifying the abnormalities regarded as significant in order to reach any useful conclusion as to prognosis". Thus it seems possible that more objective, quatitative assessment of premalignant lesions could help in the understanding of their nature and subsequent behaviour. Therefore, some of the available methods for quantifying morphological features of histological sections will be considered.

2.3 QUANTITATIVE METHODS IN HISTOLOGY

The value of precise quantitation has long been established in the physical sciences but has only fairly recently been recognised as being relevant to the study of anatomy and histology. The description of structural features by using quantitative data is known as morphometry. The major reasons for the unpopularity of morphometry have been the laborious and tedious nature of many of the techniques available for quantitation and their relative

inaccuracy. For example, basement membrane length has been determined by superimposing fine nylon thread on sections (Renstrup 1963); planimeters have been used to measure surface areas on photographic prints (Barrington and Meyer 1969) and cell sizes have been measured directly from such prints (Meyer and Gerson 1964). Abrams and Shear (1977) measured the volumes of multinucleate giant cells by the somewhat cumbersome method of tracing enlargements of serial paraffin sections and construction of three-dimensional wax models. The volumes of the models were then determined by Archimedes principle.

However, the problem of measuring the volume of a component of an organ or cell is similar to that of determining the relative volume of each mineral in a particular sample of rock. Both these problems can be solved by the application of the Delesse theorem (Delesse 1847) which states that "in a rock composed of a number of minerals, the area occupied by a given mineral on a surface of a section of the rock is proportional to the volume of the mineral in the rock". Thus the relative volume, or volume density V_v can be estimated directly from the Delesse relationship $V_v = A_a$, where A_a is the area density of profiles on section, provided the sections are a representative random sample of the material under investigation. This principle forms one of the bases of a technique for determining morphometric data known as stereology. Stereology involves a geometric analysis of structures and textures and includes methods that allow

direct derivation of metric or three-dimensional properties of structures from two dimensional sections on the basis of geometrico-statistical reasoning (Weibel 1969). Delesse originally determined A_a by tracing profiles onto heavy paper, cutting them out and weighing them. An alternative method was the use of a polar planimeter but this was frequently inaccurate. The problem of measuring areas in sections was greatly simplified when Rosiwal (1898) showed A_a could be determined by linear integration. Thus if lines of known length, L are randomly placed on a section, $A_a = L_l$ where L_l is the fraction of the lines enclosed in the profiles. This method, however, is still tedious and the finding that A_a could be determined by superimposing a regular point lattice on the section and determining the fraction P_p of all the points enclosed by the profile, was a major advance in stereology (Glagoleff 1933). Thus the related methods available for the estimation of V_v are:

$$V_v = A_a = L_l = P_p$$

Stereological methods can also be used to determine profile lengths and surface areas by using an intercept point - counting technique using a grid of parallel lines (Weibel 1969). The length is a function of the number of intercepts counted (I) and the distance between the grid lines (d) and can be determined by the formula:

$$L = \frac{\pi}{2} \times I \times d$$

This technique is very useful in estimating surface

and basement membrane lengths.

Stereological techniques are based on the assumption that the areas quantified are representative of the internal structure of the tissue under examination. To fulfil this requirement the sampling procedure should be random at all stages, including selection of blocks, sections and areas of sections finally counted (Weibel 1969). This necessary condition that structures should be randomly orientated with respect to the plane of section offers few problems in an organ such as the liver where there is a fairly uniform distribution of cells. However, many organs show preferential orientation of their constituent elements, a property known as anisotropy. Oral epithelium shows a high degree of structural anisotropy with the component cells forming characteristic strata. Sampling of randomly orientated sections of such a tissue would yield limited information as it is often not possible to distinguish between basal cells and stratum spinosum cells in such sections. However, Weibel (1969) states that anisotropic tissues can be sampled stereologically if they are sectioned in a plane which contains the axis of anisotropy. In oral epithelium this plane is perpendicular to the surface.

When designing a stereological investigation it is important to establish criteria which make the identification of the particular structural elements unequivocal. The minimum number of criteria necessary to accurately define such elements is used for practical reasons.

In addition, sufficient material must be sampled. Obviously the larger the sample examined the more accurate the information obtained, but it is important to strike a balance between the accuracy required and the effort involved in collecting data. One technique for determining minimal sample size consists of recording a large number of fields, counting the average per field and forming progressive means for increasing sample sizes (Schroeder and Münzel-Pedrazzoli 1970). The tissue area needed to bring the progressive mean into the range of $\bar{+}$10 per cent is considered to be an acceptable level of accuracy in biological systems (Weibel 1969).

Stereological techniques have recently been used to characterise the morphological features of normal and diseased oral mucosa. Schroeder and Münzel-Pedrazzoli (1970) discussed the application of stereology to the study of human gingiva. Schroeder and his co-workers have since described a number of morphological characteristics, in normal gingiva and in human and experimental gingivitis (Schroeder 1970; Schroeder and Münzel-Pedrazzoli 1973; Schroeder, Münzel-Pedrazzoli and Page 1973; Schroeder and Graf de Beer 1976). MacDonald (1973) examined the cell compartments of carcinogen-treated hamster tongue at light microscopical level and the proportion of basal lamina formed by hemidesmosomes using the electron microscope. Franklin and Craig (1978a & b) used stereology to determine nucleo-cytoplasmic ratios in normal and turpentine-treated cheek pouch

and Meyer et al (1976) used stereology to estimate the size of the nucleus and nucleolus in human cases of leukoplakia. Recently Klein-Szanto (1977) has determined the stereological baseline data of normal human epidermis from various regions of the body.

2.4 AIMS AND DESIGN OF PRESENT STUDY

The aim of the present study was to investigate quantitative morphological aspects of early experimental oral carcinogenesis in an attempt to define more fully the histological features of premalignant epithelial lesions.

The author first used a relatively standard model of experimental oral carcinogenesis, the hamster cheek pouch. The concentration of carcinogen employed was lower than that used in most previous investigations but the experimental model described was basically a repetition of previously published work. A description of the model is included to show that the techniques employed resulted in the formation of malignant tumours. Using this model sequential changes in the preneoplastic epithelium were assessed using stereological techniques and the experimental findings are recorded in Chapter 3. Familiarity with the cheek pouch system led the author to judge that although it is a useful model of epithelial carcinogenesis it fell short of the requirements of an ideal model of intraoral carcinogenesis. The deficiencies in the cheek pouch model are discussed at length.

Chapter 4 records the author's first attempt to produce tumours in a truly intraoral location. The site chosen was the ventral lingual mucosa and the carcinogen used was 4 nitroquinoline N-oxide. This model was unsuccessful and the significance of the results is discussed.

Chapter 5 records a further attempt to produce tumours

in the hamster ventral lingual mucosa. The method used was a modification of a technique that had recently been published involving roughening the experimental area with a root-canal barbed broach before painting on the carcinogen dimethyl benzanthracene. Although no neoplasms were produced in the experimental period there was histological evidence of progressive epithelial atypia.

Chapter 6 shows how a modification of the technique used in Chapter 5 resulted in the formation of intraoral squamous cell carcinomas. The model was then used to study quantitative morphological features of preneoplastic lesions using stereological techniques and the findings are discussed in Chapter 7.

CHAPTER THREE

CARCINOGENESIS IN THE HAMSTER CHEEK POUCH WITH PARTICULAR REFERENCE TO QUANTITATIVE HISTOLOGICAL CHANGES IN PRENEOPLASTIC EPITHELIUM

3.1 INTRODUCTION

The hamster cheek pouch system is reported to be a reliable model of oral carcinogenesis (see Section 1.3.3). Although many workers have subjectively described the histopathological changes in the pouch epithelium following carcinogen application there is a paucity of quantitative information, especially during the preneoplastic stages. Assessment of such changes might prove to be of value in extending knowledge of premalignant lesions.

The aim of the present study was to quantify early sequential changes in hamster cheek pouch epithelium following carcinogen applications. The parameters to be examined were changes in total epithelial thickness and the thickness of epithelial compartments, together with changes in cell numbers and cell sizes within individual epithelial compartments. The concept of cell compartments is discussed in Section 2.1.

It was hoped to correlate the compartmental analysis with cell kinetic data using the in vitro labelling technique developed by Warnakulasuriya (1976). However, this part of the investigation was unsuccessful due to negative

chemography (Rogers 1973) and this aspect was not pursued in the present study.

3.2 MATERIALS AND METHODS

3.2.1 Animals

The animals used were a line bred strain of Syrian golden hamsters (Mesocricetus auratus) which were 10-12 weeks old at the start of the experiment. To minimise the effects of sex hormone variations male hamsters* were used. Hamsters were ear-marked to aid identification and housed two per cage. They received standard laboratory chow and water ad libitum. Each animal was weighed weekly as this gave a simple guide to general health and well being.

3.2.2 Cheek Pouches

The hamster cheek pouches are a pair of balloon-like structures located beneath the dermis along the lateral side of the head and neck. They consist mainly of dense fibrous and elastic tissue and each is suspended by a thin muscle slip that is inserted into the lumbar fascia in the mid-dorsal line (Keyes and Dale 1944). The orifices of the buccal pouches open into the oral cavity. The pouches have no adnexal structures and appear to be used only for food storage.

3.2.3 Anaesthesia and Restraint

In order to examine and treat the cheek pouch mucosa, it is necessary to immobilise the animal with its mouth

*obtained from Coombehurst Breeding Establishment, Basingstoke, Hampshire.

held open and the cheek retracted. The device described by Moss, Collins and Cole (1965) was found to be effective for this purpose (Fig 3.1). Unlike these workers the author did not find it possible to manipulate the unanaesthetised animal, so the hamsters were lightly anaesthetised in a small jar with ether. A fine wire mesh covered the cotton wool in the bottom of the jar to prevent the animals from becoming soaked in ether. Following removal from the jar unconsciousness persisted for about a minute which usually gave sufficient time to manipulate the animal. The anaesthetised hamster was placed in the detached chamber of the restrainer with its head through the hole in the top and the chamber was mounted on the base. The mouth was held open by elastic bands around the incisor teeth leaving both the operator's hands free. Retractors inserted into the pouches allowed exposure of the medial wall (Fig 3.2).

3.2.4 Carcinogen

9, 10-dimethyl-1, 2-benzanthracene (DMBA) has been the most widely used carcinogen in studies of hamster cheek-pouch carcinogenesis. Morris (1961) found a 0.5 per cent concentration to be the most effective.

In the present investigation it was decided to use acetone as the solvent in an attempt to localise the neoplastic changes to a predetermined area of mucosa. A 0.25 per cent solution of DMBA in acetone was employed. This relatively weak concentration was selected to increase the latent period (Morris 1961) and thus extend the period

during which premalignant changes might be expected. In addition, this concentration decreases the likelihood of ulceration with its concomitant problems in interpretation due to scarring, but does not significantly decrease the tumour yield (MacDonald 1978), relative to the more generally used concentration.

A stock solution of 10 ml of carcinogen was prepared and stored in a dark bottle and one ml aliquots were removed for each painting period.

3.2.5 Carcinogen Application

The carcinogen was applied to an area of the medial wall of the pouch about 1 cm square near the anterior opening, using the technique described by MacDonald (1978). The applicator was a 1 cm wide artist's oil painting brush in which the bristles had been trimmed to 1 cm in length. The brush was dipped into the carcinogen solution and then wiped against the edge of the bottle to remove excess carcinogen. The brush was laid on the medial part of the pouch with the junction of the metal part of the handle and the bristles lying on the prominent raphe and vein found on the anterior aspect of the pouch (Fig 3.3). The brush was then lifted off the mucosa perpendicularly and withdrawn from the pouch. A gentle stream of air from a chip syringe was directed over the area to evaporate the acetone and leave a crystalline residue of carcinogen adherent to the desired area of mucosa.

Work by Sabes, Chaudhry and Gorlin (1963) suggested

that the time of day at which carcinogen was applied could affect the tumour yield. Therefore in this experiment all animals were painted with carcinogen at some time between 9.00 am and 10.30 am.

3.2.6 Safety Precautions

Although the effects of DMBA on man are uncertain it is a potent carcinogen for many animals. Therefore, it was essential to protect both operator and assistants from the possible risks associated with exposure. Surgical masks and gloves were used during all animal manipulations and after use instruments were thoroughly washed in acetone followed by running water.

There is little information regarding the spread of chemical carcinogens in laboratories and the consequent risks to laboratory personnel. However, Darlow, Simmons and Roe (1969) studied the dissemination of Bacillus globigii spores which had been applied to the clipped skin of mice. They concluded that if the spores had been a chemical carcinogen the amount present in the animals' bedding and in the atmosphere could be a potential hazard to laboratory personnel.

It was felt that the use of an intraoral site would limit atmospheric spread of the carcinogen and reduce the potential risks. Animals were housed in solid-based boxes with peat and straw bedding and these boxes were cleaned weekly. The technicians working with the animals were informed of the potential risks and advised to wear masks and gloves when changing the boxes.

3.2.7 Experimental Procedures

30 hamsters were used in the present investigation. They were divided into six groups each of five animals. Animals in the first group were killed at the start of the experiment and were used as untreated controls. The remaining animals received carcinogen applications to each cheek pouch three times per week for a maximum of six weeks. Thereafter, animals were examined weekly under light ether anaesthesia and the appearances of the cheek pouches were recorded. Animals were weighed weekly to give a simple guide to their general health and well-being.

Five animals were killed at each of 3, 6, 9, 12 and 15 weeks and in the case of the groups killed at 3 and 6 weeks, 3 days after the last carcinogen application. Animals were killed by ether inhalation and the medial wall of the pouch was exposed by cutting through the lateral wall of the pouch and the skin of the cheek with scissors (Fig 3.3). Representative samples of the treated area of the pouch and any visible lesions were taken from the right cheek pouch to illustrate the progression of the lesions and demonstrate the properties of the model. A standard sample of the treated area was taken from the left cheek pouch for quantitative analysis. Quantitative studies were only undertaken on normal hamsters and animals killed at 3, 6 and 9 weeks as papillomas appeared regularly in the treated site after this time (see Section 3.3.1).

A standard sample was taken from the treated area of mucosa in the left cheek pouch by making two parallel incisions 3 mm apart and about 10 mm long in an anteroposterior direction with a scalpel (Fig 3.4). A scalpel-cut at right angles to the original two incisions and close to and parallel with the prominent vein at the opening of the pouch was made. The sample was raised by carefully holding one of the corners with fine forceps and freeing the tissue with scissor cuts parallel to the epithelial surface. The sample was freed at the base by a scissor cut. Great care was necessary in handling the sample as it readily rolled into a tube shape which made orientation of the tissue for block-taking very difficult. In the original protocol of the experiment it was hoped to label the biopsy tissue with tritiated thymidine using an in vitro technique. This aspect of the experiment was not successful and was not pursued as part of the present thesis. Therefore, the details of the labelling techniques and tissue culture which were developed by Warnakulasuriya (1976) will not be considered here. However, the handling of the biopsy specimens which were used for quantitative morphological analysis, will be discussed.

The necropsy sample, a portion of tissue approximately 10 mm x 3 mm x 1 mm was carefully laid out on a small strip of filter paper with the sticky, mucoid connective tissue surface towards the paper. The filter paper was then laid on a small sheet of wax prior to block trimming.

For tissue culture it was necessary that blocks of 1 mm or less in thickness should be used. Preliminary investigations, however, showed that when single blocks of cheek pouch of this size were cultured they tended to twist and distort to such an extent that orientation of the tissue at the blocking out stage was difficult and frequently impossible. A technique was devised which largely overcame this problem. Two slices each one millimetre thick were made at right angles to the epithelial surface to give blocks 3 mm x 1 mm x 1 mm in size, but the specimen was not completely divided and remained attached by the muscular and gelatinous mucoid layer deep to the corium. The third cut was made completely through the specimen and the filter paper. This was repeated along the specimen so that there were three small squares of filter paper each containing a portion of mucosa approximately 3 mm x 3 mm, each of these pieces being divided into 3 mm x 1 mm strips still adherent at their deep margin. The specimens, still adherent to the filter paper, were placed in tissue culture medium 199* in sterile Bijou bottles and transferred to the laboratory in vacuum flasks containing crushed ice.

In the laboratory the specimens were incubated in medium 199 at 37^{o}C and labelled by the technique of Warnakulasuriya (1976). The tissue used in the stereological analysis was incubated for a maximum of one and a half hours. Warnakulasuriya (1976) found that when

*Gibco, Biocult, Paisley, Scotland

blocks were treated in this manner no differences could be detected in the morphological or stereological features between normal and cultured oral mucosa.

Tissue removed from pouches was fixed in Bouin's fluid for 2-3 hours and then transferred to 70 per cent methanol. The filter paper was carefully removed and the blocks trimmed into 3 mm x 1 mm x 1 mm strips under a dissection microscope. Care was taken that the methanol did not evaporate as this dries out and hardens the specimen. Tissue was processed and paraffin embedded using the cycle in Appendix 1. It was necessary to use a dissecting microscope to block out the specimens so that sections could be accurately orientated at 90^{o} to the mean epithelial surface (Section 2.3).

The face of the paraffin block was trimmed to about 4 mm x 4 mm and serial sections of the biopsy specimen were cut on a Leitz rotary microtome set at 3 μm. 32 serial sections were taken from each block and arranged on slides as shown in Fig 3.5. Specimens from the right cheek pouch were sectioned at 6 μm from three levels of each block. Sections were stained with haematoxylin and eosin.

It was felt possible that the techniques of taking the necropsy specimen and specimen preparation might result in a loss of superficial squames from the stratum corneum. Care was taken at the necropsy stage to avoid touching the keratinized surface and at all subsequent stages the material was handled with minimal agitation.

When histological sections were examined, in many instances red blood cells were present on the keratinized surface strongly suggesting that loss of surface squames had not occurred. Accordingly, no special precautions, other than those already noted, were taken to avoid such loss.

3.3 STEREOLOGICAL ANALYSIS

3.3.1 Sampling Procedure

This part of the study was concerned with quantification of preneoplastic epithelium. As examination of samples from the cheek pouch showed that neoplasms were frequently present in animals at 12 and 15 weeks only animals which had been treated for 9 weeks or less were used in the analysis.

As discussed in Section 2.3, in order to fulfil the requirements of stereological theory it is necessary that sampling of material should be random at all stages of the investigation. Therefore, the necropsy sample from the left cheek pouch of each animal was divided into three main pieces (see Section 3.2.7). Each of these three main pieces was further subdivided to give three small blocks ie. nine blocks in total. One small block from each of the three main blocks was randomly selected and an orientation section was cut. If preservation and orientation were satisfactory these blocks were used for analysis. If for any reason a block was unsatisfactory further blocks were examined as necessary. The middle

section of each short ribbon (Section 3.2.7) was then used for point-counting. If this particular section was unsatisfactory one on either side was used instead. Thus for each animal three blocks of the necropsy specimen were examined and four levels of each block were then counted.

3.3.2 Selection of Area for Counting

The slide was mounted on the revolving stage of a Leitz Ortholux microscope and projected onto a teaching head. Two vertical lines were drawn on the screen of the projection head to define a column of epithelium of known width (Fig 3.6). The specimen was aligned so that it was orientated with the mean epithelial surface at right angles to the vertical lines on the projection head. To avoid bias in the selection of the first field for counting the specimen was moved half a column width from the right hand edge of the section. This procedure also ensured that any damaged areas at the edge of the specimen, which was the site of the biopsy incision, were not included in the analysis. Two fields were analysed in each section. The second field was selected by taking the section out of focus and moving the stage a small but arbitrary distance to the side before refocussing the specimen and realigning it. This constituted a systematic random sampling technique (Weibel 1969).

3.3.3 Identification of Epithelial Compartments

The cheek pouch mucosa of the hamster is divisible

into progenitor, maturation and keratinized compartments (MacDonald 1971). Distinction between the keratinized and maturation compartments was readily made but distinction between the maturation and progenitor cell compartments was less easy. The distinction was made subjectively by consideration of features of cell size, nucleo-cytoplasmic ratio, orientation, location and staining characteristics. Progenitor cells were smaller and had a denser staining nucleus with a small amount of cytoplasm giving a higher nucleo-cytoplasmic ratio than maturation compartment cells. Nuclei of maturation compartment cells were larger and showed paler, vesicular staining. Progenitor cells tended to be elongated at right angles to the basement membrane, whereas maturation cells were flattened and orientated with their long axes at right angles to the progenitor cells and parallel to the surface (Fig 3.7). In the normal cheek pouch the progenitor cell compartment is restricted to a single basal layer of cells. In carcinogen-treated mucosa, most of the cells fulfilling the suggested morphological criteria for progenitor cells were in the deepest three layers of cells and the presence of mitoses and of labelled cells in autoradiographs confirmed this distribution. Using the above criteria it was found that cell compartments could be delineated with a high degree of reproducibility both when sections were examined after an interval by the same observer or when sections were assessed by other research workers in the laboratory. It was found easier to do the point-counting if lines of

demarcation were drawn between the compartments onto the teaching head screen with a fine felt-tip pen. Once the outlines had been demarcated in this way the section could be taken out of the plane of focus and the areas determined. This was undertaken by a point-counting technique using a circular transparent perspex grid superimposed over the section image on the screen (Fig 3.8). The grid consisted of two sets of parallel lines at right angles to each other and 5 mm apart. Such a grid is anisotropic and could correspond to a periodic distribution of elements in the specimen. Therefore, it was rotated to three positions for each count (Weibel, Kistler and Scherle 1966). As the amount of material available for analysis was limited each field needed to be accurately counted. However, when the 5 mm grid was used it was found that there was no statistically significant difference between point counts with the grid in three positions and six positions. Therefore, rotation of the grid to three positions for each field was judged to be satisfactory in the present experiment. The angular distance between each counting position was 60^{o} and the position was marked on the rim of the projection head. The point-counts were recorded on a standard haematology type laboratory counter (Fig 3.6). Using this system the sizes of epithelial compartments in columns of epithelium of known width could be calculated, after taking into account the various magnification factors.

3.3.4 Counting of Total Nucleated Cells

The total number of viable nucleated cells in each column of epithelium was counted. As sections were 3.5 μm thick, nuclei appeared mainly as a monolayer with minimal overlapping of cells. Cells were counted as either progenitor or maturation cells using the criteria described previously.

3.3.5 Correction to Cell Counts

When cells are counted in sections there is usually an overestimate of numbers due to the presence of nuclear fragments. To determine the true numbers of nuclei in the tissue corrections have to be made which take account of the presence of these nuclear fragments. This is especially important when there are two populations of cells of differing sizes, as cells with larger nuclear diameter will have relatively more nuclear fragments in a given volume of tissue. A satisfactory method to determine such correction factors is that described by Abercrombie (1946). To apply this method it is necessary to determine the average nuclear diameter in the cell compartment and average section thickness.

Measurement of Nuclear Diameter

To obtain the average nuclear diameters it was necessary to measure a representative sample of nuclei throughout the thickness of the epithelium. The column defined by the two lines drawn on the teaching head of the microscope was found to be too broad for this purpose. Therefore a 25 square graticule was inserted into one of

the eye-pieces and used to define a column of five squares perpendicular to the mean epithelial surface and running throughout the thickness of the epithelium. The nuclear diameters of cells in the progenitor and maturation compartments in this column were then measured using a graticule in the other eye-piece with the specimen under x100 oil immersion lens. All the nuclei in this column were measured so that there would be no bias in favour of measuring larger cells (Warnakulasuriya 1976). The widest horizontal width of each nucleus was recorded in μm by measuring the distance between two imaginary vertical lines touching the widest points of the nucleus. Over 100 nuclei in each compartment were measured for each group.

Measurement of Section Thickness

The rotary microtome used to cut the sections was set at 3 μm. In a previous investigation Warnakulasuriya (1976) found that this microtome cut sections of average thickness of nearer 3.5 μm by measuring section thickness by a through-focus photometric technique and this was the thickness used for the cell count correction in this study.

3.3.6 Application of the Correction Factor

Corrections to cell counts were made by using the formula proposed by Abercrombie (1946).

$$N = n \times \frac{T}{T+D}$$

N - corrected cell count

n - crude cell count

T - section thickness

D - average nuclear diameter

The data, in addition to giving information about changes in cell numbers during carcinogenesis was also used to derive values for changes in individual cell section areas.

By noting the number of points falling on each compartment in the epithelium it was possible to calculate the area of each, and by dividing these areas by the column width a mean thickness for the total epithelium or individual compartments, was obtained. By using the corrected cell numbers obtained by applying Abercrombie's correction to the crude nuclear counts and the area measurements obtained from point counting it was possible to estimate mean cell section areas. This measurement was a slight overestimate of cell size as the intercellular spaces were included in the measurements.

3.3.7 Statistical Analysis

Comparisons between groups of animals were made using non-parametric statistical methods because the small numbers in individual groups meant it was not possible to satisfy the assumptions required for parametric methods. All probabilities cited in the results were derived from Mann-Whitney U tests.

3.4 RESULTS

3.4.1 Characteristics of the Experimental Model

Macroscopical

The normal cheek pouch has a thin, pinkish lining through which the underlying blood vessels are frequently visible. It is dry due to the lack of salivary glands (Figs 3.2 and 3.3).

During the first week of painting the cheek pouch mucosa with DMBA no macroscopical changes were detected. During the second and third weeks of carcinogen application the pouches became red and shiny and in two animals there was frank ulceration. This had healed by the end of the fourth week and there was extensive scarring with reduction in the depths of the two affected pouches. These animals were assigned to the group which was killed at 15 weeks and were not included in the quantitative analysis. Macroscopical evidence of ulceration was not seen in any other animals during the period of carcinogen application.

Between the third and sixth weeks of treatment the redness of the pouches gradually subsided and areas of diffuse opacity and later whiteness began to appear. These developed into small, white flecks and the treated mucosa appeared rough and granular (Fig 3.9). By 6 weeks small (less than 2 mm) papillomas were seen in two pouches outside the treated area of mucosa (Fig 3.10). In other animals thick white plaques were present on the treated mucosa. Carcinogen applications were discontinued after 6 weeks and by 9 weeks the papillomas described

previously were approximately 3 mm in diameter. The treated area showed whitish flecks.

By 12 weeks, 7 of the 10 remaining animals had one or more papillomas in their pouches. Papillomas started as small, pinkish, soft nodules but in many cases grew rapidly into fungating, exophytic tumours (Figs 3.11 and 3.12).

One animal died at 13 weeks and the remaining four animals had multiple papillomas in both pouches at 15 weeks when the experiment was ended.

Routine macroscopical post-mortem examinations were made on all animals at the time of killing. There was no evidence of macroscopical metastases to regional lymph nodes, lungs or liver.

Histological

Normal cheek pouch consists of a thin stratified squamous epithelium 3-4 cell layers thick. The epithelium is uniform in thickness and has no rete ridges. It is orthokeratinized and the surface of fixed specimens often has a wavy or corrugated appearance. The progenitor cell compartment is a single layer of basal cells in contact with the basement membrane (Fig 3.13).

The subepithelial connective tissue consists of bundles of loose connective tissue and blood vessels and separates the epithelium from the muscular wall of the pouch. There are no adnexal structures.

In those animals killed after 3 weeks of carcinogen applications the epithelium in all specimens showed

hyperorthokeratosis. Most specimens showed acanthosis but there were frequently areas of epithelial atrophy. In one block incipient ulceration was present (Fig 3.14). However, there was no evidence of frank ulceration or scarring in the animals killed at this time. There was mild to moderate inflammatory cell infiltration of the corium.

At 6 weeks there was further and more marked hyperorthokeratosis and acanthosis, but the areas of atrophy described in the previous group were much less frequent. Inflammatory cells were present in the corium but the reaction was generally less severe than that seen at 3 weeks. Occasional blocks showed mild epithelial atypia (Fig 3.15) and in one animal a small papilloma which exhibited moderately severe epithelial atypia, was seen (Fig 3.16). Mild scarring of the corium was present in one block from each of two animals.

At 9 weeks inflammation was less severe although scattered mononuclear cells were still present in the corium. In the treated area of mucosa there was persistent hyperorthokeratosis and acanthosis. Focal areas of epithelial atypia were seen in blocks from each of four animals. Two animals had papillomas and these showed severe epithelial atypia (Fig 3.17).

In specimens removed at 12 and 15 weeks areas of severe epithelial atypia and papilloma formation were present in all the pouches examined. Well differentiated squamous cell carcinoma was present in 3 out of 5 animals at 12

weeks and 3 out of 4 animals at 15 weeks. Malignant tumours invariably arose by invasion of the stalk or base of papillomas. Examples may be seen in Figs 3.18 and 3.19.

3.4.2 Results of Stereological Analysis

Normal

The values for the stereological parameters measured can be seen in Tables 3.1 and 3.2. The total mean epithelial thickness was 33.3 μm SD 3.5 μm and this was made up of a cellular compartment 26.6 μm SD 3.6 μm and a keratinized compartment 6.7 μm SD 0.8 μm thick. The cell areas derived by dividing the compartment areas by the cell numbers (after applying Abercrombie's correction) per field gave an average area of 100.8 μm^2 SD 9.5 μm^2 in the progenitor cell compartment and 442.4 μm^2 SD 60.9 μm^2 in the maturation compartment.

Table 3.3 shows the average nuclear diameters obtained by the eyepiece graticule measurements. The average nuclear diameter was 5.7 μm SD 1.2 μm for progenitor cells and 8.4 μm SD 1.9 μm for maturation cells. These were the diameters used in the calculation of Abercrombie's correction (see Section 3.3.6).

Statistical correlations were made using Mann Whitney U tests. Comparisons were made between all groups and the normal animals and between the different experimental groups. For the sake of clarity only those probabilities which appear to be relevant are cited in the following results.

Mean Epithelial Thickness

Fig 3.20 shows changes in total mean epithelial thickness. After 3 weeks of carcinogen applications there was a nearly two fold increase in mean epithelial thickness (P=0.004). By 6 weeks there was a further significant increase in mean epithelial thickness (P=0.004). At 9 weeks, that is after a carcinogen-free interval of 3 weeks there had been no further significant change in mean epithelial thickness.

Compartment Analysis

Fig 3.21 shows changes in the mean thickness of individual compartments within the epithelium. It can be seen that the increase in thickness of the epithelium at 3 weeks was due mainly to an increase in the size of the keratinized and maturation compartments (P=0.008) with a relatively minor, but still significant increase in the size of the progenitor cell compartment (P=0.016). By 6 weeks the progenitor cell compartment was twice the normal thickness (P=0.004) and there were further increases in the thickness of the maturation compartment (P=0.008) and the keratinized compartment (P=0.004). Although the total thickness of epithelium was unchanged at 9 weeks there were changes in the relative sizes of the various compartments. Thus there was a reduction in the thickness of the keratinized compartment and a corresponding increase in the thickness of the cellular compartments, mainly due to an increase in progenitor cell compartment thickness (P=0.008).

Cell Numbers

The cell numbers in each compartment both before and after applying Abercrombie's correction can be seen in Table 3.2, and changes in the corrected cell numbers are illustrated in Fig 3.22. At 3 weeks the number of cells in the progenitor and maturation compartments had fallen, especially the latter (P=0.004). By 6 weeks the number of maturation cells had increased to normal levels but the number of progenitor cells was significantly above control levels (P=0.004). No further changes had occurred at 9 weeks.

Cell Sizes

The changes in cell size, expressed as mean cell section area in μm^2 are shown in Fig 3.23. At each of the three experimental periods there was a marked increase in the sizes of cells in the progenitor cell compartment as compared with controls (P=0.004) and this appeared to be progressive. The size of cells in the maturation compartment was also significantly above control levels from 3 weeks onwards. The apparent decrease in the relative sizes of maturation cells after 3 weeks was not significant due to wide variations between individual animals.

3.5 DISCUSSION

3.5.1 The Experimental Model

The sequential changes seen in the carcinogen-treated pouches were similar to those described by previous

workers (Salley 1957a; Morris 1961; MacDonald 1973). However, the gross ulceration and scarring typical of the early response of animals painted with a 0.5 per cent solution of DMBA were seen in only two animals. Neither of these animals was included in the experimental groups used for quantification. Salley (1957a) described the histological changes preceding the development of neoplasia in carcinogen-treated cheek pouch as inflammation, degeneration, regeneration and hyperplasia. In the present study after 3 weeks of carcinogen applications the appearance was similar to the inflammatory phase described by Salley but subjectively was less severe in most animals. However, the later phases of epithelial degeneration and regeneration were not seen. There was subjective evidence of cell death and cellular degeneration in some of the tissues removed at 3 weeks but this was not severe in most cases. Specimens taken at 6 weeks and 9 weeks corresponded to the hyperplastic phase described previously. There was no subjective difference in the degree of acanthosis or hyperkeratosis between animals examined at 6 and 9 weeks, but foci of epithelial atypia were more commonly present in the latter group.

In previous investigations the latent period for development of tumours has varied widely in individual experimental groups and has appeared to be related to such factors as the frequency and number of carcinogen applications and the concentration of the carcinogen (Morris 1961). In the present experiment the first tumours

appeared in two animals at 6 weeks and by 15 weeks papillomas were present in all surviving animals. Invasion was seen in the base, or stalk of some papillomas. Squamous carcinomas were all well-differentiated or moderately well-differentiated.

Although early reports (Salley 1954; 1957a) suggested that papillomas did not regress when carcinogen application was discontinued, Morris and Reiskin (1966) and Levij, Rwomushana and Polliack (1968) described spontaneous regression of small papillomas, showing that transition from squamous papilloma to carcinoma is not inevitable. However, there was no evidence of tumour regression in the present experiment, though admittedly the number of animals allowed to survive to the stage of papilloma formation was small.

3.5.2 Stereological Analysis

Normal Animals

The absolute value for the thickness of epithelium measured in sections is dependent on such technical variables as fixation, processing and section cutting. In the present investigation these variables were controlled as much as possible by using the same batch of fixative for all specimens, processing with the same automatic tissue-processing cycle and cutting the sections on the same microtome. However, it is interesting to note that the absolute value for the mean epithelial thickness of epithelium excluding keratin obtained in the present study ie, 26.6 μm is closely similar to that

obtained in a recently reported investigation of hamster cheek pouch by Franklin and Craig (1978b). Although these workers fixed the pouches in formol-acetic-alcohol the mean epithelial thickness obtained by a similar stereological method to that used in the present study was 27.02 μm. A higher value of 31.84 μm was obtained by estimating epithelial thickness by a series of direct measurements with an eyepiece graticule. It was concluded that the stereological method of thickness measurement was the more accurate due to the greater number of points and larger area of tissue sampled.

When sections are of finite thickness it is necessary to apply certain correction factors when estimating true numerical density of cells by counting nuclear profiles. The presence of fragments of cells, in which the central part of the nucleus is not in the plane of section, but are counted as nuclear profiles, gives an overestimate of true cell numbers. This overestimate due to counting nuclear fragments would be acceptable when comparing populations of cells of similar sizes but it is not acceptable when there is a significant difference in cell size. This is due to the fact that cells with a larger nuclear diameter have relatively more nuclear fragments in a given volume of tissue and cell counts would be biased towards this subpopulation.

The method used to correct for nuclear fragments in the present experiment was based on that described by Abercrombie (1946). The correction factor is derived

from the nuclear diameter and section thickness (Section 3.3.6). To measure the nuclear diameter Abercrombie (1946) suggested that the block should be recut at right angles to the original plane of section. This enables the diameter of nuclei to be measured at right angles to the plane of section. In the present study it was felt that such a procedure was impractical due to the small sizes of the blocks and it was assumed, therefore, that the horizontal width of nuclei would approximate closely to the required measurement. It was felt sufficient to measure the nuclear diameter only in a horizontal plane rather than by measuring and averaging the long and short nuclear axes. This was thought to be justified as, in sections orientated perpendicular to the surface, only the diameters of nuclei in a plane at right angles to the surface of the section determine the number of nuclear fragments present, and the widest horizontal width was taken to approximate to this measurement. All cells in a column passing through the full thickness of the epithelium were measured rather than selecting cells with a central, or widest diameter nucleus as advocated by Dunnill (1968) and Mackenzie (1970). It was considered that such selection might cause bias in favour of measuring larger cells.

The Holmes effect (Holmes 1921) is another factor which can cause errors in area estimation of structures in sections of finite thickness. The Holmes effect is due to the opacity of components in a section making it

impossible to detect overlapping boundaries within the section and leads to over-estimation of their size. The Holmes effect corrections were not applicable in the present study as nuclear profile areas were not determined, the area measurements being restricted to compartments.

Experimental Animals

Some of the quantitative changes found in this investigation are shown in a series of illustrative biopsies in Fig 3.24; all the sections are at the same magnification.

At 3 weeks the epithelium was in the inflammatory phase described by Salley (1957a). However, subjectively the degree of inflammation in the present experiment was less than that described by Salley. There was a twofold increase in total mean epithelial thickness, much of which was due to the nearly threefold increase in the thickness of the stratum corneum. The increase in the size of the cellular compartment was due to increases in the sizes of individual progenitor and maturation cells, their numbers, in fact being reduced. Many cells showed histological features of cellular degeneration and cell death and it is probable that much of the increase in cell size at this time was due to intracellular oedema in damaged cells. The cell-size measurements were over-estimated as intercellular spaces were included in the area measurement of the compartments. Subjectively there was an increase in the width of intercellular spaces in some specimens at 3 weeks but this was not usually marked. Major (1970) found that

in carcinogen-treated skin the intercellular spaces accounted for less than 10 per cent of the epithelial area.

At 6 weeks the epithelium was further increased in thickness but the inflammatory reaction was less severe. There had been a further considerable increase in the thickness of the stratum corneum. The increase in the thickness of the cellular compartment appeared to be due to a recovery in the number of cells in the maturation compartment which had returned to control levels but were considerably larger than normal maturation cells. There were increases in the numbers and to a lesser extent sizes of cells in the progenitor compartment.

At 9 weeks, that is 3 weeks after the painting of carcinogen had ceased, the overall mean epithelial thickness remained unchanged. However, there was a change in the relative proportions formed by the different compartments, the keratinized compartment having a decreased thickness and the cellular compartment a correspondingly increased thickness. This suggested that much of the early increase in the thickness of the stratum corneum was due to a hyperplasiogenic, or irritant action of the carcinogen rather than a neoplasiogenic action. The increase in the thickness of the cellular compartment appeared to be due to a progressive increase in the sizes and numbers of progenitor cells. This is the quantitative expression of one of the suggested features of epithelial atypia in oral mucosa; namely basal cell hyperplasia

(Smith and Pindborg 1969). In the hamster cheek pouch this might be more appropriately called progenitor cell hyperplasia and hypertrophy.

3.5.3 General Discussion

This study was the author's first experience with experimental oral carcinogenesis and the cheek pouch model was selected as it appeared to be the most widely used and reliable model in previous publications. However, experience of the model led the author to question its suitability for studies of this nature. Other workers have criticised the use of the cheek pouch model in studies of 'intraoral' carcinogenesis and their objections together with personal observations will be discussed.

A major objection to the cheek pouch is that the truly intraoral nature of this tissue is debatable. Kolas (1955) maintained that the cheek pouch could not be considered to be representative of the oral cavity proper as it was not subjected to the same environmental influences as the rest of the mouth. However, the pouch appears to be equally susceptible to carcinogenesis over the whole of its surface including the area adjacent to the oral cavity proper, which is presumably subjected to the same environmental influences. Stormby and Wallenius (1964) also objected to the cheek pouch on the basis of the major anatomical and histological differences between it and other oral mucosae. Salley (1957b) drew attention to the fact that the cheek pouch is derived

embryologically from the primitive buccal cavity and maintained that the histology of the pouch, apart from the absence of adnexal structures, is identical to the rest of the oral cavity. However, this assertion is incorrect. In addition to being considerably thinner than the rest of the oral mucosa and having a unique submucosal connective tissue, the cheek pouch has a single layer of progenitor cells in contact with the basement membrane. In the oral mucosa proper there is a suprabasal dividing cell population.

Smith (1968) found that the histochemical features of human and cheek pouch premalignant lesions were closely similar and considered that objections to the use of the pouch in regard to the intraoral nature were unimportant. In the standard model of cheek pouch carcinogenesis, in which a 0.5 per cent solution of DMBA is used (Morris 1961), neoplastic transformation is very rapid. Homburger (1972) has drawn attention to the possibility that many of the so-called features of premalignancy described in such models could be epiphenomena related to a hyperplasiogenic or irritant action of the carcinogen, rather than a truly neoplasiogenic effect. From the quantitative studies undertaken in the present experiment it would appear that much of the hyperkeratosis seen in the early stages of carcinogen treatment is a reversible reaction and probably related to an irritant action of the carcinogen.

A further objection to the cheek pouch is that it is

a site of immunological privilege. For example, it will accept heterografts of both normal and neoplastic tissues (Billingham, Ferrigan and Silvers 1960; Williams, Evans and Blamey 1971). The relationship between this unusual characteristic and the development and behaviour of chemically induced neoplasms is not well understood, but Smith (1968) believes it is possible that this property could in part be responsible for the high degree of susceptibility of the pouch to chemical carcinogens. Finally, a somewhat mundane but no less valid objection is the technical difficulty of handling the tissue. The pouch mucosa is very thin and has a gelatinous, sticky, submucosal layer and this makes the taking of biopsy specimens and the trimming of blocks difficult.

It was judged, therefore, that the standard hamster cheek pouch model was far from 'ideal' as a model of intraoral carcinogenesis. The cheek pouch still has potential as a model of 'epithelial' carcinogenesis, especially in relationship to epithelio-mesenchymal reactions due to its relatively simple morphology and the absence of adnexal structures.

3.6 CONCLUSIONS

Sequential histological changes in hamster cheek-pouch epithelium following applications of the carcinogen dimethyl-benzanthracene have been assessed using stereological methods. The most significant progressive change following cessation of carcinogen applications appeared to

be an increase in the size and the number of cells in the progenitor compartment. Much of the marked increase in the thickness of the stratum corneum seen in the early stages of treatment appeared to be due to a hyperplasiogenic rather than a neoplasiogenic action of the carcinogen.

The stereological techniques described in this chapter, although time consuming did yield morphological details that escaped subjective evaluation. The application of similar techniques to a more acceptable model of oral carcinogenesis might yield valuable information.

ILLUSTRATIONS AND TABLES

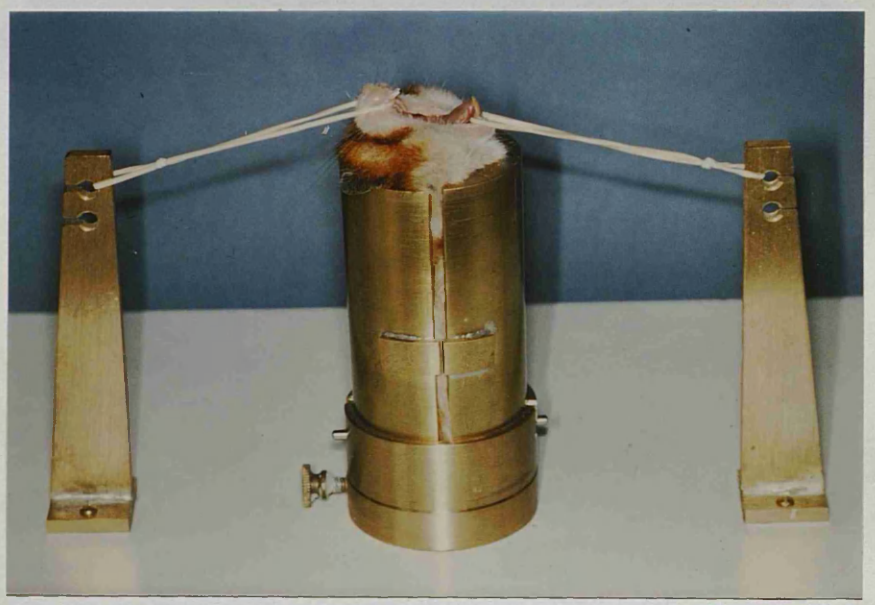


Fig 3.1 Anaesthetised hamster in restraining device.

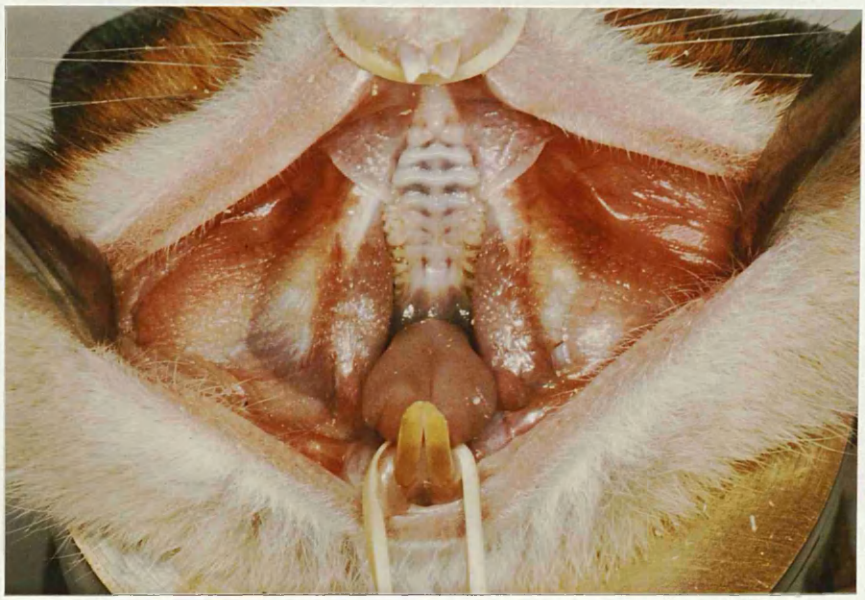


Fig 3.2 Hamster in restraining device with cheek pouches exposed.

Fig 3.3 Normal cheek pouch exposed by cutting through the lateral wall.

Fig 3.4 Area of medial wall of pouch from which the necropsy sample was taken.

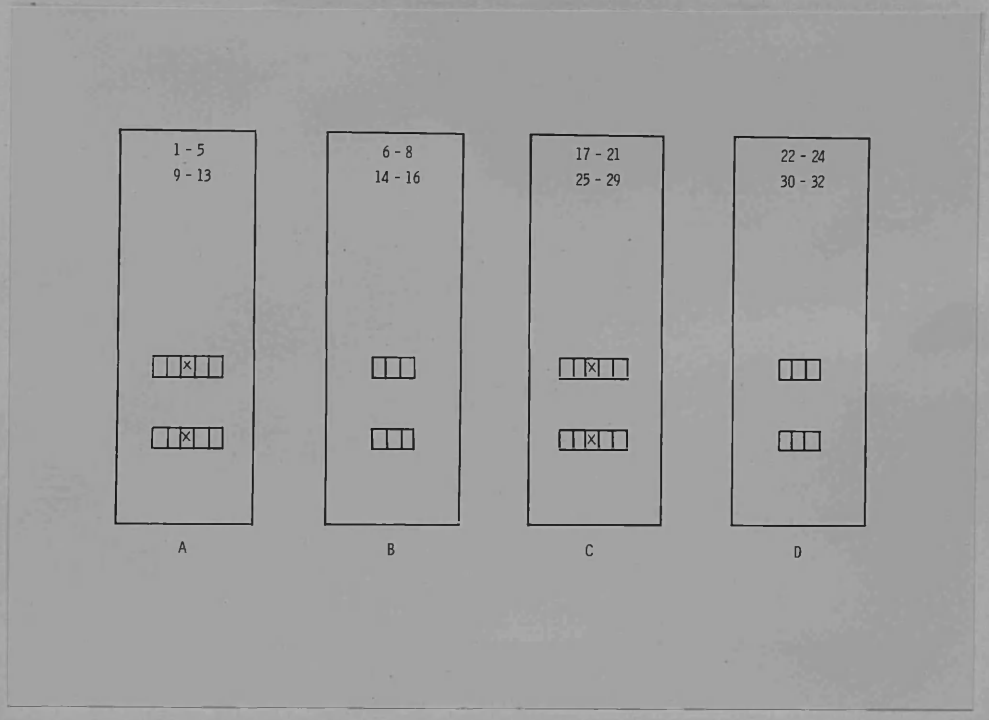


Fig 3.5 Arrangement of serial sections. The sections marked x on slides A and C were used for counting and slides B and D were retained as spares.

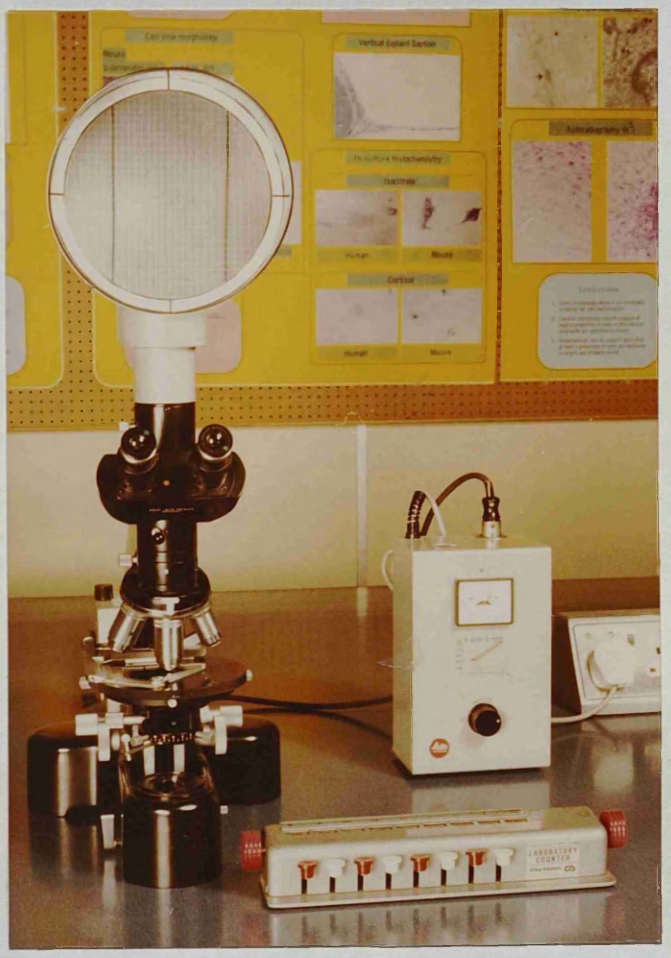


Fig 3.6. Arrangement of equipment for stereological quantification. The Leitz Ortholux microscope has a circular transparent grid on the projection screen. A laboratory counter was used to record data.

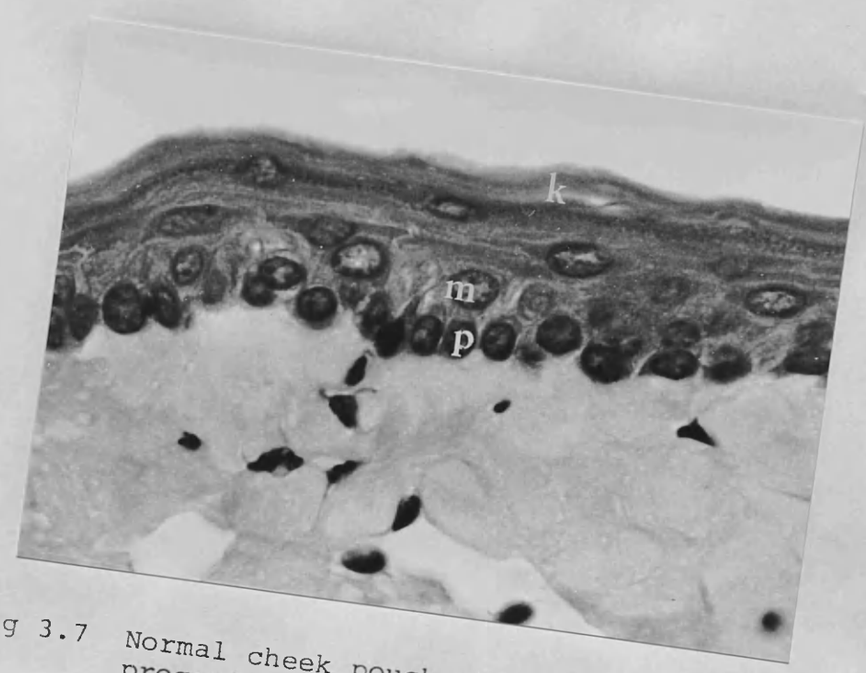


Fig 3.7 Normal cheek pouch mucosa showing progenitor (P), maturation (M) and keratinized compartment (K) x 1150

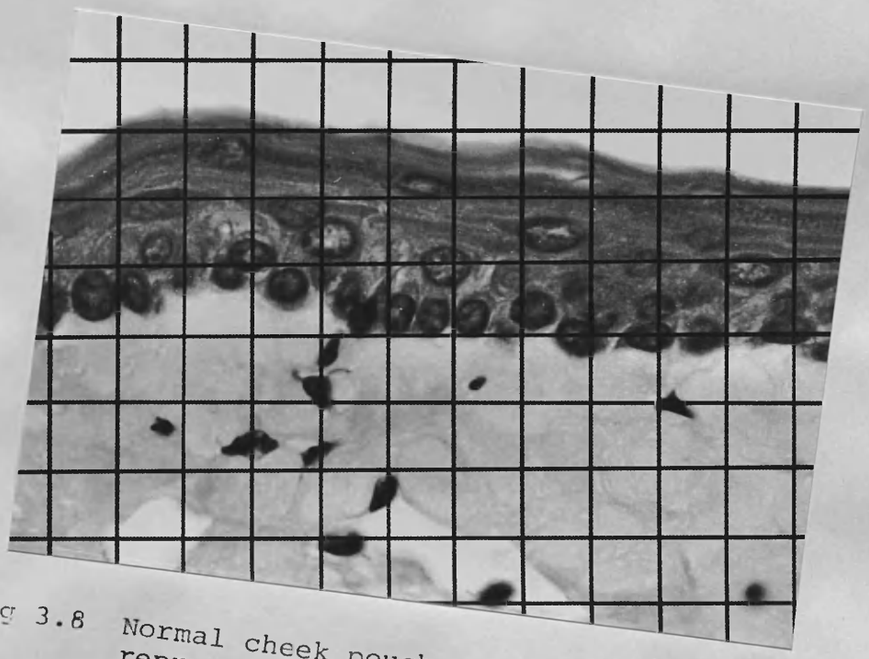


Fig 3.8 Normal cheek pouch mucosa with diagrammatic representation of the point counting grid in position.

Fig 3.9 Areas of white hyperkeratotic plaques at 6 weeks.

Fig 3.10 Extensive white plaques and early papilloma formation at 6 weeks.

Fig 3.11 Multiple papillomas at 12 weeks.

Fig 3.12 Large exophytic tumour at 15 weeks.

Fig 3.13 Normal cheek pouch mucosa x 600

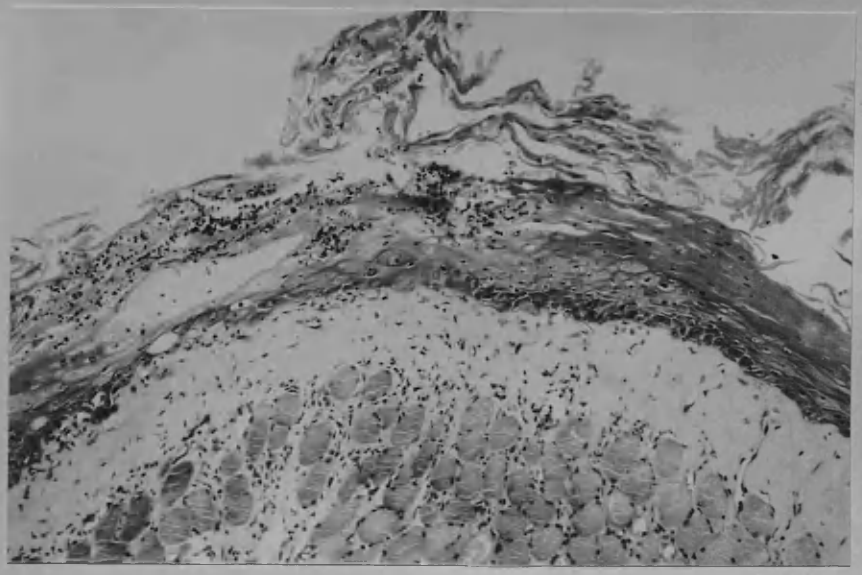


Fig 3.14 Incipient ulceration after 3 weeks of DMBA treatment x 160.

Fig 3.15 Mild epithelial atypia at 6 weeks x 256

Fig 3.16 Papilloma formation at 6 weeks x 160

Fig 3.17 Large papilloma with areas of severe dysplasia x 40

Fig 3.18 Large papilloma with invasion of base at 15 weeks x 9

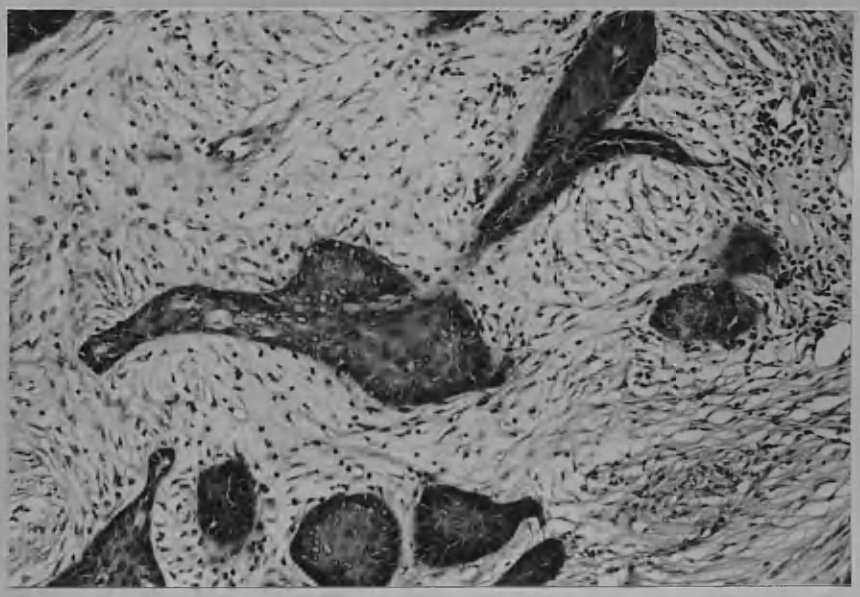


Fig 3.19 Well differentiated squamous cell carcinoma invading fibroblastic stroma x 160

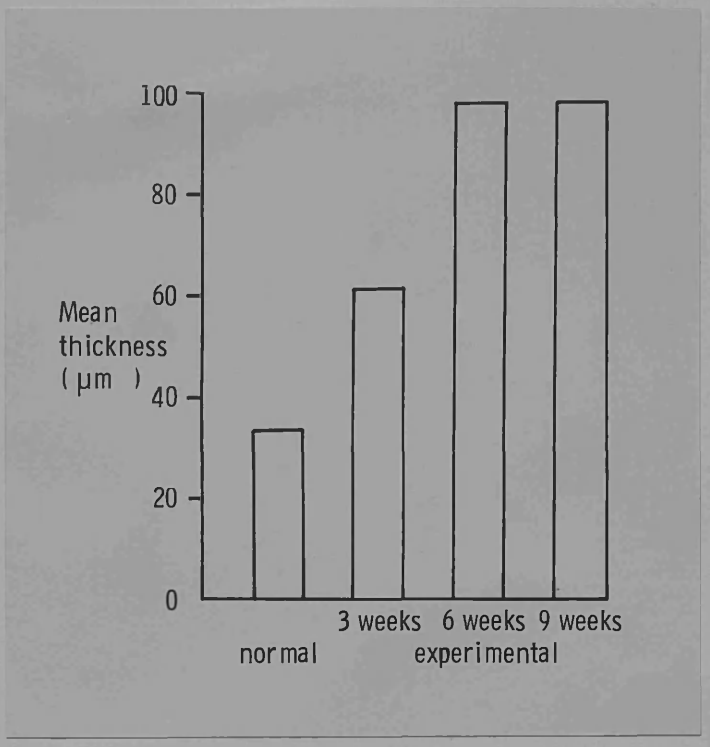


Fig. 3.20 Mean epithelial thickness following applications of DMBA

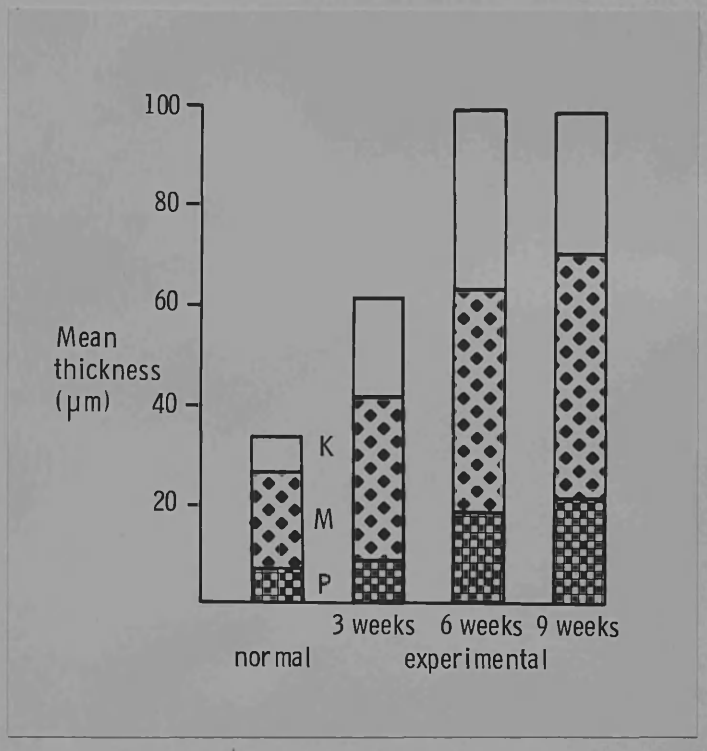


Fig. 3.21 Mean thickness of epithelial compartments. The proportion of the total thickness of each compartment is indicated by P, progenitor; M, maturation and K, keratinized compartments.

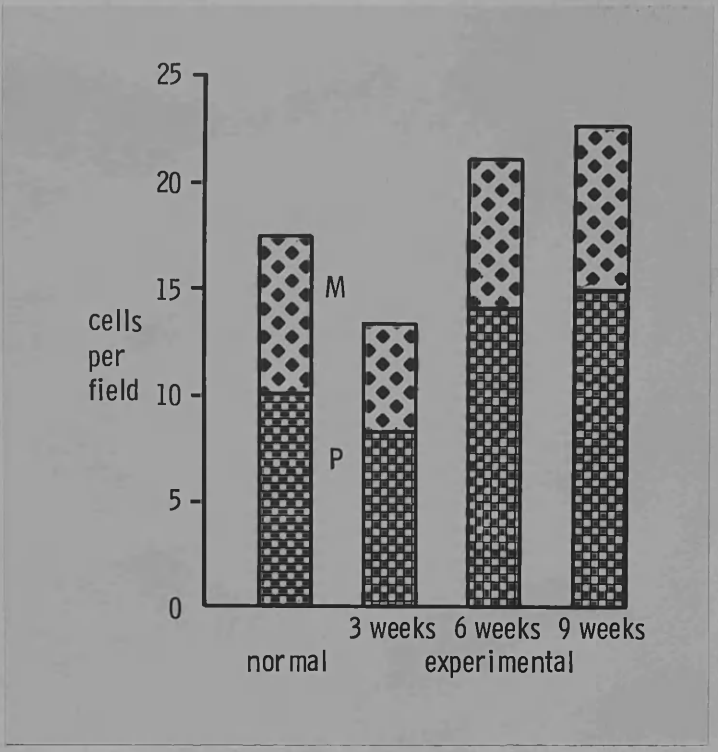


Fig. 3.22 Corrected cell numbers per field.

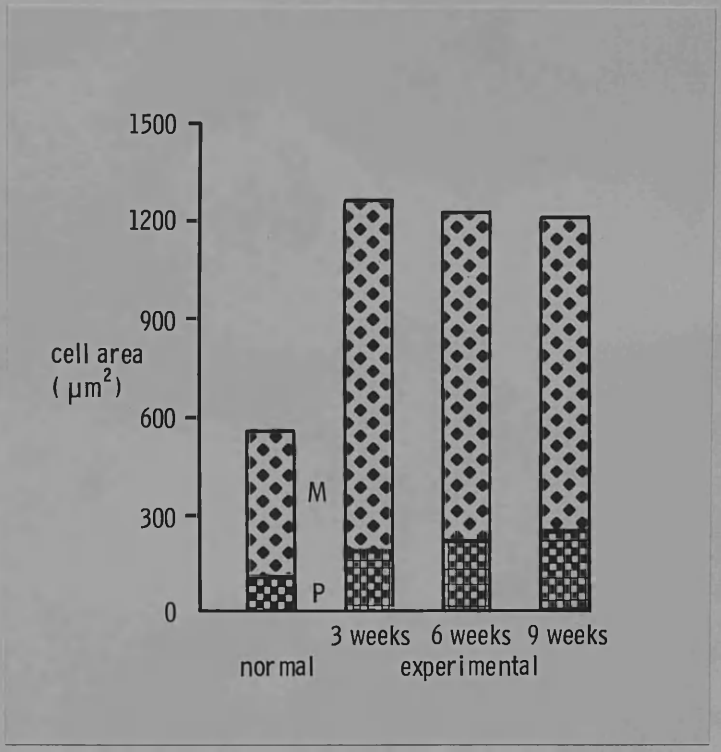


Fig. 3.23 Cell size expressed as mean section area.

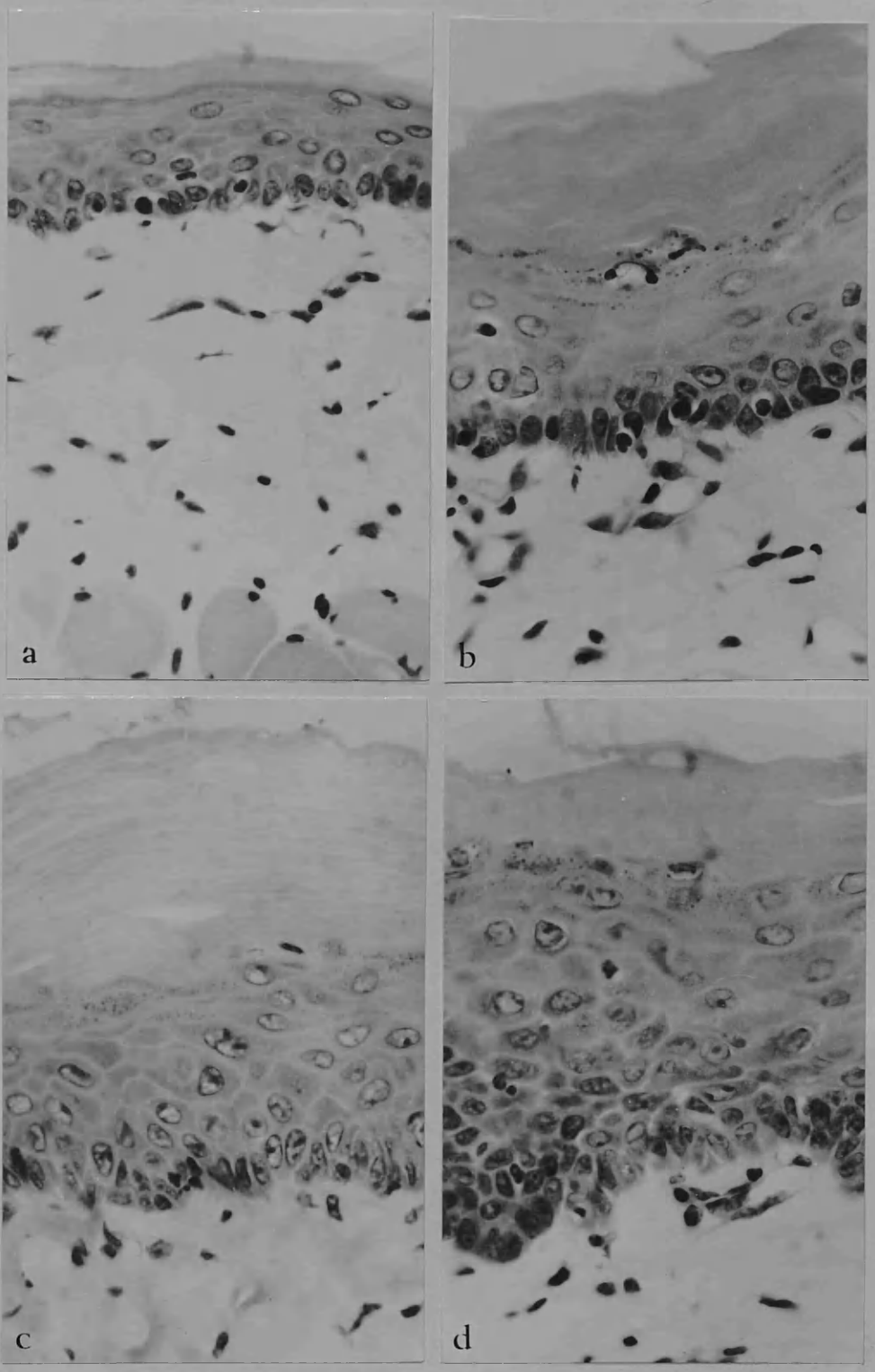


Fig 3.24 Typical areas from normal and DMBA treated cheek pouch. All photomicrographs are at the same magnification (x 400) (a) Normal (b) 3 weeks (c) 6 weeks (d) 9 weeks.

Animals	T_p	T_m	T_k	T_{total}
Normal				
1	6.6	24.9	7.3	38.8
2	5.4	16.9	7.0	29.3
3	6.3	20.3	7.5	34.1
4	6.3	20.1	5.6	32.0
5	7.2	18.9	6.0	32.1
x	6.4	20.2	6.7	33.3
SD	0.7	2.9	0.8	3.5
3 weeks				
6	9.2	35.1	21.8	66.1
7	10.3	29.2	19.0	58.5
8	6.4	20.7	15.4	42.5
9	8.2	42.5	20.9	71.6
10	8.5	36.1	20.0	64.6
x	8.5	32.7	19.4	60.7
SD	1.0	8.2	2.5	11.3
6 weeks				
11	24.2	46.1	41.1	111.4
12	17.2	43.4	47.7	108.3
13	14.3	45.3	29.7	89.3
14	15.6	41.8	34.0	91.4
15	16.1	44.3	35.1	95.5
x	17.5	44.2	37.5	99.2
SD	3.9	1.7	7.0	10.0
9 weeks				
16	25.3	49.9	41.4	116.7
17	19.1	50.3	23.4	92.8
18	24.6	51.2	25.0	100.8
19	16.2	45.4	27.6	89.2
20	17.7	45.0	21.8	84.5
x	20.6	48.4	27.8	96.8
SD	4.1	2.9	7.9	12.6

Table 3.1 Mean epithelial thickness derived from point counts and column width. Mean and standard deviations are included in the tables for comparative purposes but non-parametric tests were used for statistical analysis. Abbreviations used in tables are included in Appendix 2.

Animals	P.C.	M.C.	$C_{P.C.}$	$C_{M.C.}$	A_p	A_m
Normal						
1	28.3	27.3	10.1	7.9	98.1	507.6
2	24.5	25.7	9.3	7.5	93.5	362.7
3	28.0	28.2	10.6	8.2	95.3	398.8
4	26.8	23.3	10.2	6.6	100.0	489.4
5	25.8	23.0	9.8	6.7	117.3	453.7
$\bar{x}$	26.7	25.5	10.0	7.4	100.8	442.4
SD	1.6	2.3	0.6	0.7	9.5	60.9
3 weeks						
6	27.0	25.8	9.5	6.4	155.8	881.3
7	23.7	17.3	8.3	4.3	161.4	1090.7
8	10.8	9.5	3.8	2.4	271.0	1383.3
9	25.3	21.8	8.9	5.5	148.8	1243.6
10	30.8	26.4	10.8	6.6	126.8	878.7
$\bar{x}$	25.5	20.2	8.3	5.0	172.7	1095.5
SD	8.9	7.0	2.7	1.7	56.5	222.3
6 weeks						
11	43.0	25.3	15.9	6.8	244.6	1089.7
12	33.5	25.5	12.4	6.9	223.3	1011.5
13	38.2	27.2	14.1	7.3	163.1	998.6
14	37.3	23.8	13.8	6.4	181.8	1050.0
15	37.1	25.0	13.8	6.8	188.4	1048.5
$\bar{x}$	37.8	25.4	14.0	6.8	200.2	1039.6
SD	3.4	1.2	1.3	0.3	33.0	35.9
9 weeks						
16	47.5	27.7	16.2	7.5	251.2	1070.6
17	46.7	35.0	15.9	9.5	193.0	851.5
18	46.7	31.8	15.8	8.6	249.0	956.9
19	34.0	24.2	11.6	6.5	225.0	1121.5
20	39.2	25.0	13.3	6.8	213.5	1064.7
$\bar{x}$	42.8	28.7	14.6	7.8	226.3	1013.0
SD	6.0	4.6	2.0	1.3	24.5	108.4

Table 3.2 Uncorrected and corrected cell numbers and cell sizes in the progenitor and maturation cell compartments.

Animals		P	M
Normal	$\bar{x}$	5.7	8.4
	SD	1.2	1.9
3 weeks	$\bar{x}$	6.5	10.4
	SD	1.5	2.0
6 weeks	$\bar{x}$	6.0	9.5
	SD	1.5	2.0
9 weeks	$\bar{x}$	6.7	9.6
	SD	1.3	1.6

Table 3.3 Nuclear diameters in μm in progenitor (P) and maturation (M) compartments used in the calculation of Abercrombie's correction.

CHAPTER FOUR

HAMSTER TONGUE CARCINOGENESIS: EXPERIMENT 1

The effects of 4-nitroquinoline N-oxide on the ventral lingual mucosa: a pilot study

4.1 INTRODUCTION

The hamster cheek pouch has been the most widely used site for the experimental study of oral carcinogenesis. However, several investigators have questioned the suitability of this site and, as discussed in Section 3.5.3 personal experience led the author to judge that the cheek pouch system, although a simple and reliable model of epithelial carcinogenesis, fell short of the requirements of an ideal model of intraoral carcinogenesis. It was therefore decided to attempt to devise a more suitable experimental model of carcinogenesis in a truly intraoral site.

Although investigators have attempted to induce neoplasms in truly intraoral sites success has been limited (see Chapter 1), the tongue proving to be highly resistant to the action of chemical carcinogens. Most investigators have used the powerful fat-soluble polycyclic hydrocarbon dimethyl benzanthracene (DMBA) or the water-soluble carcinogen 4-nitroquinoline N-oxide (4NQO).

The first description of the use of 4NQO to produce intraoral neoplasms was by Fujino, Chino and Imai (1965)

who painted mouse labial mucosa with a 0.25 per cent solution of 4NQO "every weekday" for between 180 and 570 days. Forty-seven per cent of animals surviving 180 or more days of the experiment developed labial carcinomas. The tumour yield rose to nearly 80 per cent when in addition to applications of 4NQO a metal wire inserted between the lower incisors was used to injure continually the labial mucosa. Five per cent of the animals also developed lingual carcinomas.

When the palates of rats were painted thrice weekly with a 0.5 per cent solution of 4NQO palatal carcinomas were produced in all experimental animals within a period of seven months (Wallenius and Lekholm 1973a). In addition, 75 per cent of the animals developed squamous cell carcinomas of the dorsal surface of the tongue where it had come into contact with the treated area of the palate.

The efficiency of 4NQO in producing high yields of lingual carcinomas in relatively short periods of time and the fact that mechanical irritation of the tissues was not a necessary precursor of malignancy seemed to make 4NQO a suitable carcinogen to investigate for use in studies of intraoral carcinogenesis. The object of this pilot study can be summarized as follows:-

1. To attempt to produce epithelial neoplasms in the ventral lingual mucosa of the hamster by regular painting with the water-soluble carcinogen 4NQO.

2. To test the reliability and ease of the above method for the production of extra-pouch, intraoral malignancies.

3. To assess the value and limitations of a biopsy technique coupled with subsequent observation of the contralateral half of the tongue.

4.2 MATERIALS AND METHODS

4.2.1 Animals

The same line-bred strain of hamsters that was used in the experiment described in Chapter 3 was used in the present study. The animals were aged between eight and ten weeks at the start of the experiment and were housed and fed as in the previous experiment.

4.2.2 Site of Carcinogen Application

The carcinogen was applied to the ventral surface of the tongue. There were several reasons for selecting this area.

Firstly, in man over 70 per cent of intraoral squamous cell carcinomas occur in an area of mucosa accounting for less than 20 per cent of the total oral mucosal area (Moore and Catlin 1967). This region of higher incidence of tumours has been called the "drainage area" since it consists of the mucosa of and immediately adjoining the linqual sulcus, the floor of the mouth including the ventral lingual mucosa, and the retromolar region. Carcinogens present in the mouth are most

likely to drain these areas before being swallowed.

Secondly, work by MacDonald (1973) on carcinogenesis in the drainage area of the hamster suggested that the ventral lingual mucosa was the site within this area most susceptible to chemical carcinogens.

Finally, in order to reduce the number of animals used and to follow sequential changes in individual animals it was felt desirable to take a biopsy sample from the treated area of mucosa after several weeks of carcinogen application and to kill the animals after a further period of observation. In the hamster tongue there is a sharp demarcation between the papillated anterior and lateral margins of the tongue and the thinner lining epithelium of the ventral mucosa. This delineates a relatively large experimental area and facilitates the taking of a standard biopsy.

4.2.3 Carcinogen

4-nitroquinoline N-oxide is a water-soluble chemical the carcinogenic effect of which was first demonstrated by Nakahara, Fukuoka and Sugimura (1957). Although it has been reported that quinone-preparations cause destruction of cell nuclei and poisoning of laboratory animals even when applied locally on the skin (Ahlström 1951), Wallenius and Lekholm (1973a) found that 4NQO dissolved in propylene glycol did not cause clinical signs of poisoning or toxic injury to internal organs when applied intraorally.

4NQO* was dissolved in propylene glycol to a

* Fluka A G Chemische Fabrik, Buchs 56, Switzerland

concentration of 0.5 per cent.

4.2.4 Anaesthesia and Manipulation

Animals were anaesthetized in an ether chamber as described in Section 3.2.3 and immobilised in the restrainer with their mouths held open by elastic bands. The tongue was grasped with tweezers and the mucosa quickly dried with a short blast of air from a chip syringe. The 4NQO was applied to the mucosa by a single stroke of a number three camel hair brush in a postero-anterior direction, using a wiped-brush technique (Morris 1961).

The amount of carcinogen applied was estimated by measuring the increase in weight of 20 glossy papers treated with a single stroke of the brush. The average amount of carcinogen applied at each stroke was 0.11 mg.

The same safety precautions were applied as in the previous experiment (Section 3.2.6).

4.2.5 Biopsy Technique

Biopsy specimens from the ventral surface of the tongue were obtained from animals in the experimental groups. The technique for biopsy was similar to that described by MacDonald (1973). Animals were anaesthetised by intraperitoneal barbiturate and immobilised in the restrainer used for the painting procedure. The tip of the tongue was held between forceps by one operator and gently rolled towards the palate to apply slight tension to the mucosa and add support to the tongue. A biopsy consisting of half the experimental area, a strip of tissue some 4 x 2mm was taken by a second operator. With a

number 15 scalpel blade vertical incisions were made in the midline of the tongue and about 2mm laterally, close to the junction of the ventral surface and the lateral border of the tongue. These incisions were joined by scalpel across the tip. The corner of the mucosa towards the tip of the tongue was held gently in fine forceps and a mucosal flap was raised from the underlying tissues using fine scissors. The base was cut with the scissors to free the biopsy, giving a portion of tissue approximately 4mm x 2mm x 1mm. There was surprisingly little haemorrhage, provided great care was taken to avoid damaging the large sublingual veins.

Post-operative healing was usually rapid and uneventful. One animal, however, died a week after the biopsy, probably as a result of secondary haemorrhage.

The specimens obtained at the time of killing were removed in a similar manner to that used in the biopsy but animals were not allowed to recover from the anaesthetic.

4.2.6 Experimental Design

Twenty animals were used in this investigation. Five animals were untreated normal controls. These animals were killed at the start of the experiment and the ventral lingual mucosa was removed in a manner identical to that used in the biopsy procedure. The remainder were to be painted with carcinogen twice weekly for 10 weeks. During this period three animals died (see Section 4.3.2) and the remainder were divided into two groups and treated as follows:-

Group A

(5 animals) A biopsy was taken from the left half of the ventral lingual mucosa (Section 4.2.5). The animals were then weighed and their tongues examined at weekly intervals for a further 15 weeks.

Group B

(7 animals) These animals received twice weekly painting for an additional 10 weeks and were then subjected to biopsy in a manner identical to that used for Group A. The hamsters were then weighed and their tongues examined weekly for a further five weeks (see Fig 4.1).

Since histological examination of biopsy specimens showed little apparent reaction in either group the decision was made to apply the powerful promoting agent croton oil* to establish whether initiation had occurred. Croton oil is a potent promoting agent for the production of skin tumours in animals painted with initiating agents such as urethane (Salaman and Roe 1964) and polycyclic hydrocarbons (Berenblum and Shubik 1947) and has been shown to have a co-carcinogenic effect intraorally (Silberman and Shklar 1963). In the present study a 0.5 per cent solution of croton oil in acetone was prepared and at the 25th week from the start of the experiment the surviving animals from Group A (4 animals) and Group B (5 animals) were painted twice weekly in a manner similar to that used for the

* Sigma Chemical Company

application of carcinogen. After 10 paintings with croton oil the tongues were observed at weekly intervals for an additional six months before the animals were killed by ether inhalation and their tongues removed.

4.2.7 Laboratory Procedures

Two blocks cut at right angles to the surface were obtained from each biopsy or necropsy specimen. These were fixed in 10 per cent buffered formol saline, and post-fixed with mercuric chloride. Tissues were processed on an automatic tissue processor using a standard cycle and paraffin-embedded sections were cut on a Leitz rotary microtome set at 6 μm. Three short ribbons of three levels were obtained from each block. Sections were stained with haematoxylin and eosin.

4.3 RESULTS

4.3.1. Normal Animals

The ventral lingual mucosa is sharply demarcated from the papillated lateral and anterior margins of the tongue (Fig 4.2). Histologically the ventral mucosa has a regular epithelio-mesenchymal junction with short rete ridges. The epithelium is orthokeratinized and has no papillae (Fig 4.3).

4.3.2. Experimental Animals

No macroscopical changes were seen during the period of carcinogen application. After 10 weeks of applications most animals showed histological evidence of mild epithelial hyperplasia and slight increase in keratin

thickness. At 20 weeks there had been little further change apart from occasional scattered foci of chronic inflammatory cells in the superficial corium.

During the period of application of croton oil the treated mucosa became reddened and oedematous but did not ulcerate. At 40 weeks an animal from Group A developed a one millimetre diameter whitish nodule in the treated area of mucosa. This remained static in size and at the time of killing was seen to be a papilloma (Fig 4.4). There was no epithelial atypia in the papilloma or in the adjacent epithelium. From the 33rd week one animal from Group B developed an area of diffuse greyish-white opacity in the treated area. This area did not change in appearance during the subsequent observations and histology of tissue taken at the time of killing revealed areas of severe epithelial atypia with prominent dyskeratosis and acantholysis (Fig 4.5). Small parts of the biopsies taken at the time of killing from clinically normal areas of two other animals from Group B showed similar but less florid histological features. In the other surviving animals from both experimental groups the treated areas of mucosa were not histologically distinguishable from the mucosa of untreated controls at the termination of the experiment.

There was a high mortality rate (nearly 50 per cent) over the duration of the experiment. Four animals were lost when they failed to recover from the anaesthetic after painting or intraoral observations. Three animals were

found consistently to lose weight and were clearly failing to thrive. These animals were killed prematurely in order to prevent suffering. Routine post-mortem examinations of such animals usually showed intense venous congestion of the lungs but no other significant macroscopical or histological features in the major organs.

4.4 DISCUSSION

The present study indicates that the hamster tongue is relatively refractory to the action of the water-soluble carcinogen 4NQO. In contrast, Wallenius and Lekholm (1973a) have shown the rat palate and dorsal surface of the tongue to be susceptible to 4NQO. In the present study the most striking feature of the response of the hamster lingual mucosa to 4NQO was the virtual lack of reactivity in most experimental animals. The finding of a small papilloma and several areas of epithelial atypia however, suggests the possibility that more frequent or prolonged applications of the carcinogen might have eventually given rise to malignant neoplasms.

Wallenius and Lekholm (1973a) attempted to explain the increased efficiency of 4NQO over DMBA in rat oral tumourigenesis by suggesting that the salivary layer present on the oral mucosa was relatively protective against fat-soluble DMBA but not against water-soluble 4NQO. In vitro experiments by Wallenius and Lekholm (1973b) appeared to show that 4NQO easily penetrated the salivary layer, but subsequent work using xerostomic rats (Lekholm

and Wallenius 1976) showed a protective action of saliva in vivo whether the oral mucosa was treated by water- or fat-soluble carcinogens.

The results of the present investigation illustrate variability of tissues by species and site in their response to chemical carcinogens. They also suggest that it is an individual species or site susceptibility rather than a fundamental difference in the reactions between fat-soluble and water-soluble carcinogens and the salivary layer which accounts for the known efficiency of 4NQO in producing oral cancer in the rat.

This investigation was a pilot study and did not allow the distinction to be made between a weakly carcinogenic action of 4NQO or croton oil. However, the prolonged latency, variability of the response and high mortality would render this a poor experimental model.

4.5 CONCLUSIONS

Applications of 4NQO biweekly for up to 20 weeks followed by applications of the promoting agent croton oil can lead to the development of papillomas and areas of epithelial atypia in hamster ventral lingual mucosa. However, the long latency, very variable response and high mortality would make this a poor experimental model. The biopsy technique described seemed to be a useful method of following sequential changes after carcinogen application. The technique was relatively simple and was associated with a low operative mortality.

ILLUSTRATIONS

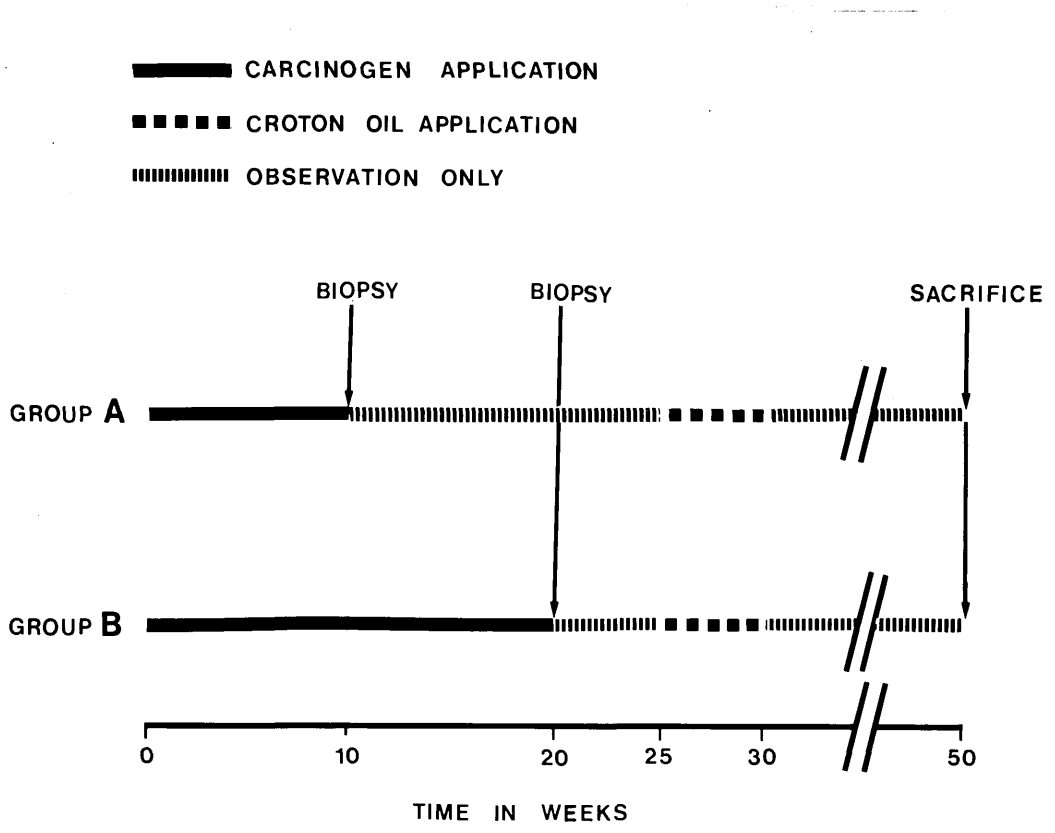


Fig. 4.1 Diagrammatic representation of the protocol used in hamster tongue carcinogenesis Experiment 1.

Fig. 4.2 Normal ventral lingual mucosa

Fig. 4.3 Histology of normal ventral surface of hamster tongue x 400

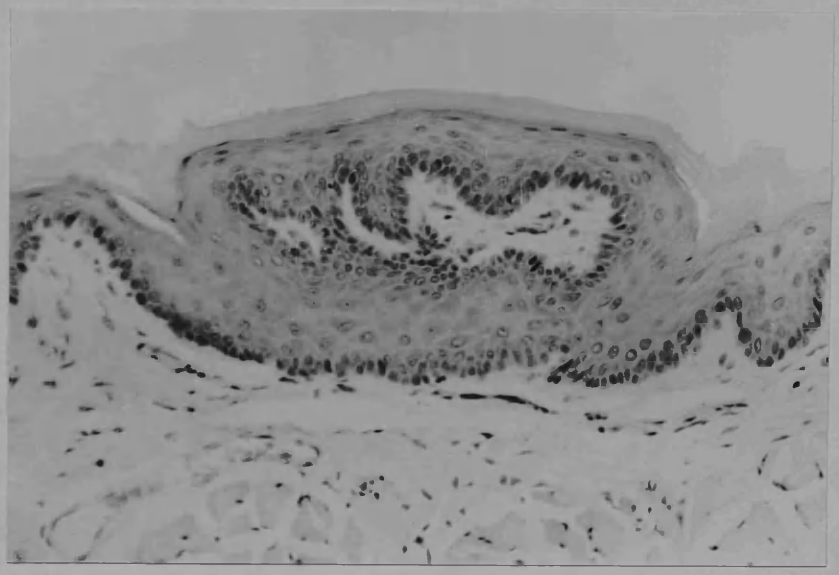


Fig. 4.4 Small lingual papilloma x 145

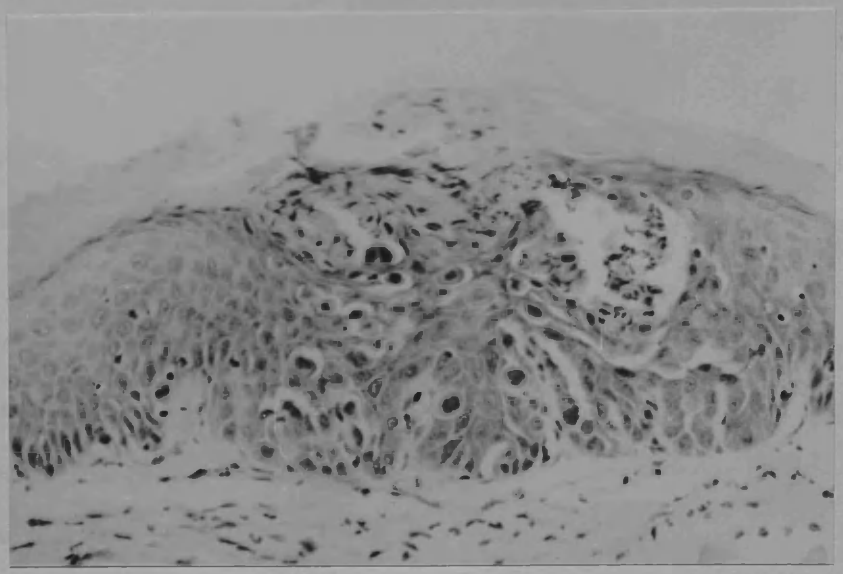


Fig. 4.5 Area of severe epithelial atypia showing dyskeratosis and suprabasal acantholysis x 210

CHAPTER FIVE

HAMSTER TONGUE CARCINOGENESIS: EXPERIMENT 2

The effects of DMBA and mild mechanical irritation on the ventral lingual mucosa: a pilot study

5.1 INTRODUCTION

The experiment reported in Chapter 4 described how an attempt to produce lingual carcinomas in the hamster with the water-soluble carcinogen 4-nitroquinoline N-oxide was unsuccessful. It was decided therefore to investigate other models of lingual carcinogenesis that have been developed using dimethyl benzanthracene (DMBA).

Dachi (1967) produced lingual carcinomas in 4 out of 15 hamsters after DMBA dissolved in dimethyl sulphoxide was applied to the tongue thrice weekly for 30 weeks. He did not specify the exact area of the tongue to which the carcinogen was applied but noted that large tumours frequently developed in adjacent parts of the oral mucosa and perioral skin and that these tumours were often responsible for the animal's death. The experimental mortality was 60 per cent.

A model of lingual carcinoma in the hamster was developed by the Japanese workers Fujita et al (1973a; 1973b) who first scratched and ulcerated the mucosa with a barbed broach and then painted the treated area with a DMBA - acetone solution. When the lateral border of

the middle third of the tongue was treated in this manner all the experimental animals developed lingual carcinomas within 13-25 weeks. In addition 12 per cent of animals showed cervical lymph node metastases.

5.1.1 Object of Present Study

The technique described by Dachi (1967) would appear to be of limited value in a study of intraoral carcinogenesis due to the prolonged latency, development of neoplasms outside the treated area and the high mortality. On the other hand, the technique described by Fujita et al (1973a; 1973b) would seem to have many attractions as a model of experimental lingual carcinoma. However, examination of the photographs in the papers of Fujita et al indicated that the 'scratching' procedure they had used was in fact extremely destructive. It was felt that scratching to this extent should not be repeated because of the problems in histological interpretation that this degree of preliminary ulceration could present. In addition, these workers gave no indication of the histological changes preceding the development of neoplasia.

Therefore, the object of the present investigation was to attempt to produce neoplasms of the ventral mucosa of the hamster tongue by a combination of light scratching of the mucosa, avoiding macroscopical ulceration, and painting with DMBA. A biopsy technique was to be employed to monitor the early histological changes.

5.2 MATERIALS AND METHODS

5.2.1 Animals

The animals were 8-10 weeks old male Syrian hamsters of the same line-bred strain used in the previous experiments.

5.2.2 Experimental Techniques

The carcinogen was a 0.5 per cent solution of 9, 10-dimethyl-1, 2-benzanthracene (DMBA) in acetone. Animals were anaesthetised with ether and restrained as in the previous experiment and the ventral lingual mucosa was quickly dried with cotton wool pledgets.

5.2.3 Carcinogen Application and Scratching Procedure

A fine barbed broach (Fig 5.1) was used to scratch lightly the ventral lingual mucosa prior to carcinogen application. It was found difficult to scratch the mucosa in an antero-posterior direction and it was therefore scratched lightly five times in the right-to-left direction and five times in a left-to-right direction. The broaches soon became clogged with organic matter making them ineffective and had to be discarded after three or four animals had been treated. The carcinogen was applied to the ventral lingual mucosa by a single stroke of a number three camel-hair brush in a postero-anterior direction. A short blast of air from a chip syringe was used to evaporate the volatile acetone leaving a crystalline residue of carcinogen adherent to the desired part of the tongue. The average amount of carcinogen applied with each stroke was 0.09 mg. Applications were made twice weekly

at some time between 9.00 am and 10.30 am. The safety precautions described in the previous two experiments were again observed.

5.2.4 Experimental Design

26 animals were used in this investigation. At the outset it was not known what the response to the planned treatment was likely to be. The experiment was modified during its course as circumstances dictated.

Experimental Animals

There were 12 hamsters in this group. The original plan was to kill two of these animals during the early weeks of carcinogen application to monitor the progression of any lesions. Biopsy samples were to be taken from five animals after 10 weeks of twice weekly scratching and carcinogen applications. An additional group of five animals would then be subjected to a biopsy after a carcinogen-free interval of several weeks. Both groups were to be observed until either neoplasms developed or a reasonable period had elapsed from the end of the painting procedure.

One animal failed to recover from the anaesthetic after 2 weeks of carcinogen applications and the ventral lingual mucosa was taken for histological examination. At 8 weeks one of the animals was killed and the ventral lingual mucosa removed. One hamster died at 10 weeks, over a weekend period. This reduced the number of experimental animals and necessitated a slight modification in the experimental design. Therefore, at 12 weeks, 2 weeks

after the last experimental manipulation, a biopsy consisting of half the treated area of mucosa was taken from four animals using the technique described in Section 4.2.5. At 17 weeks a biopsy consisting of half the treated area of ventral lingual mucosa was taken from the remaining five hamsters of the experimental group. At 25 weeks the surviving experimental animals (8 hamsters) were killed by an overdose of intraperitoneal barbiturate and the ventral lingual mucosa was removed.

Control Animals

Five animals were untreated controls and were killed at the start of the experiment. The lingual mucosa was removed in a manner similar to that used in the biopsy procedure.

Five animals were used to assess the effects of scratching during the early stages of the experiment. The lingual mucosa was scratched twice weekly in the same manner as the experimental group and one animal was killed each week for five weeks, and the ventral lingual mucosa removed for histological examination.

A further four animals received twice weekly scratching of the ventral lingual mucosa followed by acetone applications, in a manner similar to that used to apply the carcinogen, for a maximum of 10 weeks. Animals from this group were killed at 8, 12, 17 and 25 weeks.

5.2.5 Laboratory Procedures

The laboratory procedures were similar to those described in Section 4.2.7.

5.3 RESULTS

5.3.1 Normal Animals

The normal ventral lingual mucosa showed appearances identical to that described in Section 4.3.1 and illustrated in Figs 4.2 and 4.3.

5.3.2 Scratch Controls

There were no subjective macroscopical or histological changes in the ventral lingual mucosa of animals that had been treated for 1, 2, 3 or 4 weeks. There was mild epithelial hyperplasia and mild hyperorthokeratosis in the treated area of mucosa in the specimen taken after 5 weeks of scratching.

5.3.3 Scratch/Acetone Controls

There was mild epithelial hyperplasia and mild hyperorthokeratosis in the specimens taken at 8 and 12 weeks but the subsequent samples were not histologically distinguishable from untreated tissue.

5.3.4 Scratch/DMBA Animals

During the first few weeks of scratching and DMBA applications the treated area of mucosa became reddened but there was no macroscopical evidence of ulceration.

In the animal which failed to recover from the anaesthetic after the fourth application of carcinogen there was slight hyperorthokeratosis and occasional small tears were seen in the superficial keratin. Subjectively there appeared to be an increased number of mitotic figures. A patchy chronic inflammatory cell infiltration was present in the superficial corium.

From about the fourth week of treatment onwards the

ventral lingual mucosa began to develop areas of diffuse, white opacity and became more difficult to roughen with the barbed broach. By the eighth week most animals showed patchy leukoplakia in the treated areas. Fig 5.2 shows a necropsy sample taken at this time. There was mild epithelial atrophy and irregular hyperkeratosis. There was a florid chronic inflammatory cell infiltration of the corium.

At 12 weeks, that is 2 weeks after the last application of carcinogen,most biopsy specimens showed evidence of mild basal cell hyperplasia and increased numbers of mitotic figures. Dense chronic inflammatory cell infiltration was frequently present in the corium (Fig 5.3).

At 17 weeks, the chronic inflammatory cell infiltration tended to be more patchily distributed. The epithelium showed moderate acanthosis and hyperorthokeratosis. Some areas showed mild epithelial atypia (Fig 5.4).

At the time of killing (25 weeks) most animals had irregular, thick, leukoplakic patches on the ventral lingual mucosa. The histological appearances varied considerably both between individual animals and between different areas of the mucosa in the same animal. The range of histological appearances is illustrated in Fig 5.5 to Fig 5.7. Areas of atrophy and of acanthosis were present. Basal cell hyperplasia, often of a marked degree, was a frequent observation. Occasional areas of epithelium showed acantholysis similar to that described in the previous two experiments.

5.4 DISCUSSION

Previously published work has shown that regular scratching and ulceration of hamster lingual mucosa followed by applications of DMBA leads to relatively rapid tumour formation in a high percentage of animals (see Section 5.1).

In the present study the lingual mucosa was only lightly scratched with a barbed broach prior to carcinogen applications. The instrument was used merely to roughen the surface of the epithelium and there was no macroscopical or histological evidence of ulceration in any of the experimental or control animals. In some specimens examined in the early stages of treatment occasional small tears were present in the superficial keratin but there was no evidence of mechanical disruption of keratinocytes despite the fact that the barbs of the broaches used, although fine, were considerably longer than the thickness of the keratin layer.

5.5 CONCLUSIONS

Light scratching of hamster ventral lingual mucosa with a barbed broach followed by applications of DMBA bi-weekly for a maximum of 10 weeks resulted in the development of epithelial dysplasia, which appeared to be progressive. No tumours had developed by 25 weeks when the experiment was terminated.

ILLUSTRATIONS

Fig 5.1 Fine dental root canal barbed broach used for lightly scratching the ventral lingual mucosa x 15

Fig 5.2 Atrophy and irregular hyperkeratosis at 8 weeks x 250

Fig 5.3 DMBA treated ventral lingual mucosa at 12 weeks showing mild acanthosis and basal cell hyperplasia x 256

Fig 5.4 DMBA treated ventral lingual mucosa showing an area of marked acanthosis x 256

Fig 5.5 Mild epithelial hyperplasia with acantholysis and dyskeratosis x 256

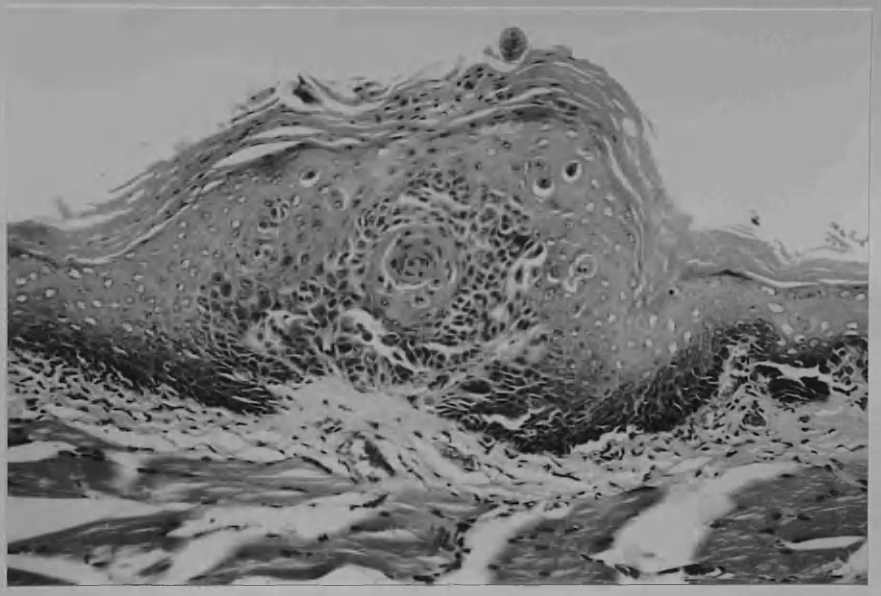


Fig 5.6 Area of moderately severe atypia x 160

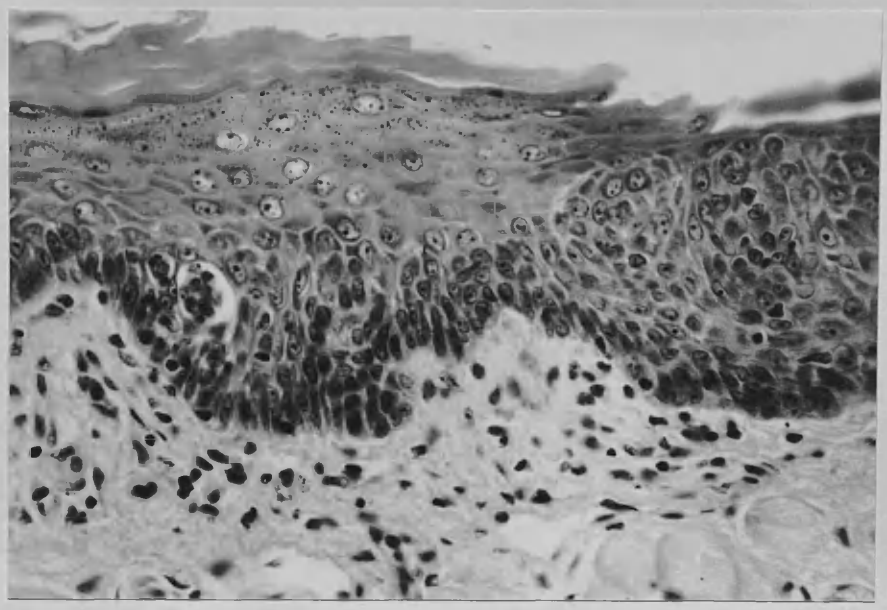


Fig 5.7 Severe atypia with extensive basal cell hyperplasia x 400

CHAPTER SIX

HAMSTER TONGUE CARCINOGENESIS: CHARACTERISTICS OF THE EXPERIMENTAL MODEL

6.1 INTRODUCTION

The pilot study described in Chapter 5 showed that epithelial dysplasia could be produced in hamster ventral lingual mucosa by a combination of scratching with a barbed broach and applications of DMBA. Although no tumours were produced the epithelial dysplasia appeared to be progressive following cessation of treatment. This led the author to judge that slight modification of the experimental manipulation would lead to the development of invasive neoplasms. The decision was made, therefore, to apply the carcinogen more frequently and, if necessary, for a longer period. A standard biopsy was to be taken as in the previous two experiments. This biopsy was to be used to monitor the progression of any lesions and in addition was to be used for morphometric quantification of preneoplastic epithelium. The characteristics of the experimental model will be described and discussed in this chapter. The quantitative studies using biopsy material will be considered in Chapter 7.

6.2 MATERIALS AND METHODS

The animals were line-bred male Syrian golden hamsters which were 8-10 weeks old at the start of the experiment.

They were caged individually and received food and water ad libitum. Forty hamsters were used in the experiment. They were divided into three groups and treated as follows:

Scratch/DMBA group (20 animals)

Three times weekly the animals were lightly anaesthetised and immobilised in the restrainer as previously described (Section 3.2.3). The tongue was roughened with a barbed broach as described in Section 5.2.3 before the application of a 0.5 per cent solution of DMBA in acetone. More pressure was applied when scratching the lingual mucosa than in the previous experiment but again frank ulceration was avoided.

Scratch/acetone control group (10 animals)

The tongues were scratched three times weekly as in the experimental group and painted with the acetone vehicle.

Untreated control group (10 animals)

These hamsters remained untreated until the time of biopsy.

Animals in the experimental group and the scratch/acetone control group were treated for 13 weeks and at 16 weeks, that is 3 weeks after the last period of scratching and painting with either DMBA or acetone, biopsies of the experimental area were taken from all animals including the untreated controls. The biopsy technique was a slight modification of that described in Section 4.2.5. It was found to be easier to make the scalpel incisions, especially the incision at the junction between the ventral and

lateral surfaces of the tongue if the tongue was supported by a thin sheet of dental wax. Thus a piece of wax approximately 15 mm x 5 mm x 2 mm was placed against the dorsal surface of the tongue and this, together with the tip of the tongue, was grasped in the forceps.

Intraoral examinations were undertaken at fortnightly intervals and all surviving animals were killed 28 weeks after the start of the experiment. Tongues and submandibular lymph nodes were removed and gross autopsies were performed. Specimens removed at the time of biopsy and at death were fixed in Bouin's fluid for 2 hours before being transferred to 70 per cent methanol. Paraffin processed blocks were sectioned at 3.5 μm on a Leitz rotary microtome and stained with haematoxylin and eosin.

6.3 RESULTS

6.3.1 Macroscopical

The normal hamster ventral lingual mucosa can be seen in Fig 6.1. In the present experiment it was noticed that when the carcinogen was applied it tended to run down the gutter of mucosa on either side of the median lingual fraenum into the floor of the mouth. From the third week onwards animals in the experimental group began to develop areas of diffuse, white opacity in the treated area, similar in appearance to human leukoplakia. Over the following weeks these areas became rough and granular (Figs 6.2 and 6.3). The first tumour was detected in the ventral lingual mucosa during the tenth week of treatment. Tumours were

usually pedunculated (Figs 6.4 and 6.5) but occasional sessile papillomas formed (Fig 6.6). At the termination of the experiment 14 out of 18 surviving animals in the experimental group had developed papillomas in the treated area of mucosa.

In addition to developing tumours in the mucosa which had been scratched and painted with carcinogen, 12 out of 18 animals developed papillomas in the floor of the mouth. The area on either side of the junction of the median lingual fraenum and the floor of the mouth appeared to be particularly susceptible (Fig 6.7). Indeed, the first papilloma produced in the experiment was seen in this site after nine weeks of carcinogen applications. Some animals developed multiple tumours both in the treated area and the floor of the mouth (Figs 6.8 and 6.9).

During the period of carcinogen application several animals developed epipilation of the skin over the lateral aspect of the pouch. This was probably due to carcinogen transferred from the tongue to the adjacent skin by the animals licking themselves. This was sometimes seen as the animals recovered from the anaesthetic. In all cases the epipilation was temporary, rarely lasting more than 4 weeks. At the termination of the experiment 3 out of 18 animals had small skin papillomas at the angle of the mouth.

In the control animals which were scratched with barbed broaches and painted with acetone three times weekly, areas of white opacity and granularity also appeared in the treated area of mucosa. The changes occured more

slowly and were less severe than in the experimental group. No tumours developed. The lesions slowly regressed after the experimental manipulation had ended but areas of whiteness and patches of coarse granularity were still present in 3 out of 8 animals surviving to the end of the experimental period.

Two hamsters from each group failed to recover from the anaesthetic after the biopsy procedure in the early stages of the experiment. This was probably due to hypothermia. When animals were placed close to a radiator during the recovery period no further animals were lost.

6.3.2 Histological

The normal ventral lingual mucosa was identical to that described previously (Section 4.3.1).

The histological features of the biopsy specimens from the scratch/acetone control group and the scratch/DMBA experimental group are considered in detail in Chapter 7 and are only described here briefly. In the control group there was mild hyperplasia with acanthosis and mild hyperorthokeratosis. There was no significant epithelial atypia (Fig 6.10). In the biopsy specimens taken from the experimental group there was more marked epithelial hyperplasia and hyperorthokeratosis. Variable degrees of epithelial atypia were present even in adjacent blocks taken from the same animal, but in some cases the degree of atypia was severe (Fig 6.11) and in one animal early squamous cell carcinoma was present.

In specimens taken from the experimental group at the

time of killing various appearances were seen ranging from histologically normal epithelium to deeply invasive squamous cell carcinomas. The range of epithelial atypia present is illustrated in Figs 6.12 to 6.16. Features of epithelial atypia frequently seen were focal areas of acantholysis and dyskeratosis. Subjectively, such areas appeared to be most commonly seen in the ventral lingual mucosa close to the lateral border of the tongue. Invasion was sometimes seen in areas where there was severe dysplasia but no evidence of papilloma formation in the overlying epithelium.

Papillomas were either pedunculated or sessile (Figs 6.17 and 6.18). They also showed variable degrees of dysplasia but frequently this was severe and often extended throughout the thickness of the epithelium. Squamous eddies were commonly seen in these lesions. Invasion tended to occur in the stalk of such papillomas and the tumours were frequently poorly differentiated (Fig 6.19). Many evoked a markedly fibroblastic stromal response (Fig 6.20). Invasion of a vessel, presumably a lymphatic, was seen in one specimen (Fig 6.21) but there was no evidence of metastatic tumour in any of the submandibular lymph nodes examined. Many such lymph nodes showed marked reactive hyperplasia and haemosiderin pigment deposition but this was probably related to the biopsy procedure.

6.4 DISCUSSION

The method used for producing lingual carcinomas in

this experiment was a modification of the technique of Fujita and his colleagues (1973a; 1973b) of combining applications of DMBA in acetone with trauma. The present study shows that macroscopical ulceration is not a necessary precursor of epithelial malignancy in this model. Indeed, Fujita et al (1973a) found that when DMBA in acetone was applied to the lateral border of the hamster tongue without prior scratching 4 out of 15 animals developed squamous cell carcinomas by 27 weeks. A recent study by Marefat and Shklar (1977) also suggests that trauma prior to DMBA applications is not necessary for the production of lingual carcinomas but does accelerate their development. In the present experiment, the scratching procedure, apart from potentially accelerating the appearance of tumours, had an added advantage. Scratching plus applications of the acetone vehicle led to non-neoplastically induced epithelial hyperplasia. This gave a control group of animals which could be compared with the experimental scratching plus DMBA group at the biopsy stage (see Chapter 7).

There appear to be both site and strain differences in the latent period and tumour yield in investigations using the technique of Fujita et al (1973a; 1973b) for the production of lingual carcinomas. For example, when Marefat and Shklar (1977) repeated part of the experiment of Fujita et al it was found that tumours developed more rapidly and that the eventual tumour yield was higher. In the present experiment, the tumour latency would appear to be within the range of previously published work using similar

techniques, but the final tumour yield was lower. This was possibly related to the biopsy procedure used, which reduced considerably the area of mucosa available to undergo malignant change. In addition, in the present study carcinogen applications were discontinued after 13 weeks whereas in the studies of Fujita et al (1973a; 1973b) treatment was generally continued until the animals died.

Tumours developing outside the treated area of mucosa were not uncommon in this experiment (Section 6.3.1). The area on either side of the median lingual fraenum, especially where it joined the floor of the mouth, appeared to be particularly vulnerable. Fujita et al (1973b) also noted that animals in which carcinogen had been applied to the ventral lingual mucosa, lateral border of the anterior third of the tongue or the tip of the tongue developed large carcinomas elsewhere in the oral cavity, although they gave no indication of their exact locations. However, they found that only a few of these tumours reached the size of the experimental lingual carcinomas. In the present study, all the oral tumours developing outside the experimental area were related to either the median lingual fraenum or the floor of the mouth, and in some cases were gross.

There are several possible reasons for this location. For example, the oral mucosa of these areas could have an innate, increased sensitivity to chemical carcinogens. The site is one of the lowest in the 'drainage area' (see Section 4.2.2) and carcinogens present in the mouth would tend to

pool there before being swallowed. Indeed, during the applications of carcinogen the solution was frequently seen to run down the gutter of mucosa on either side of the lingual fraenum before the vehicle evaporated. It is possible that the mechanical effect of the scratching could cause retention of carcinogen in the superficial layers of the ventral lingual mucosa followed by a slow release into the floor of the mouth. This could increase the time during which this tissue was exposed to the action of the carcinogen. This speculation could only be clarified by assessing the incidence of tumours in this site after DMBA had been applied to the tongue without prior scratching. Marefat and Shklar (1977) performed such an experiment but made no mention of tumours developing outside the immediate area of carcinogen application.

A somewhat unusual histological feature seen in this experiment was focal areas of acantholysis and dyskeratosis, often in association. Similar areas of focal acantholytic dyskeratosis have been described in hamster cheek pouch after DMBA applications (see Section 3.4.1 and MacDonald 1973) and in hamster tongue (Eveson and MacDonald 1977) and rat palate (Philipsen, Fisker and Stage 1977) after applications of 4NQO. These areas frequently appear to be surrounded by normal epithelium and the relationship of these lesions to the subsequent development of epithelial malignancy is not known.

The squamous cell carcinomas produced in this, as in previously published studies, showed a wide range of

differentiation,sometimes even within the same tumour mass. Although there was evidence of lymphatic vessel invasion, no metastases were detected in the regional lymph nodes. Fujita et al (1973b) found regional lymph node metastases in 12.9 per cent of animals when the lateral border of the middle third of the tongue was treated with carcinogen. However, as in the present study, no metastases were detected when the ventral lingual mucosa and other areas of the tongue were treated in a similar manner.

Marefat and Shklar (1977) reported epithelial dysplasia in the scratch/acetone controls used in their experiment on hamster tongue carcinogenesis. In the present experiment there was no evidence of epithelial dysplasia either subjectively or when Smith and Pindborg's (1969) technique for evaluating epithelial atypia was applied to the scratch/acetone control group. This difference may be related to the fact that Marefat and Shklar (1977) used the barbed broach to ulcerate the mucosa whereas in the study reported here ulceration was deliberately avoided.

6.5 CONCLUSIONS

Thrice weekly applications of DMBA to hamster ventral lingual mucosa preceded by scratching with a barbed broach led to tumour development in over 75 per cent of the animals by 28 weeks. Tumours were preceded by a phase resembling human leukoplakia. In addition to developing tumours in the treated area 60 per cent of hamsters developed tumours in the floor of the mouth or the area of mucosa on either side of the median lingual fraenum.

ILLUSTRATIONS

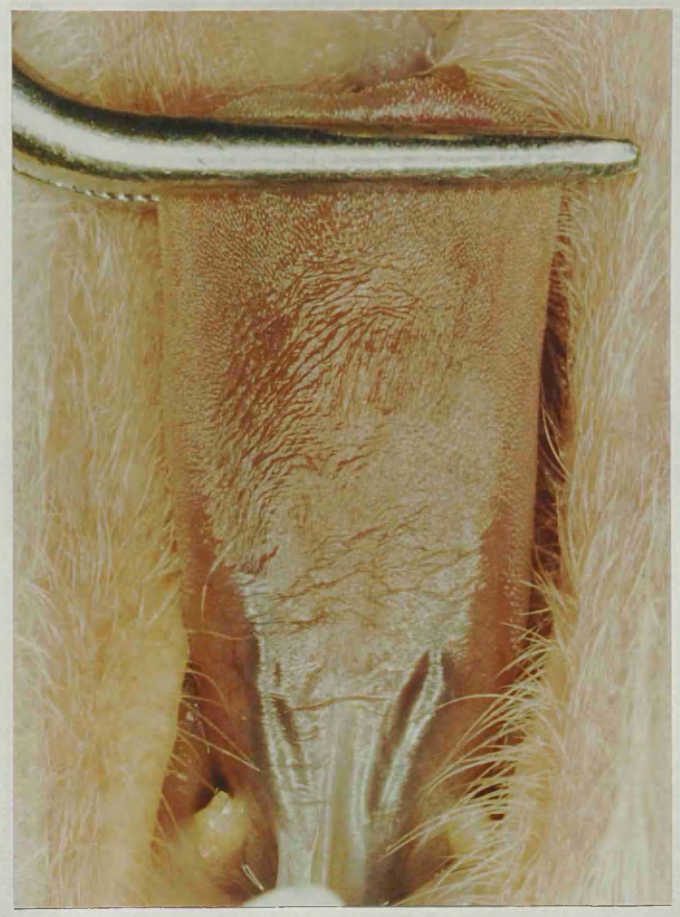


Fig 6.1 Normal ventral lingual mucosa of hamster.

Fig 6.2 Ventral lingual mucosa at 8 weeks showing diffuse whiteness and granularity.

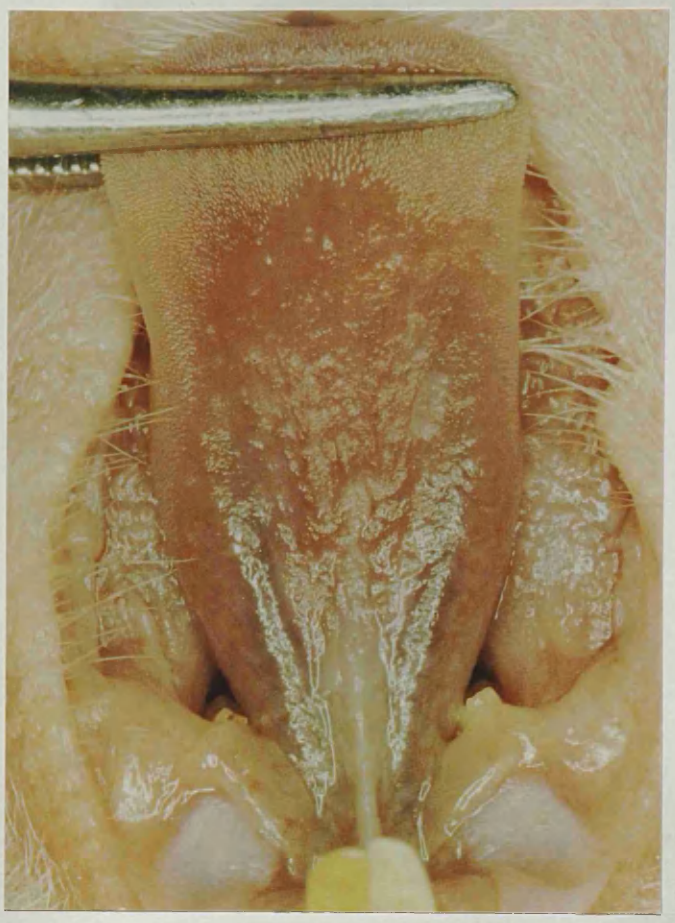
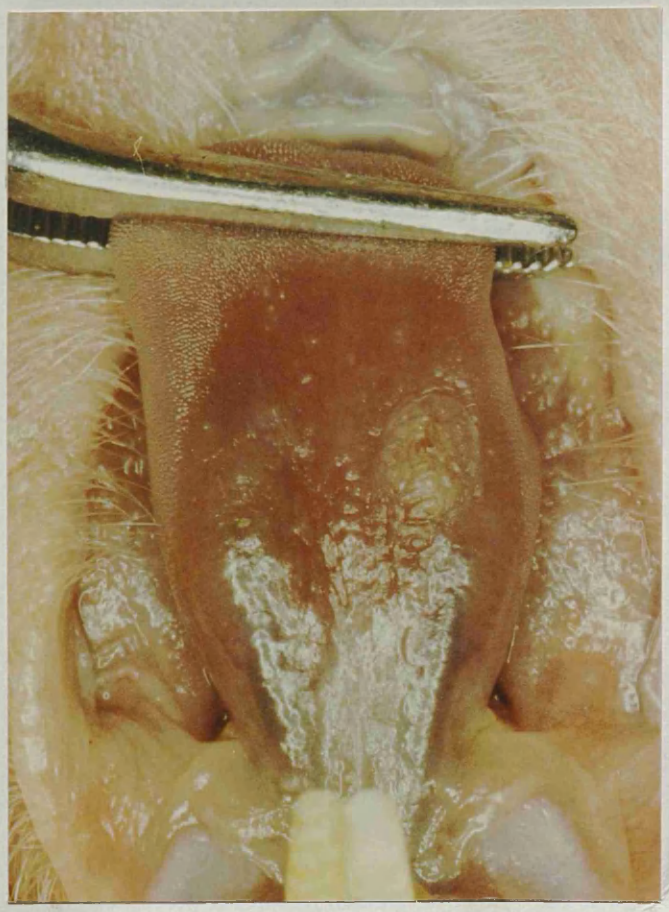


Fig 6.3 Ventral lingual mucosa at 15 weeks showing thick white plaques and thickening and opacity around the median lingual fraenum.

Fig 6.4 Ventral lingual mucosa after 11 weeks showing diffuse granular leukoplakia and early papilloma formation.

Fig 6.5 Ventral lingual mucosa at 16 weeks showing small pedunculated papilloma.

Fig 6.6 Sessile papilloma after 15 weeks of treatment.



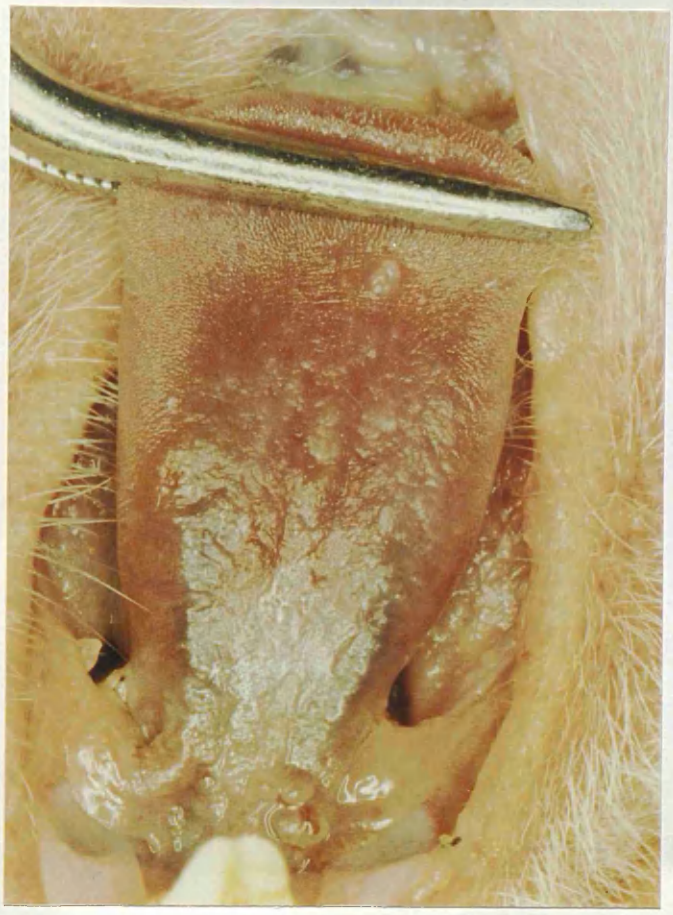
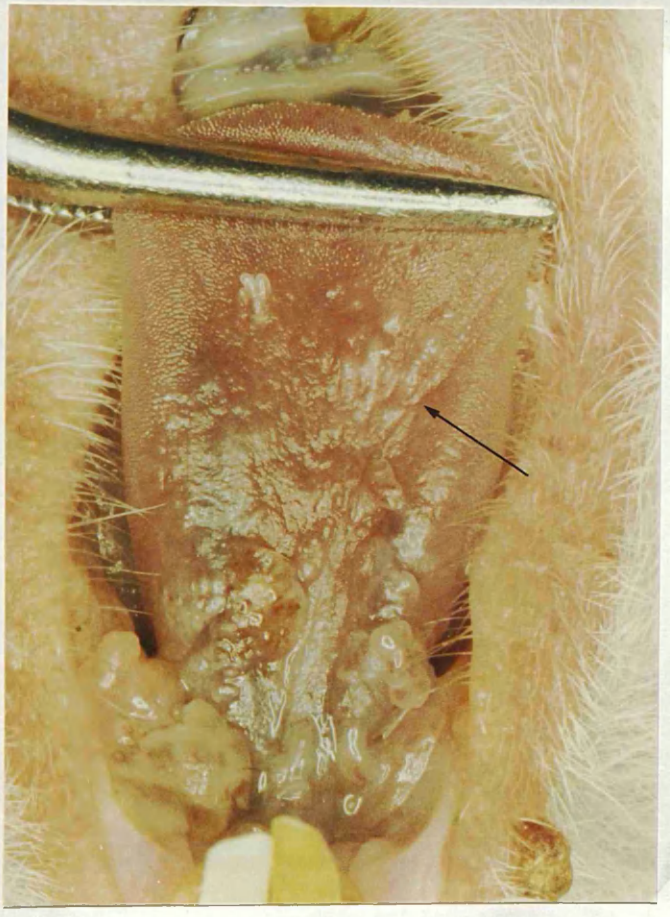


Fig 6.7 Multiple papillomas in treated area of mucosa and base of median lingual fraenum after 15 weeks.

Fig 6.8 Multiple tumours in treated mucosa of tongue and in floor of mouth at 24 weeks. Site of healed biopsy is indicated by arrow.



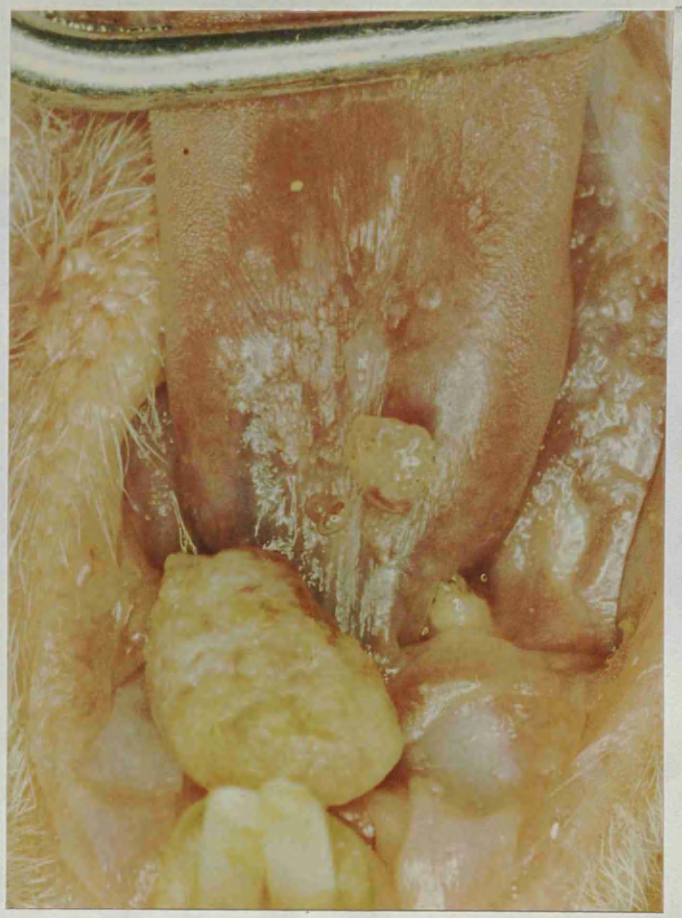


Fig 6.9 Large exophytic tumour in treated area of ventral lingual mucosa and floor of mouth at 26 weeks.

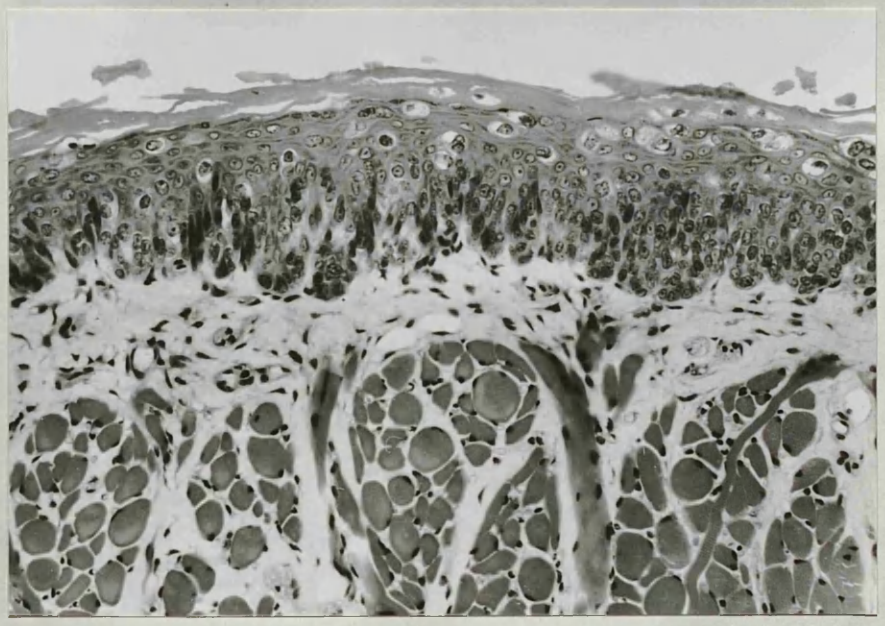


Fig 6.10 Epithelial hyperplasia in scratch/acetone control in biopsy taken at 16 weeks x 250

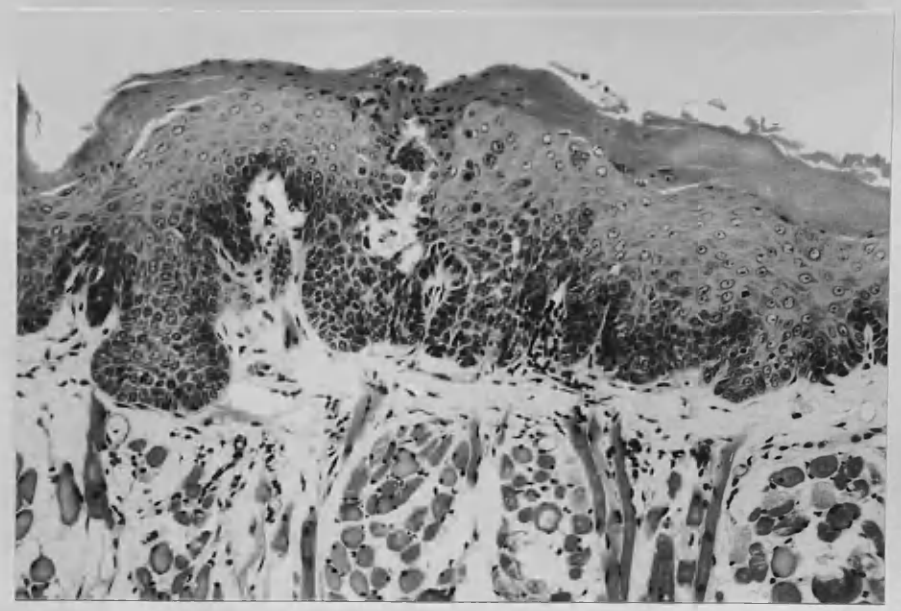


Fig 6.11a Moderately severe epithelial atypia in biopsy taken after 16 weeks x 200

Fig 6.11b Severe epithelial atypia and early invasion in biopsy taken after 16 weeks x 160

Fig 6.12 Focal areas of basal cell hyperplasia x 160

Fig 6.13 Severe epithelial atypia x 160

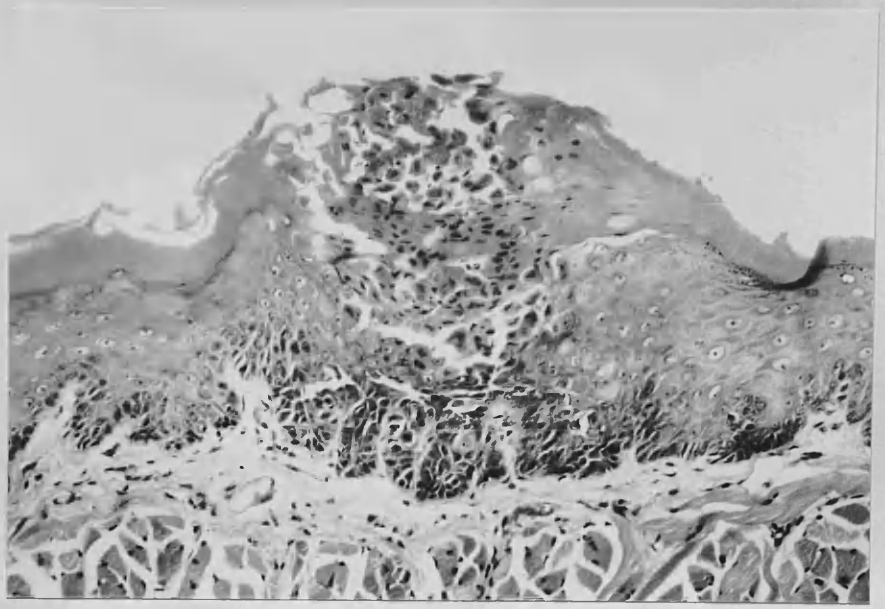


Fig 6.14 Focal area of acantholysis and dyskeratosis x 210

Fig 6.15 High power of an area of focal acantholytic dyskeratosis x 340

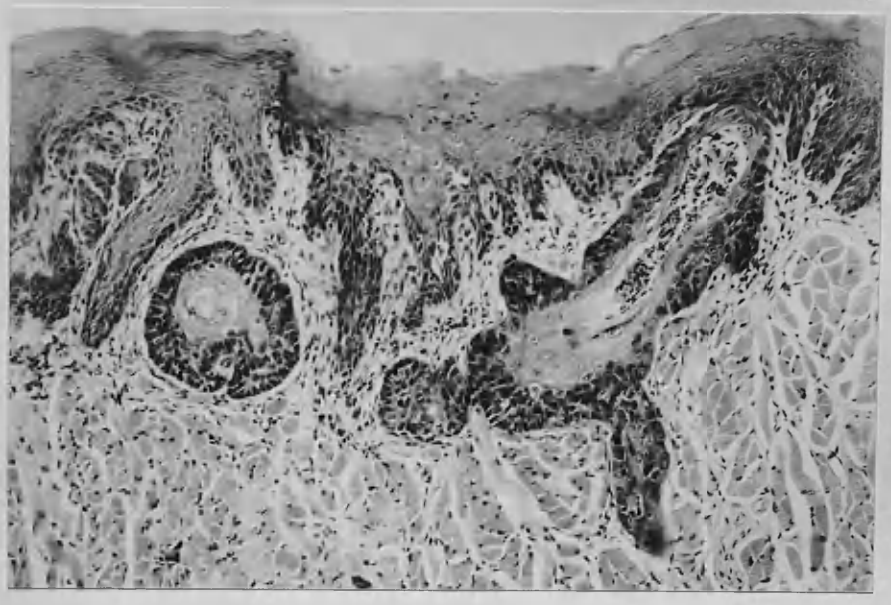


Fig 6.16 Early squamous cell carcinoma arising from an area of severe dysplasia x 160

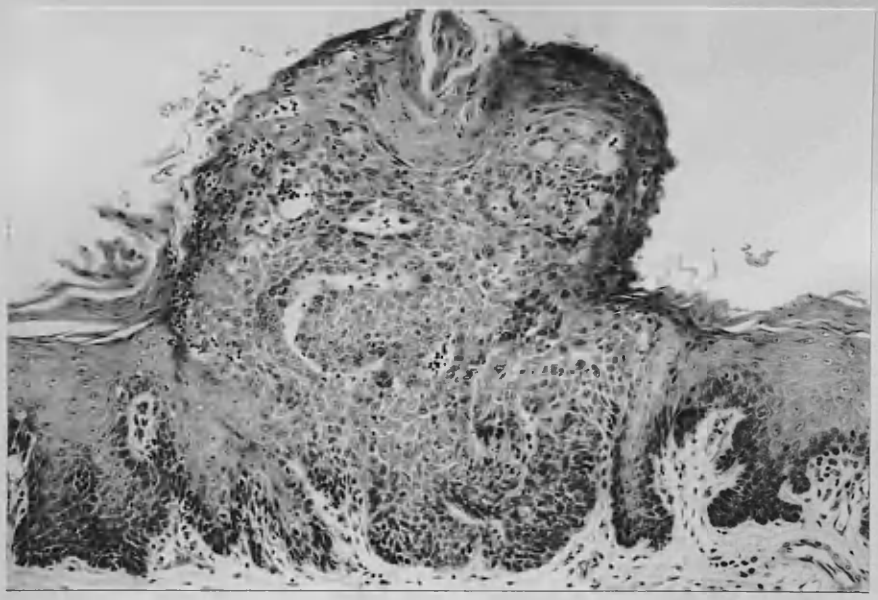


Fig 6.17 Cellular exophytic tumour with severe dysplasia x 160

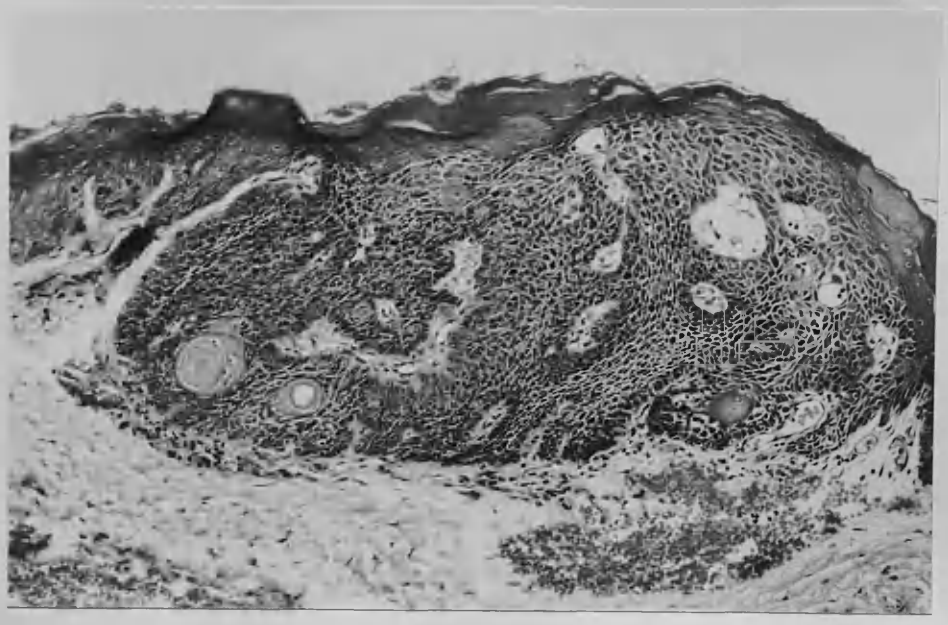


Fig 6.18 Cellular endophytic tumour with severe dysplasia and squamous eddies x 160

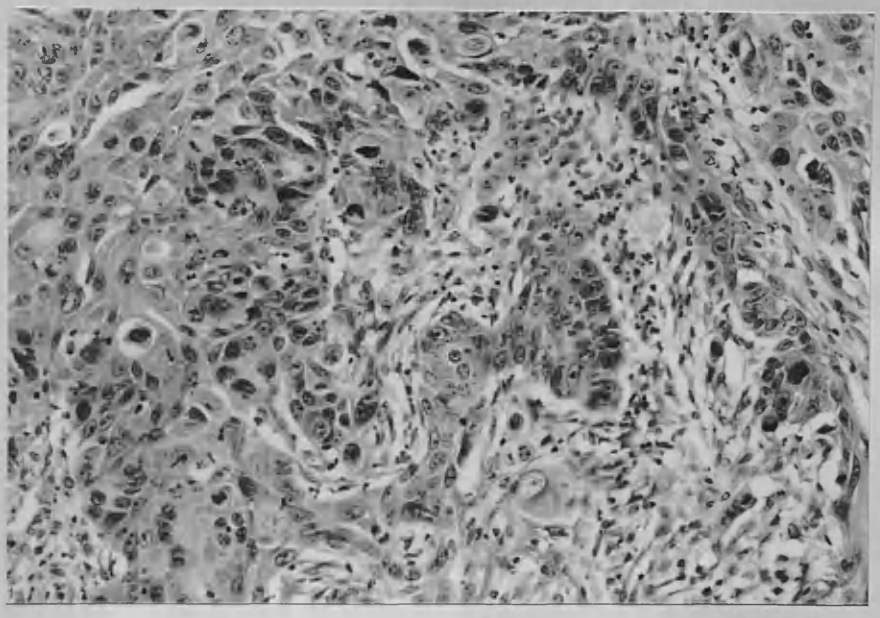


Fig 6.19 Poorly differentiated squamous cell carcinoma x 250

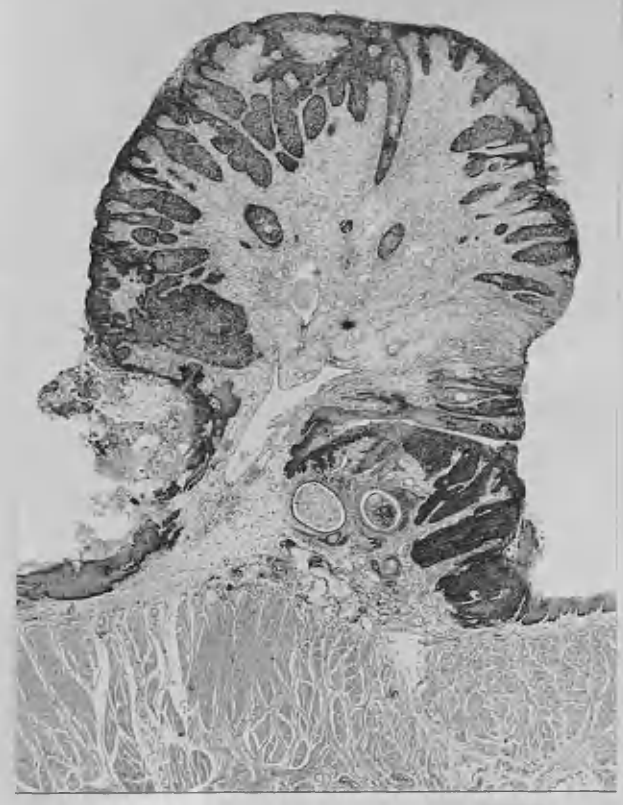
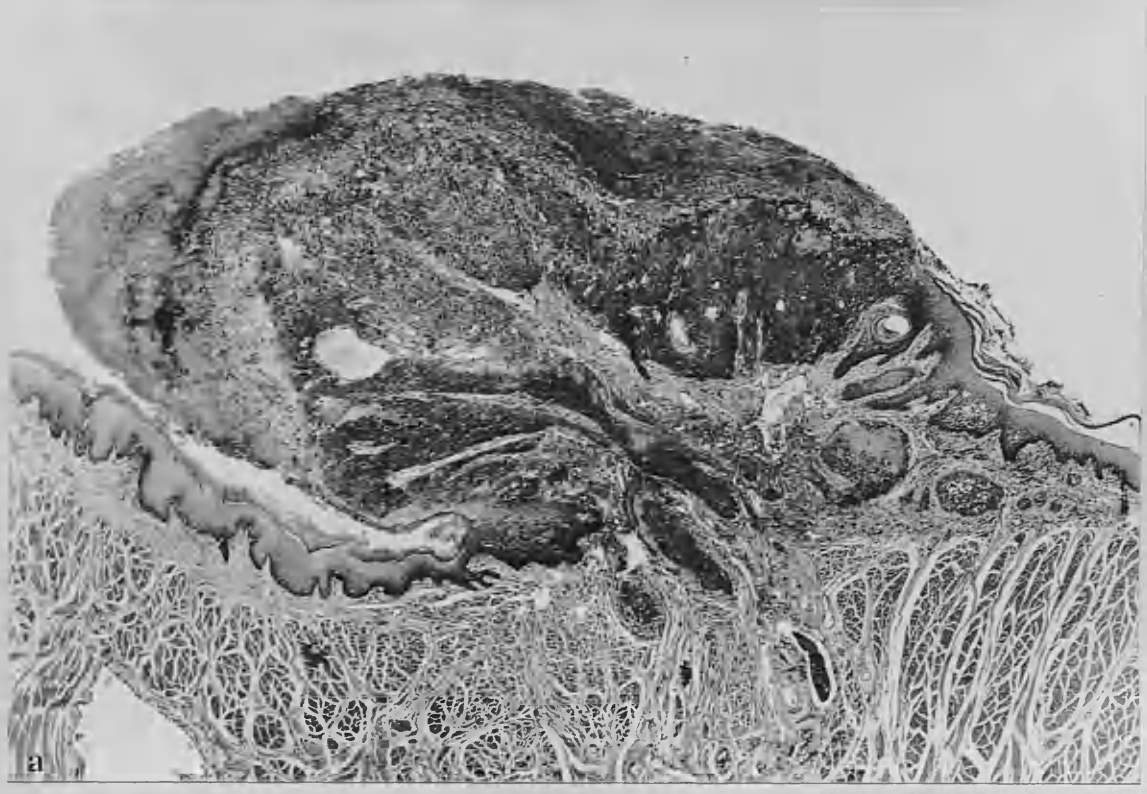


Fig 6.20a Exophytic tumour with markedly fibroblastic stroma x 32

Fig 6.20b High power showing severe dysplasia in the epithelium x 160



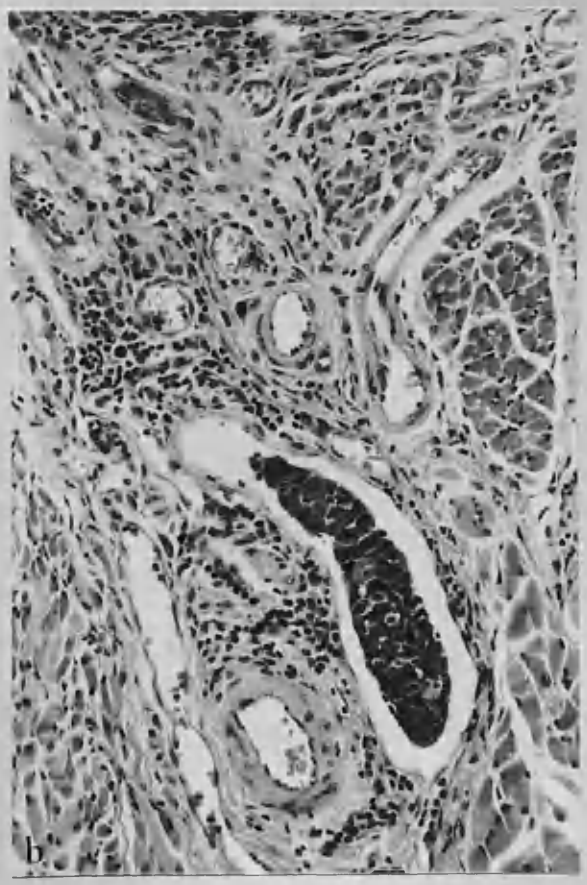


Fig 6.21

a. Large papilloma with invasion of stalk x 45

b. High power showing invasion of a vessel x 160

CHAPTER SEVEN

HAMSTER TONGUE CARCINOGENESIS: STEREOLOGICAL QUANTIFICATION OF PRENEOPLASTIC EPITHELIUM

7.1 INTRODUCTION

In Chapter 6 it was shown that a combination of mechanical irritation and applications of the carcinogen DMBA to hamster ventral lingual mucosa led to the development of squamous cell carcinomas in a significant number of animals. During that experiment biopsy specimens were taken from the treated area of mucosa (Section 6.2) to monitor the progression of the lesions. In addition this material was to be used to assess quantitative morphological changes in preneoplastic epithelium.

The object of the present experiment therefore, was to quantify morphological features of carcinogen - treated hamster ventral lingual mucosa using stereological techniques. The parameters chosen for examination were similar to those selected in the experiment on hamster cheek pouch carcinogenesis reported in Chapter 3. They included total epithelial thickness; thickness of epithelial compartments; cell numbers and cell sizes within individual epithelial compartments. In addition, the surface and basement membrane lengths and their relationships to the various epithelial compartments were also to be assessed.

7.2 MATERIALS AND METHODS

7.2.1 Animals

The material used for quantitative analysis was obtained from the biopsy specimens removed from hamsters after 16 weeks as described in Chapter 6. Thus the following groups were available (see Section 6.2):

Scratch/DMBA group (16 animals)

Scratch/Acetone control group (8 animals)

Untreated control group (8 animals)

7.2.2 Sampling Procedures

The biopsy specimens formed the primary sample for stereological purposes. The biopsy specimen, which was approximately 4 mm x 2 mm x 1 mm, was laid on a sheet of dental wax with the connective tissue surface towards the wax. Using a razor blade the specimen was divided into four blocks, each 2 mm x 1 mm x 1 mm. One block was to be used for electron microscopical observations in a related study not reported in this thesis. The other three blocks were randomly labelled A, B and C and processed for paraffin embedding. During the blocking out procedure it was necessary to use a dissecting microscope to ensure orientation of the specimen so that it could be cut at right angles to the epithelial surface. After embedding in paraffin wax a section was cut from block A and if the preservation and orientation were satisfactory this specimen was used for stereological analysis. If for any reason the specimen was unsatisfactory other blocks were examined in sequence. The faces of the paraffin blocks were trimmed to about 4 mm x 4mm.

All blocks were cut with the keratinized surface parallel to the knife edge and meeting the knife first to ensure that any distortion and compression effects should be similar in all specimens. The microtome was set at 3 μm and 32 serial sections were cut from each block and arranged on slides as shown in Fig 3.5. Every eighth section was used for point counting.

7.2.3 Selection of Area for Counting

H and E stained slides were mounted on the revolving stage of a Leitz Ortholux microscope and the image projected onto a teaching head as described in Section 3.3.2. The section was then analysed by a systematic random sampling technique. To accomplish this the section was aligned with the mean epithelial surface at right angles to the two vertical lines drawn on the teaching head screen to outline a column of known width (0.18 mm). For the purposes of this study a microscopic field was defined as a column 0.18 mm wide passing through the full epithelial thickness. The first field for point counting was selected as previously described (Section 3.3.2). The second and subsequent fields in each section were selected by taking the section out of focus and moving the stage a small, arbitrary distance to the side before refocusing and realigning the specimen. For each animal 5 to 6 fields were counted at each of 4 levels in the paraffin block giving a total of 20-24 fields per animal.

7.2.4 Identification of Epithelial Compartments

The ventral lingual epithelium is divisible into progenitor, maturation and keratinized compartments. The

distinction between the keratinized and maturation compartment was easily made. The criteria described in Section 3.3.3 which enabled a distinction to be made between the progenitor and maturation compartments of the hamster cheek pouch were applicable to the ventral lingual mucosa (Fig 7.1).

7.2.5 Counting Procedures

The areas of the progenitor, maturation and keratinized compartments were determined by using a circular transparent perspex grid, on which a regular 5 mm point lattice had been scribed using a series of intersecting parallel lines (see Section 3.3.3), superimposed over the section image on the screen of the teaching head (Fig 7.2). In addition to determining epithelial area and thickness, this system was used to estimate the surface length and basement membrane length for each field as defined in Section 7.2.3. The number intercepts between one set of parallel lines of the point lattice and the surface of the section and the junction between the epithelium and corium (Fig 7.3) were recorded for each of the three grid positions. Lengths (L) were determined by applying the formula:

$$L = \frac{\pi}{2} \times I \times d$$

where I was the number of intercepts counted for each field, and d was the distance between the grid lines. The surface length and basement membrane length for each field was then calculated after taking into account the various magnification factors and dividing by three to obtain the average from the three grid positions.

7.2.6 Cell Counts

The total numbers of viable nucleated epithelial cells in the progenitor and maturation compartments were recorded for each field. Corrected cell numbers were calculated by applying Abercrombie's correction (see Sections 3.3.5 and 3.3.6).

7.2.7 Statistical Analysis

Because of the relatively small numbers of animals in each group it was not possible to satisfy the assumptions required for parametric statistics. Therefore, comparisons between groups of animals were made using non-parametric statistics and all the probabilities cited in the results were derived from Mann-Whitney U tests. Arithmetical means and standard deviations are quoted in the results for ease of presentation and comparison but were not used in the statistical analysis.

7.3 RESULTS

7.3.1 Normal Animals

The values for the stereological parameters are recorded in Tables 7.1, 7.2 and 7.3. The total mean epithelial thickness of the ventral lingual mucosa was 73.9 μm SD 13.4 μm. This was made up of a progenitor compartment 15.6 μm SD 2.5 μm, a maturation compartment 38.4 μm SD 6.1 μm and a keratinized compartment 19.7 μm SD 4.8 μm. Cell section areas derived from compartment areas and the corrected cell numbers showed that progenitor cells had an average area of 139 μm^2 SD 18 μm^2 and

maturation cells an average area of 653 μm^2 SD 79 μm^2. The basement membrane length per field (246 μm) was longer than the surface length (226 μm) reflecting the rete ridge pattern of the epithelio-mesenchymal junction in this tissue.

The average nuclear diameter was 4.6 μm SD 0.3 μm in the progenitor compartment and 7.7 μm SD 0.8 μm in the maturation compartment (Table 7.4). These were the diameters used when applying Abercrombie's correction to calculate cell numbers.

7.3.2 Comparison of Scratch/Acetone Controls and Normal Animals

Mean Thickness

It can be seen in Fig 7.4 that the mean total epithelial thickness was increased to 93.9 μm in the scratch/acetone control group, but this was not statistically significant ($P = 0.19$). There was no significant difference in the thickness of the keratinized or maturation compartments but the progenitor cell compartment showed a highly significant increase in thickness to 23.0 μm ($P < 0.001$).

Cell Numbers and Cell Sizes

There was a significant increase in the numbers of progenitor cells per field ($P < 0.001$) in the scratch/acetone control group. There was no change in the numbers of maturation cells or the mean cell section of either progenitor or maturation cells (Figs 7.5 and 7.6). Therefore the increased size of the progenitor compartment was due to the presence of more cells.

Basement Membrane and Surface Lengths

The surface length per field of the scratch/acetone control group was unchanged. There was a small but significant ($P < 0.01$) increase in the length of the basement membrane per field from 246 μm to 276 μm (Fig 7.7). There was no change in the total number of nucleated cells per unit of basement membrane length (Fig 7.8) but the number of cells related to each unit of surface length was increased ($P < 0.001$). There was a significant increase in the number of progenitor cells per unit of basement membrane length ($P < 0.005$) but a much larger increase in progenitor cells related to a unit of surface length from 89.6 to 122.2 ($P < 0.001$). This is illustrated in Fig 7.9. There was no change in the number of maturation cells related to units of either surface or basement membrane lengths (Fig 7.10).

Nuclear Diameters

There was no significant change in the mean nuclear diameters of progenitor (4.7 μm) or maturation cells (7.4 μm) (Fig 7.11).

7.3.3 Comparison of Scratch/DMBA Group and Normal Animals

Mean Thickness

The mean thicknesses of the total epithelium and the individual epithelial compartments can be seen in Table 7.1 and Fig 7.4. The mean total epithelial thickness was increased to 122.9 μm ($P < 0.001$) and the mean thicknesses of all three compartments were considerably in excess of the untreated controls ($P < 0.001$).

Cell Numbers and Cell Sizes

The maturation cell numbers per field (11.9) were slightly increased ($P < 0.05$) and there was a highly significant increase in the number of progenitor cells per field from 20.2 to 39.9 ($P < 0.001$). A striking feature was a highly significant increase in the size of progenitor cells expressed as mean cell section areas $P < 0.001$) from 139 μm to 172 μm. The maturation cells showed a less marked increase in cell size ($P < 0.01$).

Basement Membrane and Surface Lengths

Fig 7.7 illustrates changes in surface and basement membrane lengths. There was no significant change in surface length but there was a considerable increase in basement membrane length per field ($P < 0.001$). The total number of cells per unit length of basement membrane was unchanged (Fig 7.8) but there was a highly significant increase in the total number of cells related to a unit of surface length ($P < 0.001$). There was a moderate increase in the number of progenitor cells per unit of basement membrane ($P < 0.001$) and a marked increase in the number of progenitor cells per unit of surface length ($P < 0.001$). These changes can be seen in Fig 7.9.

Nuclear Diameters

There was a highly significant ($P < 0.001$) increase in the nuclear diameters of progenitor cells of carcinogen treated mucosa (5.4 μm) when compared with untreated controls (4.6 μm) (Fig 7.11). There was a similar but less statistically significant increase in the nuclear diameters

of maturation cells ($P < 0.01$).

7.3.4 Comparison of Scratch/DMBA Group and Scratch/Acetone Control Group

Mean Thickness

There was a significant increase in the mean total epithelial thickness ($P < 0.01$) of the scratch/DMBA group (122.9 μm) compared with the scratch/acetone group (93.7 μm) (Fig 7.4). The increase in thickness of the progenitor compartment from 23.5 μm to 37.6 μm was highly significant ($P < 0.001$) but there was also a significant increase in the thickness of the keratinized compartment from 23.0 μm to 30.5 μm ($P < 0.01$).

Cell Numbers and Cell Sizes

Figs 7.5 and 7.6 illustrate changes in cell numbers and cell sizes. There was a significant increase in the numbers per field ($P < 0.001$) and sizes ($P < 0.01$) of progenitor cells but the numbers and size of maturation cells were not significantly different from the scratch/acetone controls.

Basement Membrane and Surface Lengths

The surface length per field was unchanged but there was a highly significant ($P < 0.001$) increase in basement membrane length per field from 276 μm to 378 μm (Fig 7.4). There was a significant increase in the total nucleated cells, related to a unit of surface length. The ratio of total cells to basement membrane length, however, was unchanged. Similarly, the ratio of progenitor cell numbers to basement membrane length of carcinogen treated mucosa (33.9) did not differ significantly from scratch/acetone

controls (42.7) but there was a marked increase in progenitor cells per unit of surface length from 122.2 to 174.8 ($P < 0.001$). It can be seen in Fig 7.10 that the number of maturation cells per unit of basement membrane was decreased ($P < 0.001$) but there was no significant change in the number of maturation cells related to a unit of surface length.

Nuclear Diameters

Progenitor cell nuclear diameters (5.4 μm) in carcinogen-treated mucosa were significantly greater than in the scratch/acetone control group (4.7 μm) ($P < 0.01$). In addition there was a highly significant increase ($P < 0.001$) in the diameter of maturation cells (Fig 7.11) from 7.4 μm in scratch/acetone controls to 8.4 μm in carcinogen-treated mucosa.

7.4 DISCUSSION

7.4.1 Scratch/Acetone Control Group

The mean total epithelial thickness for animals in the scratch/acetone control group was not significantly different from that in normal animals, although it had been the author's subjective judgement that the scratch/acetone treated epithelium was hyperplastic. Stereological analysis, however, revealed that the progenitor cell compartment was considerably thicker than normal. This increase in thickness appeared to be due almost entirely to an increase in the numbers of progenitor cells, with no significant increase in their sizes. The basement membrane length was

increased, reflecting an increased folding and therefore prominence of the rete ridges. If the number of progenitor cells was related to a unit of basement membrane length then there was a relatively small increase in the scratch/acetone control group due to the increased length of the basement membrane.

The merit of using surface length as an index when assessing cell production has been demonstrated by the work of Karring and Löe (1972). They assessed the reliability of various methods of expressing mitotic activity in stratified squamous epithelium and found that relating mitoses to a unit of surface length was the most reliable. The value of this index system was that it related cell production to the surface area of desquamating cells which were to be replaced by the mitoses being studied. A similar index can be used to illustrate changes in the relative sizes of the cellular compartments of epithelium.

Thus, when the number of progenitor cells in the scratch/acetone control group is related to a unit of surface length which does not alter significantly between groups, there is a much greater increase.

These results differ from those obtained by Mackenzie (1970) after short-term (7 to 14 days) frictional stimulation of oral mucosa or skin. He found that repeated daily applications of friction insufficient in severity to cause histologically detectable epithelial damage resulted in epithelial hyperplasia with increases in the thickness of the stratum spinosum, stratum granulosum and stratum corneum.

There was a 70 per cent increase in the numbers of cells in the stratum spinosum and stratum granulosum. The cells in the Malpighian region were enlarged, as were the squames in the keratinized layer.

Craig and Franklin (1977) developed a model of epithelial hyperplasia in the hamster cheek pouch by painting the mucosa thrice weekly with a 50 per cent solution of turpentine in liquid paraffin. Hyperplasia was maximal after 9 weeks of treatment. Franklin and Craig (1978b) quantified some of the morphological features of this model. Their results are not directly comparable to those obtained in the present experiment as they selected fields for analysis on the basis that the full thickness of the epithelium analysed should be at least twice the mean control thickness. No compartment analysis was undertaken in their study, but they found that there was both hypertrophy and hyperplasia of the 'average' epithelial cell. In the present study, progenitor cell hyperplasia without hypertrophy was seen in the scratch/acetone control animals. Franklin and Craig (1978b) also used intercept counts to determine the ratio of the epithelium/keratin junction (surface) to the epithelium/connective tissue junction (basement membrane). This, rather surprisingly showed a 1:1 relationship. Epithelial hyperplasia is normally associated with a greater irregularity of the rete ridge pattern as was seen in the present experiment.

7.4.2 Scratch/DMBA Group

Many of the morphological features demonstrated in

the scratch/DMBA group were similar to those in the scratch/acetone group, but were of greater magnitude.

The epithelial hyperplasia in the experimental animals noted subjectively in Chapter 6 was confirmed stereologically by the significant increase in total mean epithelial thickness. There were increases in the thicknesses of the progenitor, maturation and keratinized compartments. Although maturation cells showed hypertrophy and hyperplasia when compared with normal ventral lingual mucosa, there were no significant differences in the size or number of maturation cells when compared with scratch/acetone controls. Progenitor cells, however, showed hyperplasia and hypertrophy when compared with both normal animals and scratch/acetone controls. MacDonald (1973) in a study of carcinogen-treated hamster lingual mucosa not subjected to scratching failed to show any significant increase in total mean epithelial thickness or in the sizes of epithelial compartments after 10 weeks of treatment.

The nuclear diameters of progenitor and maturation cells were increased above those seen in both normal animals and the scratch/acetone control group.

There was a marked increase in basement membrane length per field reflecting an increase in the complexity and folding of the epithelio-mesenchymal junction.

The number of progenitor cells related to each unit of surface length was significantly above both normal and control levels but the number of maturation cells per unit of surface length, although above normal, was not

significantly different from the scratch/acetone controls.

7.5 CONCLUSIONS

Scratching hamster ventral lingual mucosa and applying acetone thrice weekly for 13 weeks did not result in significant epithelial hyperplasia in biopsy material removed at 16 weeks. However, there was a significant increase in the number of progenitor cells per defined histological field and a marked increase in the number of progenitor cells per unit of surface length. There was a small but significant increase in basement membrane length.

In the scratch/DMBA group there was significant epithelial hyperplasia with increases in the thickness of all epithelial compartments. Progenitor cells showed hyperplasia and also hypertrophy. The number of progenitor cells related to a unit of surface length was markedly increased. The basement membrane length per field was significantly above normal and control levels, reflecting an increased folding and prominence of the rete ridge pattern which had been noticed subjectively.

ILLUSTRATIONS AND TABLES

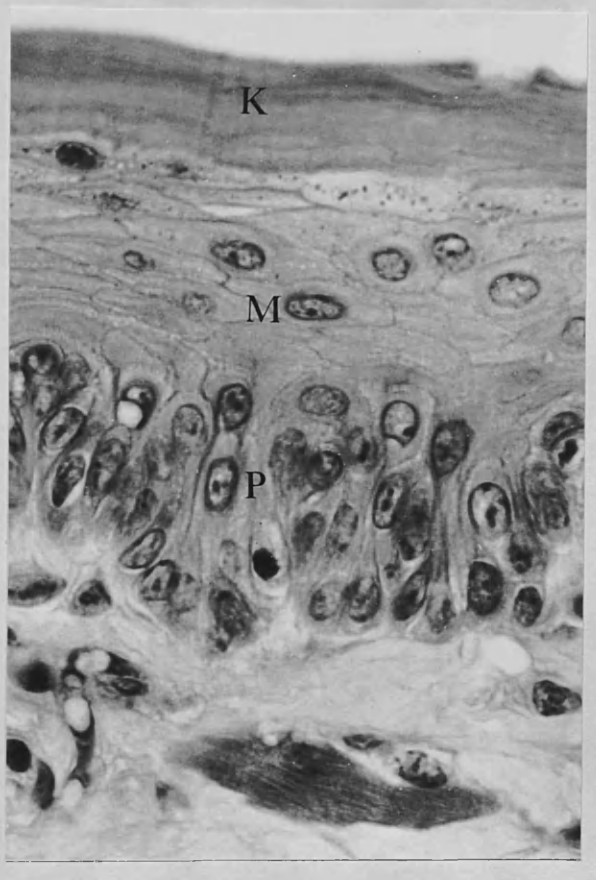


Fig 7.1 Cell compartments in hamster ventral lingual mucosa P-progenitor, M-maturation, K-keratinized compartments x 1300

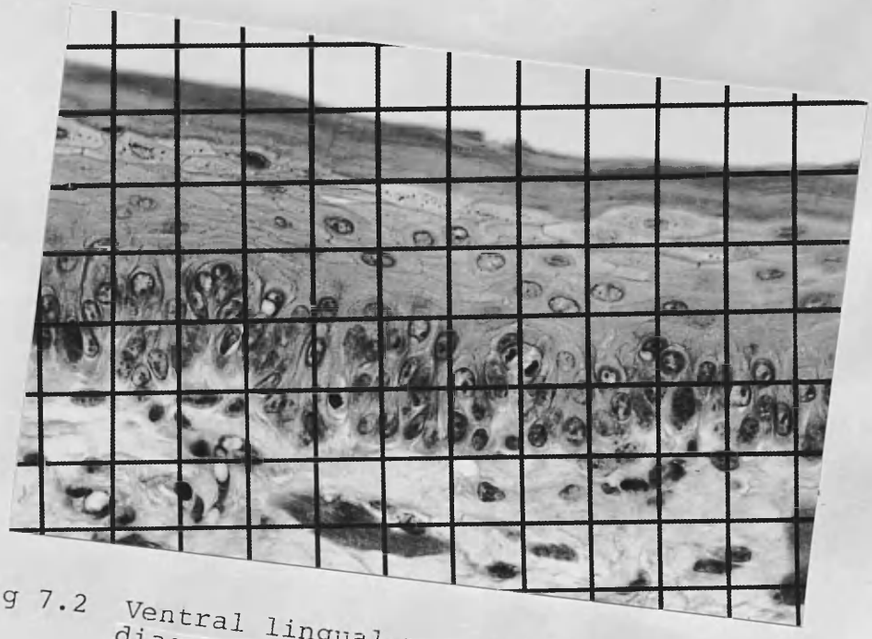


Fig 7.2 Ventral lingual mucosa with diagrammatic superimposition of grid for point-counting.

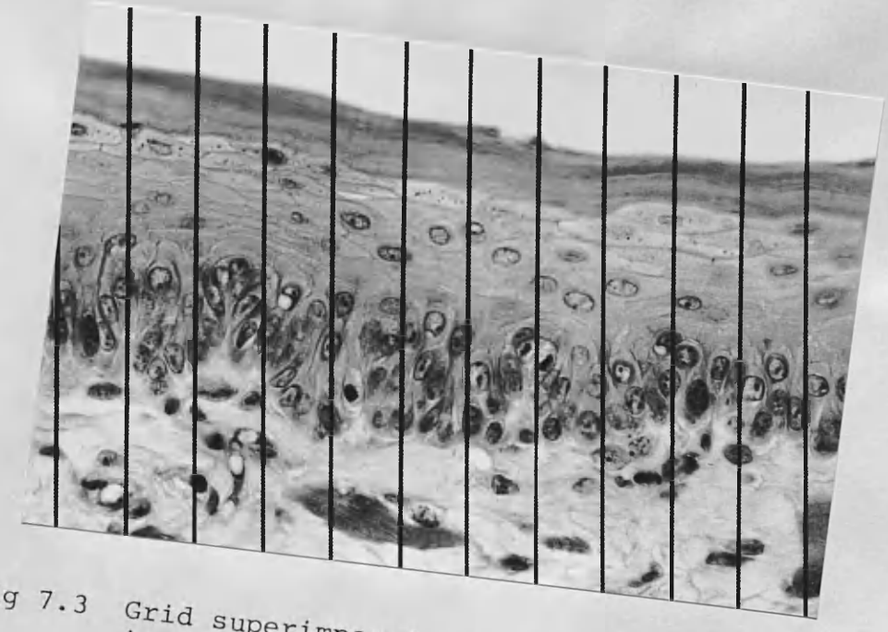


Fig 7.3 Grid superimposed to show lines used for intercept point-counting along the epithelial surface and basement membrane.

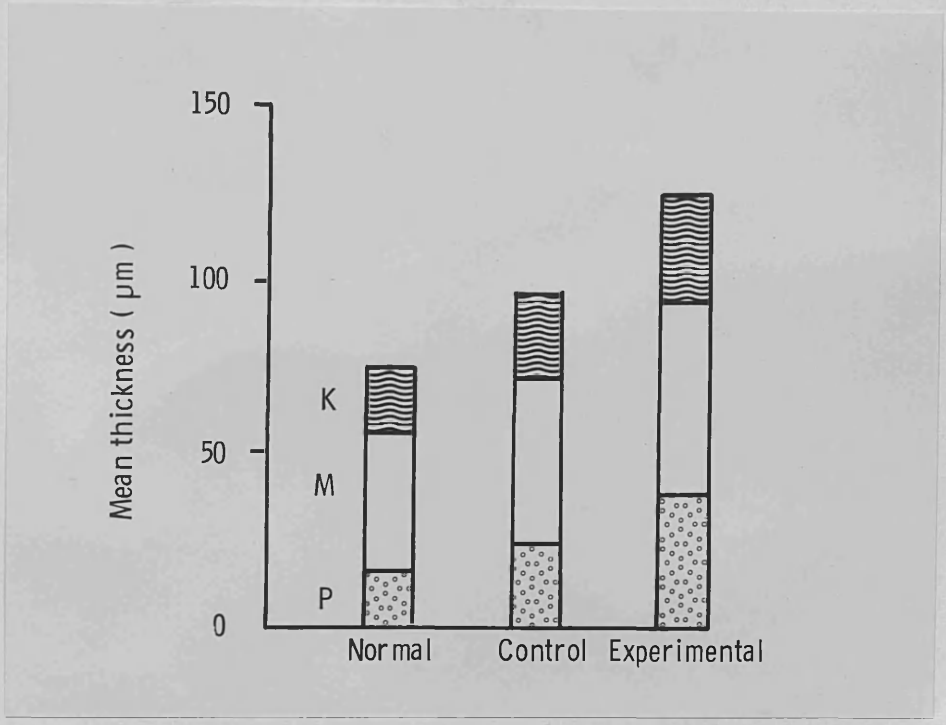


Fig 7.4 Mean total epithelial thickness and the thickness of the progenitor (P), maturation (M) and keratinized compartments (K) in normal, scratch/ acetone controls and scratch/DMBA experimental animals.

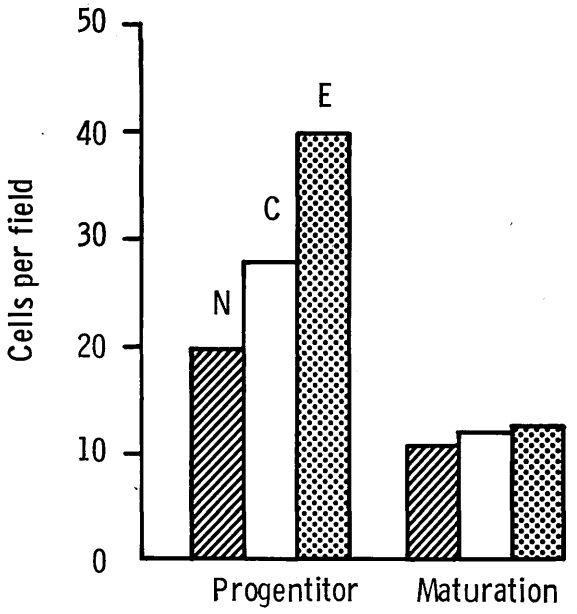


Fig 7.5 Corrected cell numbers per defined field in normal (N) control (C) and experimental (E) animals.

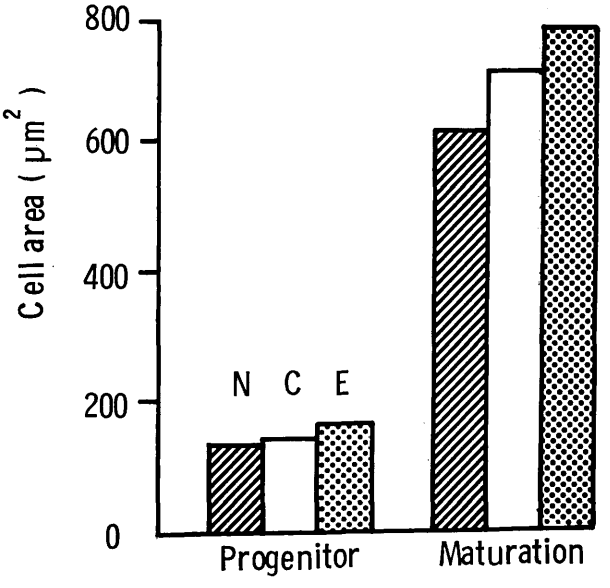


Fig 7.6 Cell sizes expressed as mean cell section areas.

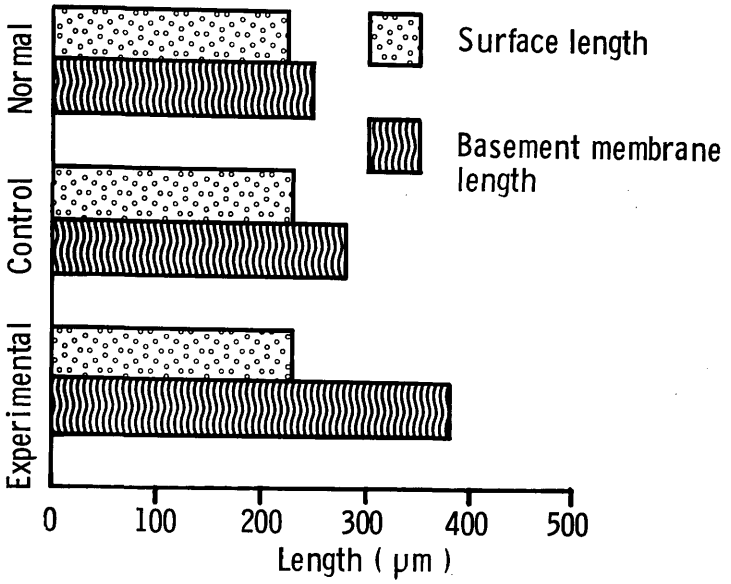


Fig 7.7 Surface and basement membrane lengths per field.

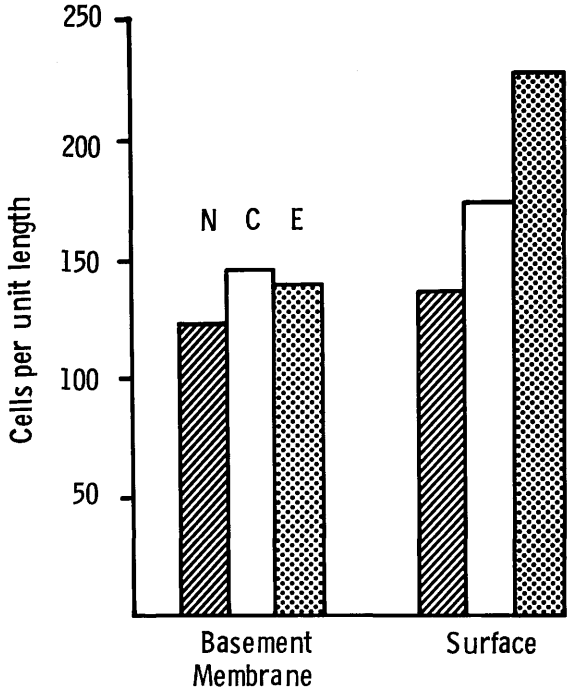


Fig 7.8 Total cells per unit length of surface and basement membrane.

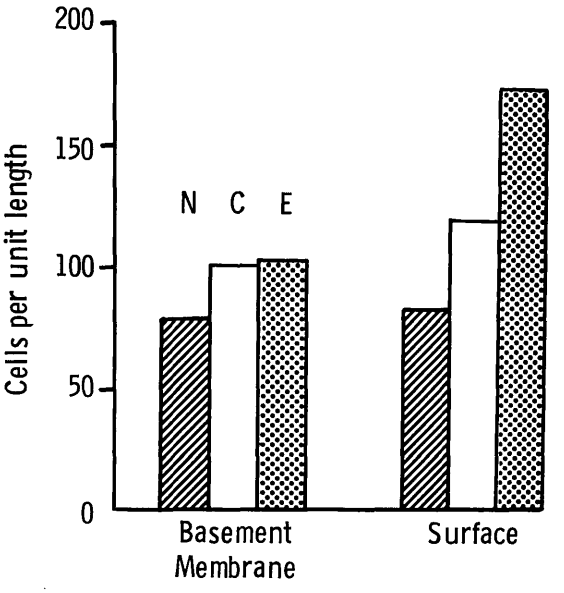


Fig 7.9 Progenitor cells per unit length of surface and basement membrane.

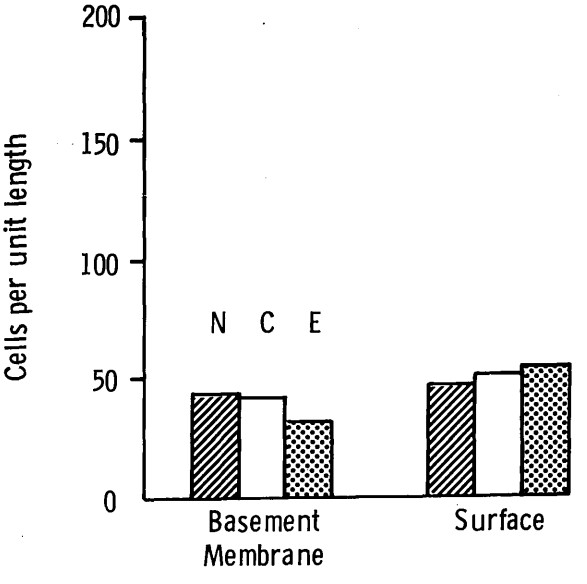


Fig 7.10 Maturation cells per unit length of surface and basement membrane.

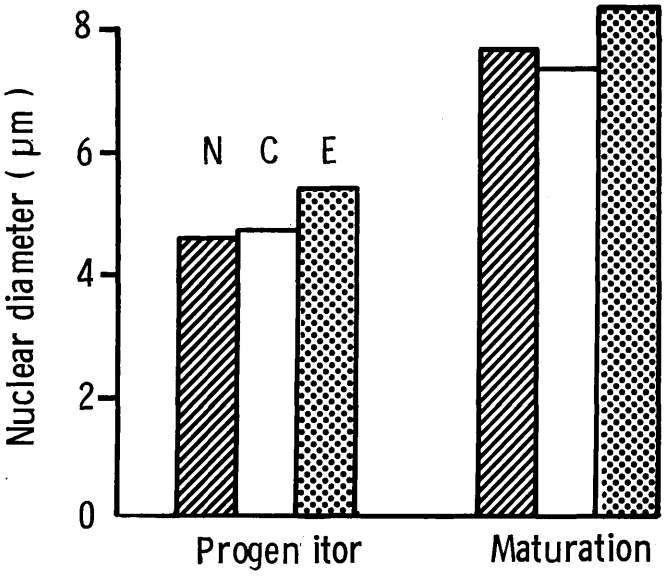


Fig 7.11 Nuclear diameters of cells in the progenitor and maturation compartments.

Animals		P_p	P_m	P_k	P_{total}	T_p	T_m	T_k	T_{total}
Normal	$\bar{x}$	28.0	69.0	35.4	132.4	15.6	38.4	19.7	73.9
	SD	4.5	11.0	8.6	24.1	2.5	6.1	4.8	13.4
Control	$\bar{x}$	42.4	84.8	41.4	168.6	23.5	47.2	23.0	93.7
	SD	8.4	20.0	12.0	40.4	4.7	11.1	6.7	22.5
Experimental	$\bar{x}$	67.6	98.6	54.8	221.0	37.6	54.8	30.5	122.9
	SD	11.9	14.6	13.0	39.5	6.6	8.1	7.0	21.7

Table 7.1 Point counts and mean epithelial thickness. Arithmetical means and standard deviations are included for comparative purposes but non-parametric tests were used for statistical analysis. Abbreviations used in the tables are included in Appendix 2, and raw data and full statistical analysis are included in Appendix 3.

Animals		P.C.	M.C.	T.N.C.	$C_{P.C.}$	$C_{M.C.}$	$C_{T.N.C.}$	A_p	A_m
Normal	$\bar{x}$	48.3	34.0	82.3	20.2	10.6	30.8	139	653
	SD	8.4	4.3	12.7	3.5	1.4	4.8	18	79
Control	$\bar{x}$	65.9	37.0	102.9	28.1	11.9	40.0	152	715
	SD	11.0	7.9	18.9	4.7	2.5	7.2	28	97
Experimental	$\bar{x}$	101.4	43.2	144.6	39.9	12.7	52.6	172	796
	SD	24.2	9.6	33.8	9.5	2.8	12.3	17	150

Table 7.2 Uncorrected and corrected cell numbers and cell areas

Animals		B.M.L.	S.L.	$\frac{C_{P.C.}}{B.M.L.}$	$\frac{C_{M.C.}}{B.M.L.}$	$\frac{C_{T.N.C.}}{B.M.L.}$	$\frac{C_{P.C.}}{S.L.}$	$\frac{C_{M.C.}}{S.L.}$	$\frac{C_{T.N.C.}}{S.L.}$
Normal	$\bar{x}$	246	226	82.1	43.2	125.3	89.6	46.9	136.5
	SD	9	4	13.4	5.5	18.9	16.2	6.3	22.5
Control	$\bar{x}$	276	229	102.1	42.7	144.8	122.2	52.0	174.2
	SD	27	3	14.4	5.2	19.6	21.1	11.1	32.2
Experimental	$\bar{x}$	378	229	105.3	33.9	139.2	174.8	55.8	230.6
	SD	54	4	17.2	6.8	24.0	42.5	12.6	55.1

Table 7.3 Basement membrane and surface length per field and the ratios of cells to basement membrane and surface length.

Animals		P	M
Normal	$\bar{x}$	4.6	7.7
	SD	0.3	0.8
Control	$\bar{x}$	4.7	7.4
	SD	0.6	0.4
Experimental	$\bar{x}$	5.4	8.4
	SD	0.3	0.5

Table 7.4 Nuclear diameters in μm in progenitor (P) and maturation compartments (M).

CHAPTER EIGHT

GENERAL DISCUSSION

Increased thickness and hypertrophy of cells appears to be a general response of epithelium to mechanical damage. For example, such changes have been reported after repeated friction (Mackenzie and Miles 1973), in healing wounds (Potten and Allen 1975), after removal of stratum corneum by Scotch-tape stripping (Pinkus 1952) and following hair plucking (Chase, Montagna and Malone 1953). Similar changes have been described in epithelia after such diverse stimuli as ultraviolet light irradiation (Rothman 1954), applications of turpentine (Craig and Franklin 1977; Tarin 1968) and chemical carcinogens (see Section 1.3.3).

The present study gives quantitative confirmation of epithelial thickening in the hamster cheek pouch and ventral lingual mucosa following applications of the carcinogen DMBA. Some of the increase in the thickness of the maturation and keratinized compartments in the cheek pouch model appeared to be due to an irritant action of the carcinogen, as the changes were partially reversed when carcinogen applications ceased. Increases in the size and numbers of cells in the progenitor compartment, however, appeared to be progressive.

In the ventral lingual mucosa mechanical irritation with a barbed broach and applications of acetone (see Section 7.3.2) did not lead to significant epithelial hyperplasia

when the tissue was analysed quantitatively, although focal areas of hyperplasia were apparent subjectively. There was a significant increase in the number, but not the size, of cells in the progenitor compartment.

In the carcinogen-treated ventral lingual mucosa there was a marked increase in the thickness of epithelium. As with the cheek pouch model there was a significant increase in the size and number of progenitor cells. In a quantitative study of oral epithelium and skin subjected to friction Mackenzie (1970) also found an increase in total epithelial thickness. However, he found that the increase in thickness of the cellular compartment appeared to be mainly due to increases in the size and number of cells in the stratum spinosum and stratum granulosum rather than cells in the basal layer (see Section 7.4.1).

Therefore, although subjectively the responses of epithelium to a wide range of stimuli may appear similar, it is probable that the various epithelial compartments contribute by different degrees to any increase in total thickness. In addition, the relative contribution that changes in cell sizes or cell numbers within compartments make to this increased thickness may also vary considerably.

It is pertinent to ask the relevance of the experimental models of oral precancerous lesions described in this and other studies to the human situation. The histological assessment of suspected premalignant oral lesions in man is highly subjective and there is little general agreement among pathologists about which features of epithelial atypia

are the most important when making a judgement about prognosis. Some workers (Kramer et al 1970; Kramer, El-Labban and Sonkadi 1974) have attempted to assess the relative importance of various histological features by computer-aided discriminant analyses (see Section 2.2). Also, the Smith and Pindborg (1969) system of epithelial atypia scoring gives a semiquantitative method of assessing such lesions.

The present study and other recent work have shown that it is possible to derive objective, quantitative data on at least some of the histological features classified as epithelial atypia. For example, changes in the basement membrane length, reflecting changes in rete ridge pattern, have been quantified by intercept-counting (see Section 7.2.5). Point-counting has been applied to the hamster cheek pouch model and the hamster ventral lingual mucosa model, showing that it is possible to quantify progenitor cell (which is equivalent to most author's 'basal cell') hyperplasia and hypertrophy. Changes in cell size are not a recognised feature of epithelial atypia and have received little attention in the assessment of suspected premalignant lesions. Franklin (1977) has shown that quantitative techniques can be used to determine changes in nucleo-cytoplasmic ratio and Thilagaratnam (1969) has quantified changes in mitotic activity in carcinogen-treated cheek pouch. At the present time other histological features of epithelial atypia have only been assessed subjectively or by semiquantitative techniques and appear to be considerably less amenable to

objective analysis.

The methods of quantitation described in this thesis are not readily applicable to routine histopathological diagnosis. However, the application of these, or similar techniques to human material could help to define more clearly those histological features of premalignant lesions which are most significant in terms of prognosis. In addition, it is possible that quantitative techniques may detect histological changes before there is any significant degree of epithelial atypia. Such knowledge could be of considerable value to the clinician with the difficult problem of managing patients with suspected premalignant lesions.

SUMMARY

The histopathological assessment of suspected premalignant oral lesions is highly subjective and there is no general agreement among pathologists about which of the features of epithelial atypia are the most important when making a judgement about likely prognosis. The aim of the present study was to investigate some quantitative morphological aspects of early experimental oral carcinogenesis in an attempt to define more fully the histological features of premalignant epithelial lesions.

In the initial experiments sequential histological changes in hamster cheek pouch epithelium following applications of the carcinogen dimethyl benzanthracene were assessed using stereological methods. The most significant progressive change following cessation of carcinogen applications appeared to be an increase in the size and number of cells in the progenitor compartment. Much of the marked increase in the thickness of the stratum corneum seen in the early stages of the treatment appeared to be due to a hyperplasiogenic rather than a neoplasiogenic action of the carcinogen. Experience with the cheek pouch model led the author to judge that it was far from ideal as a model of intraoral carcinogenesis and a number of attempts were made to find a suitable alternative.

An attempt was made to induce neoplasms in the ventral lingual mucosa of the hamster using the water-soluble carcinogen 4-nitroquinoline N-oxide (4NQO). The

carcinogen was applied biweekly for up to 20 weeks without the development of any neoplastic change. Further applications of the promoting agent croton oil was followed by the development in a small number of animals of papillomas and areas of epithelial atypia. However, the long latency, very variable response and high mortality would make this a poor experimental model.

A further attempt was made to induce neoplasms in the hamster ventral lingual mucosa by light scratching with a root canal barbed broach and applications of dimethyl benzanthracene. Treatment was continued biweekly for a maximum of ten weeks. Biopsy specimens were taken at intervals and all the surviving animals were killed at 25 weeks. No neoplasms developed but there was evidence of epithelial dysplasia which appeared to be progressive.

Therefore, in a further group of animals a similar technique was used but the frequency and duration of treatment was increased. Thrice weekly applications of DMBA to the ventral lingual mucosa preceded by scratching with a barbed broach for 13 weeks led to tumour development in over 75 per cent of animals by 28 weeks. Tumours were preceded by a stage resembling human oral leukoplakia. In addition to developing tumours in the treated area of mucosa 60 per cent of animals developed tumours in the floor of the mouth or the area of mucosa on either side of the median lingual fraenum.

Biopsy material removed from the hamster ventral lingual mucosa at 16 weeks was subjected to quantitative

analysis using stereological techniques. In carcinogen-treated mucosa there was significant epithelial hyperplasia with increases in the thickness of the progenitor, maturation and keratinized compartments. There was progenitor cell hyperplasia and hypertrophy similar to that seen in the cheek pouch model. There was a marked increase in the number of progenitor cells related to a unit of surface length. In addition, the basement membrane length per field was significantly above normal and control levels, reflecting an increased folding and complexity of the rete ridge pattern in carcinogen treated epithelium.

The relevance of the results to the histopathological assessment of human precancerous oral lesions is discussed.

APPENDICES

APPENDIX 1

TISSUE PROCESSING CYCLE

An automatic tissue processor with the following cycle was used:-

1.	70% methylated spirits	30 minutes
2.	80% methylated spirits	1 hour
3.	8% phenol in methylated spirits	2 hours
4.	8% phenol in methylated spirits	1 hour
5.	8% phenol in methylated spirits	2 hours
6.	Absolute alcohol	2 hours
7.	Absolute alcohol	3 hours
8.	50% absolute alcohol, 50% chloroform	30 minutes
9.	Chloroform	3 hours
10.	Chloroform	4 hours
11.	Paraffin wax	2 hours
12.	Paraffin wax	2 hours

APPENDIX 2

ABBREVIATIONS

A_m	Area of maturation compartment in section (μm^2)
A_p	Area of progenitor compartment in section (μm^2)
B.M.L.	Basement membrane length (μm)
$C_{M.C.}$	Corrected maturation cell numbers
$C_{P.C.}$	Corrected progenitor cell numbers
$C_{T.N.C.}$	Corrected total nucleated cell numbers
M.C.	Uncorrected maturation cell numbers
P.C.	Uncorrected progenitor cell numbers
P_k	Point count in keratinized compartment
P_m	Point count in maturation compartment
P_p	Point count in progenitor compartment
P_{total}	Point count in total epithelium
S.D.	Standard deviation
S.L.	Surface length (μm)
T.N.C.	Uncorrected total nucleated cell numbers
T_k	Thickness of keratinized compartment (μm)
T_m	Thickness of maturation compartment (μm)
T_p	Thickness of progenitor compartment (μm)
T_{total}	Thickness of total epithelium (μm)
$\bar{x}$	Arithmetical mean

APPENDIX 3

Data on individual animals and statistical analysis for hamster tongue carcinogenesis experiment in Chapter 7.

Animals	P.C.	M.C.	$C_{P.C.}$	$C_{M.C.}$	B.M.L.	S.L.
Normal	47.0	37.6	19.6	11.8	232	220
	43.2	32.5	18.0	10.2	240	229
	45.3	36.5	18.9	11.4	261	228
	45.6	35.6	19.0	11.1	247	225
	41.2	39.6	17.2	12.4	250	226
	67.5	33.7	28.1	10.5	256	225
	44.6	29.2	18.6	9.1	240	232
	51.8	27.0	22.4	8.4	242	223
Control	61.3	34.3	26.2	11.0	265	231
	59.3	48.0	25.3	15.4	308	229
	68.2	44.4	29.1	14.3	300	225
	56.3	27.7	24.0	8.9	245	231
	53.0	30.0	22.6	9.6	239	228
	82.3	34.8	35.1	11.2	269	231
	81.7	45.7	34.9	14.7	308	231
	65.3	31.0	27.9	10.0	272	225
Experi-	86.7	39.5	34.1	11.5	316	231
mental	79.0	39.2	31.1	11.5	324	234
	95.7	38.5	37.6	11.4	382	226
	81.8	42.0	32.2	12.4	331	226
	99.5	41.2	39.1	12.1	355	225
	79.2	41.3	31.2	12.1	305	229
	103.5	45.5	40.7	13.4	401	226
	93.8	40.5	36.9	11.9	366	228
	101.7	34.0	40.0	10.0	426	232
	119.0	50.2	46.8	14.8	489	225
	86.3	38.0	33.9	11.4	327	228
	87.2	37.2	34.3	10.9	357	236
	99.5	38.2	39.1	11.2	399	232
	128.5	41.3	50.6	12.2	468	225
	176.2	75.5	69.2	22.2	415	228
	105.2	48.7	41.4	14.3	383	225
N - C	P=0.001	P=0.323	P=0.001	P=0.287	P=0.014	-
N - E	P<0.001	P<0.001	P<0.001	P<0.05	P<0.001	-
C - E	P<0.001	-	P<0.001	-	P<0.001	-

Animals	$\frac{C_{P.C.}}{B.M.L.}$	$\frac{C_{M.C.}}{B.M.L.}$	$\frac{C_{T.N.C.}}{B.M.L.}$	$\frac{C_{P.C.}}{S.L.}$	$\frac{C_{M.C.}}{S.L.}$	$\frac{C_{T.N.C.}}{S.L.}$
Normal	84.5	50.9	135.3	89.1	53.6	142.7
	75.0	42.5	117.5	78.6	44.5	123.1
	72.4	43.7	116.1	82.9	50.0	132.9
	76.9	44.9	121.9	84.4	49.3	133.8
	68.8	49.6	118.4	76.1	54.9	140.0
	109.8	41.0	150.8	124.9	46.7	171.6
	77.5	37.9	115.4	80.2	39.2	119.4
	92.6	34.7	127.3	100.4	37.7	138.1
Control	98.9	41.5	140.4	113.4	47.6	161.0
	82.1	50.0	132.1	110.5	67.2	177.7
	97.0	47.7	144.7	129.3	63.6	192.9
	98.0	36.3	134.3	98.0	38.5	142.4
	94.6	40.2	134.7	99.1	42.1	141.2
	130.5	41.6	172.1	151.9	48.5	200.4
	113.3	47.7	161.0	151.1	63.6	214.7
	102.6	36.8	139.3	124.0	44.4	168.4
Experi-	107.9	36.4	144.3	147.6	49.8	197.4
mental	96.0	35.5	131.5	132.5	49.1	182.1
	98.4	29.8	128.2	166.4	50.4	216.8
	97.3	37.5	134.7	142.5	54.9	197.3
	110.1	34.1	144.2	173.8	55.6	227.6
	102.3	39.7	142.0	136.2	52.8	189.1
	101.5	33.4	134.9	180.1	59.3	239.4
	100.8	32.5	133.3	161.8	52.2	214.0
	93.9	23.5	117.4	172.4	43.1	215.5
	95.7	30.3	126.0	208.0	65.8	273.8
	103.7	34.9	138.5	148.7	50.0	198.7
	96.1	30.5	126.6	145.3	46.2	191.5
	98.0	28.1	126.1	168.5	48.3	216.8
	108.1	26.1	134.2	224.9	54.2	279.1
	166.7	53.5	220.2	303.5	97.4	400.9
	108.1	37.3	145.4	184.0	63.6	247.6
N - C	P=0.005	P=0.032	-	P=0.000	P=0.323	P=0.002
N - E	P<0.001	P<0.001	-	P<0.001	P<0.1	P<0.001
C - E	-	P<0.001	-	P<0.001	-	P<0.01

Animals	T_P	T_M	T_K	T_{total}	A_p	A_m	$P_{N.D.}$	$M_{N.D.}$
Normal	11.9	33.8	17.3	63.0	109	516	4.9	7.2
	13.6	34.5	13.1	61.2	136	609	4.8	7.8
	16.2	49.2	29.1	94.5	154	777	5.0	9.2
	14.2	39.0	22.1	75.3	135	632	4.5	8.0
	16.1	46.4	19.3	81.8	168	674	4.2	7.6
	19.6	35.7	20.7	76.0	126	612	4.8	7.4
	14.7	34.8	15.8	65.3	142	688	4.3	6.7
	18.2	33.4	19.9	71.5	146	716	4.3	7.7
Control	21.5	53.8	23.3	98.6	148	880	4.6	7.5
	29.9	58.2	19.5	120.6	213	680	5.9	8.1
	25.7	58.3	32.4	116.4	159	734	4.6	7.8
	17.9	40.6	23.0	81.5	134	821	4.6	7.8
	17.5	30.9	18.3	66.7	139	579	5.0	7.0
	22.7	42.4	17.9	83.0	116	681	4.0	6.9
	29.4	57.4	33.6	120.4	152	703	4.9	7.2
	23.7	35.5	15.9	75.1	153	639	4.3	7.2
Experi-	32.0	57.6	34.3	124.4	169	902	5.1	9.0
mental	35.2	70.6	45.9	151.7	204	1105	5.1	8.9
	34.1	51.9	28.9	114.9	163	819	5.9	9.0
	33.1	68.2	33.9	135.2	185	990	4.8	8.4
	39.3	66.9	34.4	140.6	181	995	5.4	7.4
	27.5	47.4	32.7	107.6	159	705	5.4	8.9
	35.3	54.4	24.6	114.3	156	731	5.1	8.4
	35.8	52.5	28.8	117.1	175	794	5.6	8.0
	40.9	43.1	20.2	104.2	184	776	5.2	8.3
	46.8	53.1	28.4	128.3	180	646	5.4	7.9
	30.6	44.9	20.4	95.9	162	709	5.4	7.9
	33.1	51.5	31.4	116.0	174	850	5.4	7.6
	42.4	48.1	44.4	134.9	195	773	5.3	8.6
	40.4	80.8	25.0	116.2	144	750	6.2	8.5
	54.0	59.7	27.9	141.5	140	484	5.4	3.1
	41.3	56.1	26.1	123.5	180	706	5.3	9.2
N - C	P=0.000	P=0.052	P=0.164	P=0.019	P=0.221	P=0.117	P=0.360	P=0.287
N - E	P<0.001	P<0.001	P<0.001	P<0.001	P=0.001	P<0.01	P=0.001	P<0.01
C - E	P<0.001	-	P<0.01	P<0.01	P<0.01	-	P<0.01	P<0.001

REFERENCES

REFERENCES

ABERCROMBIE, M. (1946)
Estimation of nuclear population from microtome sections.
Anatomical Record, 94, 239-247.

ABRAMS, B and SHEAR, M. (1977)
Estimation of the volumes of multinucleate giant cells.
Journal of Oral Pathology, 6, 264-267.

AHLSTRÖM, C.G. (1951)
A short review of mitotic poisons.
Acta Pathologica et Microbiologica Scandinavica, Supplement 91.

AL-ANI, S. and SHKLAR, G. (1966)
Effects of a chemical carcinogen applied to hamster gingiva.
Journal of Periodontology, 37, 36-42.

BANOCZY, J. and SUGAR, L. (1972)
Longitudinal studies in oral leukoplakias.
Journal of Oral Pathology, 1, 265-272.

BANOCZY, J., RADNAI, I and REMENYI, I. (1969)
Modszertani tapasztalataink Dunakeszi es Felsögöd lakossagan Vegzett stomato-onkologiai Szürövizsgalatok alapjan.
Forgorvosi Szemk, 62, 118-122, cited by Banoczy and Sugar 1972.

BARRINGTON, E.P. and MEYER, J. (1969)
Regional differences in gradients of dry residue density in oral epithelia of the rat: a quantitative study.
Archives of Oral Biology, 14, 1085-1099.

BAYON, H. (1912)
Epithelial proliferation induced by the injection of gasworks tar.
Lancet, 2, 1579.

BERENBLUM, I. (1954)
Carcinogenesis and tumour pathogenesis.
Advances in Cancer Research, 2, 129-175.

BERENBLUM, I. (1970a)
The study of tumours in animals. In General Pathology. ed. Lord Florey. pp 301-334. London: Lloyd Luke (Medical Books) Ltd.

BERENBLUM, I. (1970b)
The epidemiology of cancer. In General Pathology. ed. Lord Florey. pp 732. London: Lloyd Luke (Medical Books) Ltd.

BERENBLUM, I. and SHUBIK, P. (1947)
The role of croton oil applications associated with a single painting of a carcinogen, in tumour induction of the mouses' skin.
British Journal of Cancer, 1, 379-382.

BILLINGHAM, R.E., FERRIGAN, L.W. and SILVERS, W.K. (1960)
Cheek pouch of the Syrian hamster and tissue transplantation immunity.
Science, 132, 1488.

BINNIE, W.H. (1975)
Oral Cancer. In Oral Mucosa in Health and Disease. ed. Dolby, A.E. pp 301-334. Blackwell Scientific Publications.

BIZZOZERO, G. (1894)
Growth and regeneration of the organism.
British Medical Journal, 1, 728-732.

BONNE, C. (1925)
Sur la presence de papillomes sur les muqueuses d'animaux badigeonnes au goudron.
Compt. Rend Soc. de biol., 93, 907, cited by Woglom 1926.

BONNE, C. (1927)
Über Geschwulste bei teertieren.
Zeitschrift für Krebsforschung, 25, 1-22.

BRUSZT, P. (1962)
Stomato-onkologische Reihenuntersuchungen in sieben Gemeinden Südungerns.
Schwiez Monatsschrift für Zahnheilkunde, 72, 758-766, cited by Banoczy and Sugar 1972.

BUTLER, W.H. and JONES, G. (1976)
Preneoplasia: Is it an entity? In Scientific Foundations of Oncology. ed. Symington, T. and Carter, R.L. pp 1-7, London: William Heinemann Medical Books Ltd.

CAWSON, R.A. (1966)
Chronic oral candidiasis and leukoplakia.
Oral Surgery, Oral Medicine and Oral Pathology, 22, 582-591.

CAWSON, R.A. (1969)
Leukoplakia and oral cancer.
Proceedings of the Royal Society of Medicine, 62, 610-615.

CAWSON, R.A. (1973)
Induction of epithelial hyperplasia by Candida albicans.
British Journal of Dermatology, 89, 497-503.

CAWSON, R.A. (1975)
Premalignant lesions of the mouth.
British Medical Bulletin, 31, 164-168.

CAWSON, R.A. (1976)
Infections of the oral mucous membrane. In Scientific Foundations of Dentistry. ed. Cohen, B. and Kramer, I.R.H. pp 484-497. London: William Heinemann Medical Books Ltd.

CAWSON, R.A. and RAJASINGHAM, K.C. (1972)
Ultrastructural features of the invasive phase of Candida albicans.
British Journal of Dermatology, 87, 435-443.

CAZIN (1894)
Des origenes et des modes de transmission du cancer.
cited by Woglom 1926.

CHANG, K.M. (1966)
Experimental production of lesions on cheek pouch of the hamster by betel quid.
Journal of the Formosan Medical Association, 65, 125-131.

CHASE, H.B., MONTAGNA, W. and MALONE, J.D. (1953)
Changes in the skin in relation to the hair growth cycle.
Anatomical Record, 116, 75-81.

CHURCHILL, W.H. and DAVID, J.R. (1973)
Levamisole and cell mediated immunity.
New England Journal of Medicine, 289, 375-376.

COHEN, B. and SMITH, C.J. (1967)
Aetiological factors in oral cancer: experimental investigation of early epithelial changes.
Helvetica Odontologica Acta, 11, 112-124.

COHEN, B., POSWILLO, D.E. and WOODS, D.A. (1971)
The effects of exposure to chewing tobacco on the oral mucosa of monkey and man.
Annals of the Royal College of Surgeons of England, 48, 255-273.

COOK, J.W., HEWETT, C.L. and HIEGER, I. (1933)
Isolation of a cancer-producing hydrocarbon from coal tar. II. Isolation of 1, 2- and 4, 5- benzopyrenes, perylene and 1, 2- benzanthracene.
Journal of the Chemical Society, 1, 396-398.

COOKE, B.E.D. (1956)
Leukoplakia buccalis and oral epithelial naevi. A clinical and histological study.
British Journal of Dermatology, 68, 151-174.

CRAIG, G. (1977)
Personal communication.

CRAIG, G.T. and FRANKLIN, C.D. (1977)
The effect of turpentine on hamster cheek pouch mucosa: a model of epithelial hyerplasia and hyperkeratosis.
Journal of Oral Pathology, 6, 268-277.

DACHI, S.F. (1962)
Effects of polyoxyethylene sorbitan monostearate (Tween 60) upon experimental oral carcinogenesis in the hamster.
Journal of Dental Research, 41, 476-483.

DACHI, S.F. (1967)
Experimental production of carcinomas of the hamster tongue.
Journal of Dental Research, 46, 1480.

DACHI, S.F., SANDERS, J.E. and URIE, E.M. (1967)
Effects of dimethyl sulfoxide on dimethylbenzanthracene - induced carcinogenesis in the hamster cheek pouch.
Cancer Research, 27, 1183-1185.

DARLOW, H.M., SIMMONS, D.J.C. and ROE, F.J.C. (1969)
Hazards from experimental skin painting of carcinogens.
Archives of Environmental Health, 18, 883-893.

DELESSE, M.A. (1847)
Procede mechanique pour determiner la composition des roches.
Compt. Rend. Acad. Sci., 25, 544-545, cited by Weibel 1969.

DUNHAM, L.J. and HERROLD, K.M. (1962)
Failure to produce tumours in the hamster cheek pouch by exposure to ingredients of betel quid; histopathologic changes in the pouch and other organs by exposure to known carcinogens.
Journal of the National Cancer Institute, 29. 1047-1068.

DUNHAM, L.J., MUIR, C.S. and HAMNER, J.E. (1966)
Epithelial atypia in hamster cheek pouches treated repeatedly with calcium hydroxide.
British Journal of Cancer, 20, 588-593.

DUNNILL, M.S. (1968)
Quantitative methods in histology. In Recent Advances in Clinical Pathology. ed. Dyke, S.C. pp 401-416.
London: Churchill Ltd.

EDWARDS, M.B. (1976)
Chemical carcinogenesis in the cheek pouch of Syrian hamsters receiving supplementary zinc.
Archives of Oral Biology, 21, 133-135.

EINHORN, J. and WERSÄLL, J. (1967)
Oral carcinoma in cases of leukoplakia of the oral mucosa.
Acta Oto-Laryngologica, Supplement 224, 20-22.

EISENBERG, E. and SHKLAR, G. (1977)
Levamisole and hamster pouch carcinogenesis.
Oral Surgery, Oral Medicine and Oral Pathology, 43, 562-571.

ELZAY, R.P. (1966)
Local effect of alcohol in combination with DMBA on hamster cheek pouch.
Journal of Dental Research, 45, 1788-1795.

ELZAY, R.P. (1967)
Dimethyl sulfoxide and experimental oral carcinogenesis in the hamster pouch.
Archives of Pathology, 83, 293-297.

ELZAY, R.P. (1969)
Effect of alcohol and cigarette smoke as promoting agents in hamster pouch carcinogenesis.
Journal of Dental Research, 48, 1200-1205.

EVESON, J.W. and MacDONALD, D.G. (1977)
Effects of the water-soluble carcinogen 4-nitroquinoline N-oxide on hamster lingual mucosa.
Oral Surgery, Oral Medicine and Oral Pathology, 44, 600-605.

FRANKLIN, C.D. (1977)
Objective analysis of experimental epithelial hyperplasia and neoplasia.
Journal of Dental Research, 56, D48.

FRANKLIN, C.D. and CRAIG, G.T. (1978a)
Stereological quantification of histological parameters in normal hamster cheek pouch epithelium.
Archives of Oral Biology, 23, 337-345.

FRANKLIN, C.D. and CRAIG, G.T. (1978b)
Stereological quantification of histological parameters in turpentine-induced hyperplasia of hamster cheek pouch epithelium.
Archives of Oral Biology, 23, 347-353.

FUJINO, H., CHINO, T. and IMAI, T. (1965)
Experimental production of labial and lingual carcinoma by local application of 4-nitroquinoline N-oxide.
Journal of the National Cancer Institute, 35, 907-918.

FUJITA, K., KAKU, T., SASAKI, M. and ONOE, T. (1973a)
Experimental production of lingual carcinomas in hamsters by local application of 9, 10-dimethyl-1, 2-benzanthracene.
Journal of Dental Research, 52, 327-332.

FUJITA, K., KAKU, T., SASAKI, M. and ONOE T. (1973b)
Experimental production of lingual carcinomas in hamsters: Tumor characteristics and site of formation.
Journal of Dental Research, 52, 1176-1185.

GIUNTA, J.L., REIF, A.E. and SHKLAR, G. (1974)
Bacillus Calmette - Guerin and antilymphocyte serum in carcinogenesis. Effects on the hamster pouch.
Archives of Pathology, 98, 237-240.

GIUNTA, J.L. and SHKLAR, G. (1971)
The effect of antilymphocyte serum on experimental hamster buccal pouch carcinogenesis.
Oral Surgery, Oral Medicine and Oral Pathology, 31, 344-353.

GIUNTA, J.L. and SHKLAR, G. (1972)
Studies on tongue carcinogenesis in rats using DMBA with and without cyanoacrylate adhesive.
Archives of Oral Biology, 17, 617-622.

GLAGOLEFF, A.A. (1933)
On the geometrical methods of quantitative mineralogic analysis of rocks.
Trans. Inst. Econ. Min., Moskow, 59, 475, cited by Weibel 1969.

GOLDHABER, P. (1957)
The role of saliva and other local environmental factors in oral carcinogenesis.
Journal of the American Dental Association, 54, 517-524.

GOLDHABER, P., WEISBERGER, D. and SOGNNAES, R.F. (1956)
Effect of a carcinogen (3-methylcholanthrene) on oral mucosa and skin of desalivated mice.
International Association of Dental Research, 34, Abstract 56.

HAGA, I. (1913)
Experimentelle Untersuchungen über die Erzeugung atypischer Epithel-und Schleimhautwucherungen.
Ztschr. f. Krebsforsch., 12, 525, cited by Woglom 1926.

HAMILTON, E and POTTEN, C.S. (1972)
Influence of hair plucking on the turnover time of the epidermal basal layer.
Cell and Tissue Kinetics, 5, 505-517.

HAMNER, J.E. (1972)
Betel quid inducement of epithelial atypia in the buccal mucosa of baboons.
Cancer, 30, 1001-1005.

HANAU, A. (1889)
Erfolgreiche experimentelle Uebertragung von Carcinom.
Fortschr. d. Med., 7, 321, cited by Woglom 1926.

HENEFER, E.P. (1966)
Ethanol, 30 per cent, and hamster pouch carcinogenesis.
Journal of Dental Research, 45, 838-844.

HOBAEK, A. (1946)
Leukoplakia oris.
Acta Odontologica Scandinavica, 7, 61-91.

HOLMES, A. (1921)
In Petrographic Methods and Calculations. pp 310-319.
London: Murby and Company, cited by Weibel 1969.

HOMBURGER, F. (1969)
Chemical carcinogenesis in the Syrian golden hamster.
A review.
Cancer, 23, 313-338.

HOMBURGER, F. (1972)
Chemical carcinogenesis in Syrian hamsters. In Progress in Experimental Tumour Research. ed. Homburger, F. Vol. 16, pp 152-175. Basel: Karger.

IVERSEN, O.H., BJERKNES, R. and DEVIK, F. (1968)
Kinetics of cell renewal, cell migration and cell loss in the hairless mouse dorsal epidermis.
Cell and Tissue Kinetics, 1, 351-367.

JEPSEN, A. and WINTHER, J.E. (1965)
Mycotic infection in oral leukoplakia.
Acta Odontologica Scandinavica, 23, 239-256.

KARRING, T. and LÖE, H. (1972)
The reliability of various mitotic index systems in assessing mitotic activity in stratified squamous epithelium.
Journal of Periodontal Research, 7, 271-282.

KENDRICK, F.J. (1964)
Some effects of a chemical carcinogen and a cigarette smoke condensate upon hamster cheek pouch mucosa.
Health Sci., 24, 3698-3699, cited by Homburger 1969.

KENT, S.P. (1960)
Spontaneous and induced malignant neoplasms in monkeys.
Annals of the New York Academy of Sciences, 85, 819-827.

KEYES, P.H. and DALE, P.P. (1944)
A preliminary survey of the pouches and dentition of the Syrian hamster.
Journal of Dental Research, 23, 427-438.

KLEIN-SZANTO, A.J.P. (1977)
Stereologic baseline data of normal human epidermis.
Journal of Investigative Dermatology, 68, 73-78.

KOLAS, S. (1955)
Investigation of normal human saliva for possible anticarcinogenic action and chemical carcinogenesis in mucous membranes.
Oral Surgery, Oral Medicine and Oral Pathology, 8, 1192-1203.

KÖVESI, G. and BANOCZY, J. (1973)
Follow-up studies in oral lichen planus.
International Journal of Oral Surgery, 2, 13-19.

KRAMER, I.R.H., EL-LABBAN, N. and LEE, K.W. (1978)
The clinical features and risk of malignant transformation in sublingual keratosis.
British Dental Journal, 144, 171-180.

KRAMER, I.R.H., EL-LABBAN, N. and SONKODI, S. (1974)
Further studies on lesions of the oral mucosa using computer-aided analyses of histological features.
British Journal of Cancer, 29, 223-231.

KRAMER, I.R.H., LUCAS, R.B., EL-LABBAN, N. and LISTER, L. (1970)
The use of discriminant analysis for examining the histological features of oral keratoses and lichen planus.
British Journal of Cancer, 24, 673-683.

KRESHOVER, S. (1952)
The effect of tobacco on epithelial tissues of mice.
Journal of the American Dental Association, 45, 528-540.

KRESHOVER, S.J. and SALLEY, J.J. (1957)
Predisposing factors in oral cancer.
Journal of the American Dental Association, 54, 509-514.

LACASSAGNE, A. and LATARJET, R. (1946)
Action of methylcholanthrene on certain scars of the skin in mice.
Cancer Research, 6, 183-188.

LEBLOND, C.P. (1964)
Classification of cell populations on the basis of their proliferative behaviour.
National Cancer Institute Monographs, 14, 119-150.

LEBLOND, C.P., GREULICH, R.C. and PEREIRA, J.P.M. (1964)
Relationship of cell formation and cell migration in the renewal of stratified squamous epithelia. In Advances of Biology of Skin, 5. ed. Montagna, W and Billingham, R.E. pp 39-67, Oxford: Pergamon Press.

LEKHOLM, U. and WALLENIUS, K. (1976)
Experimental oral cancer in rats with xerostomia.
Odontologisk Revy, 27, 11-18.

LEVIJ, I.S. and POLLIACK, A. (1968)
Potentiating effect of vitamin A on 9,10-dimethyl 1, 2-benzanthracene carcinogenesis in the hamster cheek pouch.
Cancer, 22, 300-306.

LEVIJ, I.S., RWOMUSHANA, J. and POLLIACK, A. (1968)
Spontaneous partial regression of 9-10 dimethyl 1-2 benzanthracene-induced epithelial atypia in the hamster cheek pouch.
Israeli Journal of Medical Science, 4, 913-916.

LEVIJ, I.S., RWOMUSHANA, J.W. and POLLIACK, A. (1969)
Enhancement of chemical carcinogenesis in the hamster cheek pouch by prior topical application of vitamin A palmitate.
Journal of Investigative Dermatology, 53, 228-231.

LEVY, B.M. (1948)
The effect of a chemical carcinogen (20-methyl-cholanthrene) on the oral tissues: a preliminary report. Oral Surgery, Oral Medicine and Oral Pathology, 1, 582.

LEVY, B.M. (1958)
The experimental production of carcinoma of the tongue in mice.
Journal of Dental Research, 37, 950.

LEVY, B.M., GORLIN, R. and GOTTSEGEN, R. (1950)
A histologic study of the reaction of skin and mucous membrane to a single application of 9, 10 dimethyl-1, 2, benzanthracene.
Journal of Dental Research, 29, 678-679.

LISTGARTEN, M.A., ALBRIGHT, J.T. and GOLDHABER, P. (1963)
Ultrastructural alterations in hamster cheek pouch epithelium in response to a carcinogen.
Archives of Oral Biology, 8, 145-165.

LÖE, H., KARRING, T. and HARA, K. (1972)
The site of mitotic activity in rat and human oral epithelium.
Scandinavian Journal of Dental Research, 80, 111-119.

LUCAS, R.B. (1976)
In Pathology of Tumours of the Oral Tissues. 3rd Ed. pp 134. Edinburgh: Churchill-Livingstone.

LUCAS, R.B. (1978)
Personal communication.

LUTHRA, U.K., BHARADWAJ, V.P. and WAHI, P.N. (1969)
Alkaline phosphatase activity in DMBA induced experimental oral carcinogenesis in hamster cheek pouch.
Indian Journal of Medical Research, 57, 1677-1684.

MacDONALD, D.G. (1971)
Cell renewal in oral epithelium. In Current Concepts of the Histology of Oral Mucosa. Ed. Squier, C.A. and Meyer, J. pp 61-79. Illinois: Charles C Thomas.

MacDONALD, D.G. (1973)
Experimental oral carcinogenesis.
PhD Thesis. University of Glasgow.

MacDONALD, D.G. (1975)
Premalignant lesions of oral epithelium. In Oral Mucosa in Health and Disease. ed. Dolby, A.E. pp 335-369. Blackwell Scientific Publications.

MacDONALD, D.G. (1978)
A technique for localisation of tumours in hamster cheek-pouch carcinogenesis.
Archives of Oral Biology, 23, 573-577.

MacDONALD, D.G. and RENNIE, J.S. (1975)
Oral epithelial atypia in denture induced hyperplasia, lichen planus and squamous cell papilloma.
International Journal of Oral Surgery, 4, 40-45.

MACKENZIE, I.C. (1970)
An experimental study of the effects of mechanical stimulation on keratinizing epithelia of rodents.
PhD Thesis. University of London.

MacKENZIE, I.C. and MILES, A.E.W. (1973)
The effect of chronic frictional stimulation on hamster cheek pouch epithelium.
Archives of Oral Biology, 18, 1341-1349.

MAJOR, I.R. (1970)
Correlation of initial changes in the mouse epidermal cell population with two stage carcinogenesis - a quantitative study.
British Journal of Cancer, 24, 149-163.

MAREFAT, P. and SHKLAR, C. (1977)
Experimental production of lingual leukoplakia and carcinoma.
Oral Surgery, Oral Medicine and Oral Pathology, 44, 578-586.

MASHBERG, A., MORRISSEY, J.B. and GARFINKEL, L. (1973)
A study of the appearance of early asymptomatic oral squamous cell carcinoma.
Cancer, 32, 1436-1445.

MEHTA, F.S., PINDBORG, J.J. and HAMNER, J.E. (1971)
Oral Cancer and Precancerous Conditions in India.
Copenhagen: Munksgaard.

MEHTA, F.S., PINDBORG, J.J., GUPTA, P.C. and DAFTARY, D.K. (1969)
Epidemiologic and histologic study of oral cancer and leukoplakia among 50,915 villagers in India.
Cancer, 24, 832-849.

MELNIKOV, R.A. (1963)
The induction of tumours in monkeys and rabbits by chemical and radiation agents.
Acta Unio Int. Contra Cancrum, 19, 708-709, cited by Cohen and Smith 1967.

MESKIN, L.H. and WOOLFREY, B.F. (1964)
Radiographic localisation of labeled carcinogen: Carbon 14 labeled dimethyl-benzanthracene in the hamster cheek pouch.
Archives of Pathology, 78, 643-647.

MESROBIAN, A.Z. and SHKLAR, G. (1969)
Gingival carcinogenesis in the hamster using tissue adhesives for carcinogen fixation.
Journal of Periodontology, 40, 603-606.

MEYER, J., ALVARES, O.A., WATERHOUSE, J.P., ROED-PETERSEN, B. and PINDBORG, J.J. (1976)
Size of nucleus and nucleolus in quantitative electron microscopy on orthokeratinized leukoplakias.
Scandinavian Journal of Dental Research, 84, 158-167.

MEYER, J. and GERSON, S.J. (1964)
Comparison of human palatal and buccal mucosa.
Periodontics, 2, 284-291.

MINCER, H.H., COLEMAN, S.A. and HOPKINS, K.P. (1972)
Observations on the clinical characteristics of oral lesions showing histologic epithelial dysplasia.
Oral Surgery, Oral Medicine and Oral Pathology, 33, 389-399.

MOORE, C. and MILLER, A.J. (1958)
Effect of cigarette smoke tar on hamster pouch.
Archives of Surgery, 76, 786-793.

MOORE, C. and CATLIN, D. (1967)
Anatomic origins and locations of oral cancer.
American Journal of Surgery, 114, 510-513.

MORI, M., MIYAJI, T., MURATA, I. and NAGASUNA, H. (1962)
Histochemical observations on enzymatic processes of experimental carcinogenesis in hamster cheek pouch.
Cancer Research, 22, 1323-1326.

MORRIS, A.L. (1961)
Factors influencing experimental carcinogenesis in the hamster cheek pouch.
Journal of Dental Research, 40, 3-15.

MORRIS, A.L. and REISKIN, A.B. (1966)
Hamster cheek pouch response to varying lengths of carcinogen exposure.
Journal of Dental Research, 44, 664-667.

MOSS, R.L., COLLINS, T.A. and COLE, D.F. (1965)
A new apparatus for intraoral manipulation in the hamster.
Journal of Dental Research, 44, 668-670.

McCARTHY, P.L. and SHKLAR, G. (1964)
In Diseases of the Oral Mucosa. 2nd Ed. pp 298-300.
New York: McGraw-Hill Book Company.

McKINNEY, R.V. and SINGH, B.B. (1977)
Basement membrane changes under neoplastic oral mucous membrane.
Oral Surgery, Oral Medicine and Oral Pathology, 44, 875-888.

NAKAHARA, W., FUKUOKA, F. and SUGIMURA, T. (1957)
Carcinogenic action of 4-nitroquinoline N-oxide.
Gann, 48, 129-137.

OYAMA, S. (1935)
Experimentelle Studien über die Entstehung des Zungenkrebses.
Mitt. Med. Akad. Kioto, 15, 861-880, cited by Fujita et al 1973a.

PEACOCK, E.E. and BRAWLEY, B.W. (1959)
An evaluation of snuff and tobacco in the production of mouth cancer.
Plastic and Reconstructive Surgery, 23, 628-635.

PHILIPSEN, H.P., FISKER, A.V. and STAGE, I. (1977)
Experimental production of palatal carcinoma in rats by local application of 4-nitroquinoline N-oxide (4NQO).
Journal of Dental Research, Special Issue A, A101.

PINDBORG, J.J. (1965a)
Oral Cancer from an international point of view.
Journal of the Canadian Dental Association, 31, 219-226.

PINDBORG, J.J. (1965b)
Oral precancerous conditions in south-east Asia.
International Dental Journal, 15, 190-199.

PINDBORG, J.J. (1972)
Is submucous fibrosis a precancerous condition in the oral cavity?
International Dental Journal, 22, 474-480.

PINDBORG, J.J., BARMES, D. and ROED-PETERSEN, D. (1968)
Epidemiology and histology of oral leukoplakia and leukoedema among Papuans and New Guineans.
Cancer, 22, 379-384.

PINDBORG, J.J., MEHTA, F.S. and DAFTARY, D.K. (1970)
Occurrence of epithelial atypia in 51 Indian villagers with oral submucous fibrosis.
British Journal of Cancer, 24, 253-257.

PINDBORG, J.J. and RENSTRUP, G. (1963)
Studies in oral leukoplakias. II. Effect of snuff on oral epithelium.
Acta Dermato-Venereologica, 43, 271-276.

PINDBORG, J.J., RENSTRUP, G., POULSEN, H.E. and SILVERMAN, S. (1963)
Studies in oral leukoplakias. V. Clinical and histologic signs of malignancy.
Acta Odontologica Scandinavica, 21, 407-414.

PINDBORG, J.J., ROED-PETERSEN, B. and RENSTRUP, G. (1972)
Role of smoking in floor of the mouth leukoplakias.
Journal of Oral Pathology, 1, 22-29.

PINDBORG, J.J., SRIVASTAVA, A.N. and GUPTA, D. (1964)
Studies in oral leukoplakias. VIII. Epithelial changes in tobacco-induced leukoplakias in India.
Acta Odontologica Scandinavica, 22, 499-512.

PINKUS, H. (1952)
Examination of the epidermis by the strip method. Biometric data on regeneration of the human epidermis.
Journal of Investigative Dermatology, 19, 431-447.

POLLIACK, A., CHARUZY, I. and LEVIJ, I.S. (1969)
The effect of oestrogen on 9, 10-dimethyl-1 2-benzanthracene (DMBA) - induced cheek pouch carcinoma in castrated and non-castrated male Syrian golden hamsters.
British Journal of Cancer, 23, 781-786.

POLLIACK, A. and LEVIJ, I.S. (1969)
The effect of topical vitamin A on papillomas and intraepithelial carcinomas induced in hamster cheek pouches with 9, 10-dimethyl-1, 2-benzanthracene.
Cancer Research, 29, 327-332.

POSWILLO, D.E. and COHEN, B. (1971)
Inhibition of carcinogenesis by dietary zinc.
Nature, 231, 447-448.

POTT, P. (1775)
Chirurgical observations relative to the cataract, the polypus of the nose, the cancer of the scrotum, the different kinds of ruptures and mortification of the toes and the feet. pp 62-68. London: Hower, Clark and Pollins.

POTTEN, C.S. (1974)
The epidermal proliferative unit: the possible role of the central basal cell.
Cell and Tissue Kinetics, 7, 77-88.

POTTEN, C.S. and ALLEN, T.D. (1975)
The fine structure and cell kinetics of mouse epidermis after wounding.
Journal of Cell Science, 17, 413-447.

PROTZEL, M., GIARDINA, A.C. and ALBANO, E.H. (1964)
The effect of liver imbalance on the development of oral tumours in mice following the application of benzpyrene or tobacco tar.
Oral Surgery, Oral Medicine and Oral Pathology, 18, 622-635.

QUASTLER, H. (1960)
Cell population kinetics.
Annals of the New York Academy of Sciences, 90, 580-591.

QUASTLER, H. and SHERMAN, F.G. (1959)
Cell population kinetics in the intestinal epithelium of the mouse.
Experimental Cell Research, 17, 420-438.

RAMULU, C., RAJU, M.V.S., VENKATARATHNAM, G. and REDDY, C.R.R.M. (1973)
Nicotine stomatitis and its relation to carcinoma of the hard palate in reverse smokers of chuttas.
Journal of Dental Research, 52, 711-718.

REISKIN, A.B. and BERRY, R.J. (1968)
Cell proliferation and carcinogenesis in the hamster cheek pouch.
Cancer Research, 28, 898-905.

RENSTRUP, G. (1963)
Studies in oral leukoplakias. IV. Mitotic activity in oral leukoplakias.
Acta Odontologica Scandinavica, 21, 332-340.

RENSTRUP, G. (1970)
Occurrence of candida in oral leukoplakias.
Acta Pathologica et Microbiologica Scandinavica B., 78, 421-424.

RENSTRUP, G., SMULOW, J. and GLICKMAN, I. (1962)
Effect of chronic mechanical irritation on chemically induced carcinogenesis in the hamster cheek pouch.
Journal of the American Dental Association, 64, 770-777.

ROFFO, A. (1930)
Leucoplasie expérimentale produite par le tabac.
Revue Sudamér Médicale Chirurgie, 1, 321-330.

ROGERS, A.W. (1973)
Techniques of Autoradiography. 2nd Edition. Amsterdam: Elsevier Scientific Publishing Company.

ROSIWAL, A. (1898)
Ueber geometrische Gesteinsanalysen. Ein einfacher Weg zur ziffernmassigen Feststellung des Quantitatsverhaltsisses de Mineralbestandteile gemengter Gesteine.
Verh. K.K. geol. Reichsanst. 5-6, 143-175, cited by Weibel 1969.

ROTHMAN, S. (1954)
In Physiology and Biochemistry of Skin. pp 377.
Chicago: University of Chicago Press.

ROWE, N.H. and GORLIN, R.J. (1959)
The effect of vitamin A deficiency upon experimental oral carcinogenesis.
Journal of Dental Research, 38, 72-83.

RWOMUSHANA, J.W., POLLIACK, A. and LEVIJ, I.S. (1970)
Cervical lymph node metastasis of hamster cheek pouch carcinoma induced by DMBA.
Journal of Dental Research, 49, 184.

SABES, W.R., CHAUDHRY, A.P. and GORLIN, R.J. (1959)
The effect of tween-80 and prednisolone acetate on chemical carcinogenesis in the hamster cheek pouch.
Journal of Dental Research, 38, 713.

SABES, W.R., CHAUDHRY, A.P. and GORLIN, R.J. (1963)
Effects of cortisone on chemical carcinogenesis in hamster pouch and submandibular salivary gland.
Journal of Dental Research, 42, 1118-1130.

SALAMAN, M.H. and ROE, F.J.C. (1964)
Co-carcinogenesis.
British Medical Bulletin, 20, 139-144.

SALLEY, J.J. (1954)
Experimental carcinogenesis in the cheek pouch of the Syrian hamster.
Journal of Dental Research, 33, 253-262.

SALLEY, J.J. (1955)
Effect of mineral oil as a solvent for 9, 10-dimethyl-1, 2-benzanthracene.
Journal of Dental Research, 34, 723.

SALLEY, J.J. (1957a)
Histologic changes in the hamster cheek pouch during early hydrocarbon carcinogenesis.
Journal of Dental Research, 36, 48-55.

SALLEY, J.J. (1957b)
Experimental oral carcinogenesis. In Proceedings of 25th Year Celebration of Dental Research Fellowship Program. pp 132. New York: University of Rochester.

SALLEY, J.J. (1961a)
Epithelial carcinogenesis in the absence of accessory structures.
Oral Surgery, Oral Medicine and Oral Pathology, 14, 1478-1484.

SALLEY, J.J. (1961b)
Penetration of carcinogenic hydrocarbons into oral tissues as observed by fluorescence microscopy.
Journal of Dental Research, 40, 177-184.

SALLEY, J.J., ESHLEMAN, J.R. and MORGAN, J.H. (1962)
Effect of chronic thiamine deficiency on oral carcinogenesis.
Journal of Dental Research, 41, 1405-1413.

SALLEY, J.J. and KRESHOVER, S.J. (1959)
The effect of topical application of carcinogens on the palatal mucosa of the hamster.
Oral Surgery, Oral Medicine and Oral Pathology, 12, 501-508.

SCHROEDER, H.E. (1970)
Quantitative parameters of early human gingival inflammation.
Archives of Oral Biology, 15, 383-400.

SCHROEDER, H.E. and GRAF-DE BEER, M. (1976)
Stereologic analysis of chronic lymphoid cell infiltrates in human gingiva.
Archives of Oral Biology, 21, 527-537.

SCHROEDER, H.E. and MÜNZEL-PEDRAZZOLI, S. (1970)
Application of stereologic methods to stratified gingival epithelia.
Journal of Microscopy, 92, 179-198.

SCHROEDER, H.E. and MÜNZEL-PEDRAZZOLI, S. (1973)
Correlated morphometric and biochemical analysis of gingival tissue: morphometric model, tissue sampling and test of stereologic procedures.
Journal of Microscopy, 99, 301-329.

SCHROEDER, H.E., MÜNZEL-PEDRAZZOLI, S. and PAGE, R. (1973)
Correlated morphometric and biochemical analysis of gingival tissue in early chronic gingivitis in man.
Archives of Oral Biology, 18, 899-923.

SHAFER, W.G. and WALDRON, C.A. (1961)
A clinical and histopathologic study of oral leukoplakia.
Surgery, Gynecology and Obstetrics, 112, 411-420.

SHARAV, Y. and MASSLER, M. (1967)
Age changes in oral epithelia. Progenitor population, synthesis index and tissue turnover.
Experimental Cell Research, 47, 132-138.

SHEAR, M. (1972)
Erythroplakia of the mouth.
International Dental Journal, 22, 460-473.

SHKLAR, G. (1965a)
The precancerous oral lesion.
Oral Surgery, Oral Medicine and Oral Pathology, 20, 58-70.

SHKLAR, G. (1965b)
Metabolic characteristics of experimental hamster pouch carcinomas.
Oral Surgery, Oral Medicine and Oral Pathology, 20, 336-339.

SHKLAR, G. (1967)
The effect of cortisone on the induction and development of hamster-pouch carcinomas.
Oral Surgery, Oral Medicine and Oral Pathology, 23, 241-248.

SHKLAR, G. (1968)
The effect of manipulation and incision on experimental carcinoma of hamster buccal pouch.
Cancer Research, 28, 2180-2182.

SHKLAR, G., CATALDO, E. and FITZGERALD, A.L. (1966)
The effect of methotrexate on chemical carcinogenesis of hamster buccal pouch.
Cancer Research, 26, 2218-2224.

SHKLAR, G., TURBINER, S. and SIEGEL, W. (1969)
Chemical carcinogenesis of hamster mucosa: reaction to dimethyl sulfoxide.
Archives of Pathology, 87, 637-642.

SHTEYER, A. and LALONDE, E.R. (1974)
The effects of chronic irritation and dimethylbenzanthracene in various solvents on experimental carcinogenesis in the hamster cheek pouch.
Israel Journal of Dental Medicine, 23, 1-3.

SIEGEL, W.V. and SHKLAR, G. (1969)
The effect of dimethyl sulfoxide and topical triamcinolone on chemical carcinogenesis of hamster buccal pouch.
Oral Surgery, Oral Medicine and Oral Pathology, 27, 772-779.

SILBERMAN, S. and SHKLAR, G. (1963)
The effect of a carcinogen (DMBA) applied to the hamster's buccal pouch in combination with croton oil.
Oral Surgery, Oral Medicine and Oral Pathology, 16, 1344-1355.

SILVERMAN, S. and ROZEN, R.D. (1968)
Observations on the clinical characteristics and natural history of oral leukoplakia.
Journal of the American Dental Association, 76, 772-777.

SIMPSON, W.L. and CRAMER, W. (1943)
Fluorescence studies of carcinogenesis in skin. I. Histological localisation of 20-methylcholanthrene in mouse skin after a single application.
Cancer Research, 3, 363-369.

SMITH, C. J. (1967)
Effects of carcinogen and cortisone on mast cells in the hamster cheek pouch.
British Journal of Cancer, 21, 190-197.

SMITH, C.J. (1968)
Experimental carcinogenesis in the hamster cheek pouch: a histochemical investigation.
PhD Thesis. University of London.

SMITH, C.J. (1972)
Variations in histochemical properties of hydrolytic enzymes in oral precancerous conditions.
Journal of Dental Research, 51, 308-318.

SMITH, C.J. (1973)
Global epidemiology and aetiology of oral cancer.
International Dental Journal, 23, 82-93.

SMITH, C.J. (1976)
Neoplasia: oral aspects. In Scientific Foundations of Dentistry, ed. Cohen, B. and Kramer, I.R.H. pp 229-240. London: William Heinemann Medical Books Ltd.

SMITH, C.J. and PINDBORG, J.J. (1969)
Histological grading of oral epithelial atypia by the use of photographic standards. Copenhagen: C. Hamburgers Bogtrykkieri.

SPRAGUE, W.G. (1963)
A survey of the use of the term 'leukoplakia' by oral pathologists.
Oral Surgery, Oral Medicine and Oral Pathology, 16, 1067-1074.

STORMBY, N.G. and WALLENIUS, K. (1964)
Effect of reduced salivation on oral tumour induction in hamsters by 9, 10-dimethyl-1, 2-benzanthracene.
Odontologisk Revy, 15, 186-209.

SUNTZEFF, V., CARRUTHERS, C. and COWDRY, E.V. (1947)
The role of sebaceous glands and hair follicles in epidermal carcinogenesis.
Cancer Research, 7, 439-443.

TABAH, E.J., GORECKI, Z., RITCHIE, A.C. and SKORYNA, S.C. (1957)
Effects of saturated solutions of tobacco tars and 9, 10-dimethyl-1, 2-benzanthracene on the hamsters cheek pouch.
Proceedings of the American Association for Cancer Research, 2, 254.

TARIN, D. (1968)
Further electron microscopic studies on the mechanism of carcinogenesis: the specificity of changes in carcinogen-treated mouse skin.
International Journal of Cancer, 3, 734-742.

THILAGARATNAM, C.N. (1969)
Cell cycle studies during carcinogenesis in the hamster cheek pouch.
PhD Thesis. University of Edinburgh.

THILAGARATNAM, C.N. and MAIN, J.H.P. (1972)
Changes in cell cycle characteristics in hamster cheek pouch epithelium during treatment with DMBA.
Journal of Oral Pathology, 1, 89-102.

THOMA, K.N. (1941)
Stomatitis nicotina and its effect on the palate.
American Journal of Orthodontics, 27, 38-47.

VAN PROHASKA, J., BRUNSCHWIG, A. and WILSON, H. (1939)
Oral administration of methylcholanthrene to mice.
Archives of Surgery, 38, 328-333.

WALDRON, C. (1970)
In Thoma's Oral Pathology, 6th edition. ed. Gorlin, R.J. and Goldman, H.M. pp 801. St Louis: C.V. Mosby Co.

WALLENIUS, K. (1966)
Experimental oral cancer in the rat.
Acta Pathologica et Microbiologica Scandinavica (Supplement 180).

WALLENIUS, K. and LEKHOLM, U. (1973a)
Oral cancer in rats induced by the water-soluble carcinogen 4-nitrochinoline N-oxide.
Odontologisk Revy, 24, 39-48.

WALLENIUS, K. and LEKHOLM, U. (1973b)
Influence of saliva on epidermal cancer in rat induced by water - or fat - soluble carcinogens.
Odontologisk Revy, 24, 115-126.

WALKER, F., CARTER, J., CRAWFORD, G.P.M., LAIRD, H., LESSELLS, A.M. and POLLET, J.E. (1970)
Hamster cheek pouch mucosubstances and immunological privilege.
British Journal of Experimental Pathology, 51, 379-384.

WANTLAND, W.W. (1954)
Cited by Salley 1954.

WARNAKULASURIYA, K.A.A.S. (1976)
Cell proliferation in human oral mucosa.
PhD Thesis. University of Glasgow.

WATANABE, Y. (1970)
Oral cancer and its dental aetiological factors.
Transactions: Congress of the International Association of Oral Surgeons, 283-293.

WATTS, J. McK. (1961)
The importance of the Plummer-Vinson syndrome in the aetiology of carcinoma of the upper gastro-intestinal tract.
Postgraduate Medical Journal, 37, 523-533.

WEIBEL, E.R. (1969)
Stereological principles for morphometry in electron microscopic cytology.
International Review of Cytology, 26, 235-302.

WEIBEL, E.R., KISTLER, G.S. and SCHERLE, W.F. (1966)
Practical stereological methods for morphometric cytology.
Journal of Cell Biology, 30, 23-38.

WHO COLLABORATING CENTRE FOR ORAL PRECANCEROUS LESIONS (1978)
Definition of leukoplakia and related lesions: an aid to studies on oral precancer.
Oral Surgery, Oral Medicine and Oral Pathology, 46, 518-539.

WILLIAMS, D.E., EVANS, D.M.B. and BLAMEY, R.W. (1971)
The primary implantation of human tumours to the hamster pouch.
British Journal of Cancer, 25, 533-537.

WOGLOM, W.H. (1926)
Experimental tar cancer.
Archives of Pathology, 2, 533-576 and 709-752.

WOODS, D.A. (1969)
Influence of antilymphocyte serum on DMBA induction of oral carcinomas.
Nature, 224, 276-277.

WOODS, D.A. and SMITH, C.J. (1969a)
Ultrastructure of the dermal-epidermal junction in experimentally induced tumours and human oral lesions.
Journal of Investigative Dermatology, 52, 259-263.

WOODS, D.A. and SMITH, C.J. (1969b)
The effects of 4-hydroxyanisole on hamster cheek pouch.
Experimental and Molecular Pathology, 10, 107-114.

WOODS, D.A. and SMITH, C.J. (1970)
Ultrastructure and development of epithelial cell pseudopodia in chemically induced premalignant lesions of the hamster cheek pouch.
Experimental and Molecular Pathology, 12, 160-174.

WYNDER, E.L. and FRYER, J.H. (1958)
Etiologic considerations of Plummer-Vinson (Paterson-Kelly) syndrome.
Annals of Internal Medicine, 49, 1106-1128.

WYNDER, E.L., HULTBERG, S., JACOBSSON, F. and BROSS, I.J. (1957)
Environmental factors in cancer of the upper alimentary tract. A Swedish study with special reference to Plummer-Vinson (Paterson-Kelly) syndrome.
Cancer, 10, 470-487.

YAMAGIWA, K. and ICHIKAWA, K. (1918)
Experimental study of the pathogenesis of carcinoma.
Journal of Cancer Research, 3, 1-29.

YAMAMURA, T., NISHIDA, Y., EDA, S., SHIMONO, M., YAMANE, H., TACHIKAWA, T., KOIKE, H., ICHIKAWA, T., YOSHIDA, M., WATANABE, O. and MATSUYAMA, H. (1975)
An experimental study of intraoral carcinogenesis in rats.
Oral Surgery, Oral Medicine and Oral Pathology, 39, 87-102.

ZEGARELLI, E.V., EVERETT, F.G. and KUTSCHER, A.H. (1961)
Familial white folded dysplasia of the mucous membranes.
Oral Surgery, Oral Medicine and Oral Pathology, 14, 1436-1443.