EXFOLIATIVE CYTOLOGY AS AN AID IN ORAL DIAGNOSIS

L. W. BAILLIE, B.D.S., University of Sydney

UNIVERSITY
OF SYDNEY
DENTAL LIBRARY

A Thesis submitted in partial
requirement for the degree of
M.D.S.

Department of Oral Surgery,
Faculty of Dentistry,
University of Sydney.

1969

ACKNOWLEDGEMENTS

This thesis is a report of original investigations carried out in the Institute of Dental Research, Sydney. I express my appreciation to the Director of the Institute of Dental Research, Mr. Robert Harris, for the opportunity and co-operation which enabled me to complete this work whilst holding appointment as Clinical Pathologist. I am grateful to Dr. David Green, of the Radiotherapy Department, Royal Prince Alfred Hospital, whose constant encouragement was of great value; Mr. Brian Rumble for preparation of the colour-prints; and Mrs. L. Mackintosh and Miss N. Paterson for their care with the typing. I am indebted to Dr. Barry Way for his support and advice throughout the past two years. Finally, I must thank all those from whom I received help and encouragement during this project and, in particular my wife, Ann.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
EXFOLIATIVE CYTOLOGY AND ITS RELATIONSHIP TO ORAL DIAGNOSIS - WITH SPECIAL REFERENCE TO MALIGNANT LESIONS. A REVIEW OF THE LITERATURE.	
History of Exfoliative Cytology	4
Applications in Use	6
Oral Cancer Statistics	11
Histological Basis and Mechanisms of Cellular Exfoliation	16
Cell maturation	17
Intercellular bridges (desmosomes) and keratohyaline granules	21
Stratum corneum	22
Cellular exfoliation	25
Morphological Characteristics of Exfoliated Cells	
Normal oral cytology	29
Abnormal oral cytology	34
Virus induced cellular changes	35
Aphthous vesicals and ulceration	37
Pemphigus-like lesions	37
Pernicious anaemia	41
Radiation induced changes	42
Hereditary benign intraepithelial dyskeratosis	42
Darrier-White Disease	43
White sponge naevus	45
Other epithelial cells in oral smears	47

Contd:

	Page.
Atypical Oral Cytology	47
Cytological Changes in Malignancy	
Morphological characteristics of individual cells	
Nucleus	52
Cytoplasm	54
Alterations in cellular size and shape	55
Inter-relationship of cells	56
Carcinogenic Changes	61
Premalignant oral lesions from the cytology viewpoint	
Leukoplakia	70
Lichen planus	77
Leukoedema	78
Erythroplasia of Queyrat	79
Sub-mucous fibrosis	79
Carcinoma-in-situ	79
Cytological Aspects of Oral Carcinoma	83
Basal cell carcinoma	83
Squamous cell carcinoma	83
Malignant melanoma	85
Oral Cytological Techniques	87
Preparation	87
Procedure (normal)	88
Collection of secretions	89
Rinsing and gargling techniques	90

Contd:

	Page.
Irrigation	91
Aspiration	91
Touch preparation	93
Sponge biopsy	93
Smears from bullae and vesicles	93
Fixation	94
Drying and melting	96
Staining	97
Additional aids	97
Schiller iodine test	97
Toluidine blue supravital staining	98
Other cytological techniques	
Acridine-orange fluorescent cytology	100
Sulfhydryl activity in exfoliated cells	104
May-Grunwald Giemsa stain	106
Grading Oral Cytological Material	106
"False positive" and "False negative"	114
The Value of Oral Exfoliative Cytology	119
Advantages of cytology	120
Accuracy and reliability of oral cytology as a cancer detection method	121
Studies on a random selection of oral lesions	124
Studies on malignant lesions	127
Discussion of published results	129
Adaptability of the cytological method	130
Limitations of oral cytology	131

Contd.

	Page.
Indications and contra-indications for oral cytological smears	133
Effect of radiotherapy on oral exfoliative cytology	
General considerations	136
Radiation changes in exfoliated cells	137
Value of smears during radiotherapy	140
The clinical value of post treatment oral smears	143
EVALUATION OF EXFOLIATIVE CYTOLOGY AS AN AID IN ORAL DIAGNOSIS - EXPERIMENTAL WORK.	
Introduction	146
Materials and methods	147
Equipment	148
Clinical Proceedure	150
Staining	152
Scanning	153
Grading of smears	154
Variation in proceedure	155
Discussion of techniques	156
Results	162
Dental patients	162
Discussion	172
Establishment of an oral cytology service	176
Presentation of radiotherapy cases involved in the project	180
Discussion	186
Pilot Study of effects of radiotherapy on oral cytology	187
Discussion	195

Contd:

	Para.
Conclusion	200
ATLAS OF EXFOLIATED CELL TYPES	203
Bibliography	230

LIST OF ILLUSTRATIONS

Fig. 1. Herpes simplex.

Fig. 2. Pemphigus vegetans.

Fig. 3. Pemphigus vegetans.

Fig. 4. Familial benign chronic pemphigus.

Fig. 5. Hereditary mucosal syndromes.

Fig. 6. Typical field of normal gingival cells. X 240.

Fig. 7. Normal oral squame folded to resemble a tadpole cell. X 2,400.

Fig. 8. Inner layer parabasal cell. X 2,400.

Fig. 9. Dividing parabasal cells. These cells are not common in oral smears. X 2,400.

Fig. 10. Phagocytic histiocyte. X 1,008.

Fig. 11. Field of small histiocytes and polymorphs around degenerate keratinised cells. The vague cellular outline and vacuolated consistency of the histiocytes is obvious. X 1,008.

Fig. 12. Leptotrichia organisms are not infrequently found, particularly in smears from the tongue. X 1,008.

Fig. 13. Smears from an oral aphthous ulcer. The cells are fairly normal, although there may be some tendency for increased cell size and vacuolisation. X 1,008.

Fig. 14.) Multinucleated cells with vague cell borders and
Fig. 15.) irregular shape, in a smear from herpes simplex. X 1,008.

Fig. 16. Partially acantholytic cells from an oral bulla. The patient has recurring lesions suggestive of a benign pemphigus. X 1,008.

Fig. 17. Duct formation of ectopic gland cells in a lesion from the floor of the tooth. X2,400.

Fig. 18.) Minor salivary gland cells in smears from a traumatic ulcer
Fig. 19.) in the floor of the mouth. Histological section of a biopsy taken at the same appointment showed salivary glands very close to the surface. Note the long rectangular shape of the gland cells, with foamy vacuoled cytoplasm but definite cell outline. The nuclei are well preserved and of uniform consistency. X 1,008.

Fig. 20. Cluster of 'contaminate' respiratory tract cells in an oral smear. These cells probably originate from the nasopharynx and are found only occasionally. X 1,008.

Fig. 21. A common field from an acute oral ulceration. X 240.

Fig. 22.) Small cells with mature cytoplasm but large nuclei,
Fig. 23.) such as these, may be seen in inflammatory oral lesions, especially in non-cornified mucosa, such as in the cheek and soft palate. X 1,008.

Fig. 24.) Intermediate and superficial cells with large, fairly
Fig. 25.) dark nuclei in smears from benign acute buccal erosions. The nuclei, however, are uniform in size and shape and do not display clumps of chromatin, although nucleoli are often prominent. These lesions heal quickly after removal of irritants. X 1,008.

Fig. 26. Severe epithelial hyperplasia and inflammatory reaction to chronic irritation of the hard palate. The irritant in this case was a vacuum chamber in the palate of a maxillary denture; when the patient stopped wearing the prosthesis, the lesion slowly healed. H. & E. stain. X 300.

Fig. 27. Abnormal mitotic activity in epithelium around a traumatic ulcer in the lower labial sulcus. H. & E. stain. X 300.

Fig. 28. Malignant cells in a cytological smear from an oral carcinoma. These 'third type' cells, which characterise carcinoma in situ in vaginal smears, are not usually found in oral smears. X 1,008.

Fig. 29. Malignant cells in a smear from a very superficial erosive carcinoma of the tongue. The cells here are more typical of an oral lesion, in the degree of keratinisation, but it can be noted that the nuclei in the cells in both Figs. 28 and 29 show chromatin clumping and lack of uniformity in size and shape. X 1,008.

Fig. 30. Early erosive squamous carcinoma. H. & E. stain. X 300.

Fig. 31. Well differentiated squamous cell carcinoma of oral mucosa. H. & E. stain. X 300.

Fig. 32. Section showing acanthosis and parakeratosis in epithelium of the soft palate, as a result of pipe smoking. H. & E. stain. X 300.

Fig. 33. "True" leukoplakia in oral epithelium showing hyperkeratosis, some dyskeratosis and inflammatory reaction in stroma. H. & E. stain. X 300.

Fig. 34. Sheet of parakeratosic cells from a non-malignant oral white patch. X 1,008.

Fig. 35. Cell showing aperinuclear vacuole. These may be seen in smears from oral white lesions but are not typical of leukoplakia. X 1,008.

Fig. 36.) Degenerating cells in smears from chronic leukoplakia.
Fig. 37.) X 1,008.

Fig. 38. A group of superficial cells showing large well preserved nuclei, from an irregular elevated white lesion, resulting from a chronic candidiasis. X 1,008.

Fig. 39. Cells showing dyskaryotic features (large hyperchromatic, somewhat irregular nuclei surrounded by adequate cytoplasm) from chronic verrucous leukoplakia of the buccal mucosa. X 1,008.

Fig. 40. Large, hyperchromatic, irregular nuclei in a sheet of superficial cells from an encrusted, squamous carcinoma of the lip. Benign white lesions frequently shed clumps of cells with well preserved nuclei, but none as large, irregular and black as these (compare with Fig. 32). X 1,008.

Fig. 41. Smear of superficial cells from an oral carcinoma showing typical malignent nuclei. In some cases, lightly taken superficial smears may be of greater value than deeper scrapings. X 1,008.

Fig. 42. Smear from a deep scraping of the same lesion as Fig. 41 showing inflammatory cells and exudate, which tend to obscure the epithelial elements. X 1,008.

Fig. 43. Keratin pearl from an oral squamous cell carcinoma. X 1,008.

Fig. 44.) Malignant oral carcinoma cells. X 1,008.
Fig. 45.)

Fig. 46. 'Free' nucleus in a malignant oral smear. X 1,008.

Fig. 47. Low power field of prolific numbers of malignant cells. During the initial stages of radiotherapy, oral carcinomata tend to show greatly increased exfoliation. X 240.

Fig. 48. Cells from irradiated normal oral mucosa appear normal cytologically but tend to be increased in size, show vacuolisation and somewhat pyknotic nuclei. X 1,008.

Fig. 49. A folded multinucleated giant cell with vacuoles in a smear from irradiated oral tissue. X 1,008.

Fig. 50. Cell showing marked vacuolisation in response to radiotherapy. X 1,008.

Fig. 51. Bizarre cellular shapes resulting from radiotherapy to oral tissues. X 1,008.

Fig. 52. Low power field of aggregates of keratinised cells with fairly large nuclei from a non-malignent radionecrotic ulcer. Cytological smears reliably reflect recurrent malignancy in irradiated oral tissue by showing cells with definite malignant tendencies, even prior to clinical evidence. The atypia seen here is insufficient to indicate recurrent disease. X 240.

Fig. 53. Section of an encrusted squamous cell carcinoma of the lower lip. Obviously, smears of superficial cells of such lesions are of very little value. X 300.

Fig. 54. Low power field of cellular debris in a smear from an oral carcinoma covered by necrosis. The degenerate cells here give no indication of the nature of the underlying tumour. X 240.

Papanicolaou stain used in all colour photomicrographs.

INTRODUCTION

Exfoliative cytology has developed over the past twenty years into one of the most important diagnostic sciences in medicine. In its various forms and applications it is the basis of many papers and the literature concerning the use of exfoliative cytology in the oral cavity is extensive. In view of this, the lack of serious interest shown in this technique by oral diagnosticians is puzzling. There appears to be a general scepticism that any possible value can be achieved from studying superficial mucosal cells when the changes of real importance are taking place in the deep layers of epithelium.

A further observation also becomes apparent with increasing experience in the management of oral soft tissue lesions, and that is the infrequency with which biopsies are performed to confirm a somewhat subjective diagnosis or to check on the progress of a persistent lesion. Obviously, biopsy is contra-indicated in certain circumstances and undesirable in many others, so that routine biopsy procedure for every soft tissue lesion is impossible. Often oral carcinoma is not detected until the lesion is advanced and clinically obvious as such. The implications

of this fact seems to be that, either the patient is completely unaware of the lesion and, therefore, has sought no advice at any time, or else it has been seen and misdiagnosed. With respect to the former, it is doubtful that this occurs as often as has been claimed. Undoubtedly, many oral neoplasms are painless but most are also usually elevated or erythematous, ulcerated, and indurated, thus causing bleeding or restriction of function, which may be noticed by the patient. However, the fact that an oral lesion is unnoticed by the patient for a considerable length of time, suggests a very good reason for regular professional dental and oral examination of people of all age groups, whether edentulous or not.

There is a need, then, for an oral diagnostic aid to supplement biopsy and it would appear that exfoliative cytology could satisfy this need. In order to assess the value of this aid, an oral cytology service was established, firstly, among dental patients; and secondly among patients with an oral malignancy currently under treatment, or about to undergo treatment, or with a history of oral malignancy.

This thesis presents a review of the literature concerning oral cytology; the results of an oral cytology service instituted by the author and demonstrating the

diagnostic value of oral exfoliative techniques, and an atlas of photomicrographs of exfoliated cell types from oral lesions.

EXFOLIATIVE CYTOLOGY AND ITS RELATIONSHIP TO ORAL DIAGNOSIS - WITH SPECIAL REFERENCE TO MALIGNANT LESIONS - A REVIEW OF THE LITERATURE

History of Exfoliative Cytology

The first record of investigation of exfoliative material was made in 1847 by Pouchet[111], who noted monstrual variations in unstained vaginal smears (McGrew, 1961)[78]. In 1851, Herman Lebert[73] suggested the diagnostic value of cells exfoliated from malignant lesions (Sandler et al., 1960)[121] and in 1860 malignant cells were described by Beale[10] from the sputum of a patient with carcinoma of the throat (Stahl and Wiman, 1962)[152]. Tissue fragments had been noted in sputum from cases of respiratory tract cancer since 1841 (Walsh)[180].

After Sanders[118] in 1864 recorded finding cancer cells in urinary sediment, great interest was aroused about the diagnostic possibilities of cytology of body fluids (McGrew, 1961)[78]. In 1890, Miller[80], in a study of oral microorganisms, described epithelial cells and polymorph leucocytes in saliva (Montgomery, 1951)[83]. However, with the development, around the turn of the century, of improved techniques in histopathology, cytological techniques were neglected[78].

In 1917 the first of a series of articles by Stockart and Papanicolaou[158] appeared on cyclic variations in smears from the female genitalia. The value of vaginal smears as a diagnostic aid for uterine cancer was shown by Papanicolaou and Traut in 1941[95], and the Papanicolaou staining technique soon became widely adopted. The basic textbook of cytology — Papanicolaou's "Atlas of Exfoliative Cytology" — was first published in 1954[97].

Interest in oral cytology was renewed in 1940 when Weinman[185] applied various cytological techniques to study the keratinisation of oral mucosa. In 1949, Morrison, Hopp and Wu[88] used cytological smears in the diagnosis of nasopharyngeal neoplasms, with excellent results. They felt, however, that cytology was not a substitute for biopsy and to be reliable demanded of the cytologist complete knowledge of normal cells, attention to detail and experience with oral smears (Von Haam, 1965)[55]. The simplicity of the smear technique somewhat thwarted the development in 1950 of Gladestone's sponge biopsy technique[45] as an oral diagnostic aid. Montgomery and Von Haam[83,84,85] presented a series of 3 articles in 1951 on the exfoliative cytology of oral mucosa in health, leukoplakia and malignancy. Since that year much has been written on the subject,

Applications in the Use of Exfoliative Cytology

The chief application of exfoliative cytology generally, is for the detection of malignancy, whether directly from tissue surfaces or indirectly via body fluids, sputum specimens or lavage (Graham, 1964 - Chap. 31)[52]. It is particularly suited to regions of the body where natural access for adequate examination or biopsy is poor, hence it has become intensively used in detection of gynaecological and respiratory carcinoma, and is of great value in otorhino laryngology where several areas susceptible to malignancy are hidden (Stahl and Wiman, 1962)[152]. The differences in numerical cell counts in cervical smears from normal individuals and patients with cancer, has been reported as being highly significant and could serve as the basis of population screening (Folles, Horvath and Bostrom, 1961)[169].

Montgomery and Von Haam[85] saw the value in the application of exfoliative cytology to oral lesions in 1951, although the efficacy of mass screening of the mouth has been discounted (Selbach and Von Haam, 1963)[131]. Shapiro et al. (1964)[134] state that the recognised advantages of cytological screening in uterine cervix, where most carcinomas develop in a junctional zone between columnar and stratified squamous epithelium, cannot be properly applied to the extensive oral mucosa, almost any

part of which may develop malignancy.

Cervical cancer, unlike oral cancer, usually has a long *in situ* stage where the lesion is superficial and desquamating, rather than the early infiltration typical of oral malignancy (Hedak *et al.*, 1967)[79], (Selbach and Von Haam, 1963)[131]. Furthermore, some oral tumours may be covered by a thick keratinised layer of crust, which prevents exfoliation of diagnostic cells (Selbach and Von Haam, 1963)[131], (Shapiro, Gorlin and Jordan, 1964)[134]. In contrast, however, Helsper and Sharp (1964)[59] advocate the routine use of rinsing and gargling cytology techniques to detect early malignancy in all patients over 50 years of age. A similar technique was also found valuable in another study of 159 cases (83 normal and 76 pathological) (Watanabe and Shiraishi, 1961)[181].

Virtually all workers in the field have found cytology valuable as a cancer detection method in numerous oral situations, such as examination of clinically innocuous lesions (Sandler, 1962)[123], (Shapiro, Gorlin and Jordan, 1964)[134], (Silverman and Ware, 1960)[141]; (Screening of extensive lesions (Gardner, 1965)[43], (Shapiro, Gorlin and Jordan, 1964)[134], (Silverman and Ware, 1960)[141], or follow-up of patients recently treated for head or neck cancer

(Sandler et al., 1960)[121], (Shapiro, Gorlin and Jordan)[134]. Silverman, Becks and Farber (1958)[139] state that cytology is an excellent adjunct to biopsy.

The application of cytological techniques to the investigation of material from fistulae or sinus tracts has been found of value in the present project, as was found by others (Sandler, Freund and Stahl, 1959)[120]. Usually such tracts are the result of infection but in some patients, especially in those who have been previously treated for a carcinoma, the infective process may be associated with residual or recurrent neoplasm. A quick means of getting cytological diagnosis, for example, during surgery, has been suggested (Pavla Nevak, 1967)[99] and could be applicable to special situations in oral surgery.

The applicability of the technique in dental practice for the early recognition of oral cancer is clear. In a Japanese study it was found that 56 per cent of patients with oral cancers (including some with sarcoma) were first seen by the dentist (Watenabe et al., 1962)[182]. The necessity for continuing the education of the dental profession in the value of cytology and early oral cancer detection was brought out by Stahl et al. (1967)[156],

who provided dental practitioners in New York with a cytology service for three years.

Oral cytology has been found valuable and reliable in animal experiments on carcinogenesis, especially using the hamster buccal pouch, where cytology will reflect the progress of the lesion, particularly in the early stages (Levij, Pollak and Thorgeirsson, 1967)[75], (Stahl, 1963)[154].

Exfoliative cytology is not limited generally or in the oral situation to playing a supporting role in the detection of malignancy. Study of the pigmentation of human epidermis has been facilitated by exfoliative cytology (Kligman, 1968)[70]. The diagnosis of ophthalmic lesions such as allergic inflammation, bacterial, fungal and viral infections, keratinisation, papilloma, as well as dyskeratosis and malignant lesions, may be guided by cytology with reasonable accuracy. Naib, Clepper and Elliot (1967)[90] studied cytologically the effects of respiratory viruses on respiratory tract cells, and noted the consistent finding of true or syncytial giant cells and intra-nuclear or intra-cytoplasmic inclusion bodies. Intra-cytoplasmic inclusions were observed (with loss of cilia) in para-influenza virus infection; and in adenovirus, cytomegalo-virus and herpes simplex

infections the cellular inclusions were intra-nuclear. These workers claim that the cellular changes are more clearly seen in exfoliated cells than in tissue section and conclude that the cytological examination of repiratory cells may be of definite value in the diagnosis of respiratory infections (1968)[91].

The importance to newly born infants of the recognition of viral cellular changes in smears from prenatal mothers has been stressed (Nowakovsky et al., 1968)[94]. Herpes genitalis produces ballooning degeneration of cells, multi-nucleated giant cells, intra-nuclear eosinophilic inclusion bodies and multi-nucleated syncytia of cells in vaginal smears.

Similar diagnostically significant effects are seen in oral smears from herpetic and other viral lesions of the mouth (Cooke, 1958)[29]. Pathognomonic cellular changes in exfoliated cells have been established in smears from oral pemphigus, white sponge naevus, benign hereditary intra-epithelial dyskeratosis and Darrier's disease (Ficke and Blozis, 1966)[168], (Feathers and Griffin, 1963)[183]. Furthermore, it is likely that study of epithelial cells in aspirated fluids from mandibular and maxillary cysts can give an early indication of the nature of the lesion.

The effects, cytologically, of dentures on palatal and buccal mucosa have been studied by Suad et al. (1966)[166], who suggests that the exfoliative method can indicate the effects of different impression techniques, denture materials and denture adaption.

Finally, the use of buccal smears in the determination of chromosomal sex in individuals is well known (Fleke and Blozis, 1966)[168]. The hetero-chromatic x-chromosome may be seen as a small, well-defined body (Barr body) on the linear surface of the nuclear membrane during interphase of cells from female buccal smears. It is rarely seen in male buccal cells, but is present in 20-30 per cent of cells from the female cheek (Silverman, 1965)[144].

Oral Cancer Statistics

Oral carcinoma is a serious problem and presents an unique challenge to dental practice. Failure to recognise malignancy during routine oral examination may be disastrous for the patient, and detrimental to the clinician, but early detection of such lesions is a highly rewarding health service. The incidence of oral cancer varies with different parts of the world, being, for example,

2-2.7 per cent of all malignant tumours in Canada, but 31.9-47 per cent in India (Saxena and Allt, 1967)[127]. It has been suggested that cancers of the mouth and lip constitute 10 per cent of all malignancies in men (Weathers and Griffin, 1963)[183]. In Victoria (Australia) between 1961 and 1965, there were 300-350 cases per year, approximately 12 per 100,000 head of population, this constituting 3 per cent of all malignant disease (Ames, 1967)[2]. In areas of Australia further north, where there is increased actinic radiation, the incidence would be higher. Tan (1967)[165] recorded 5,754 cases over a 6-year period throughout Australia, representing 9.1 per 100,000 head of population. It is probable that these figures are somewhat less than the actual incidence of oral cancer, as they were obtained from major hospitals, but obviously it is impossible to assess the number of cases that were treated by local excision (usually squamous cell carcinoma of the lip) in private medical practice or private hospitals. However, on the basis of Tan's study, the average annual incidence of oral carcinoma (New South Wales) is approximately 119 cases, although between 1959 and 1964 there has been a general increase in rate per 100,000 population of patients with oral cancer. The

mortality rate from oral malignancy in the United States has been recorded(Gardner, 1965)[143] as:-

3.19 per 100,000 head of population in 1936;

2.0 per 1,000,000 head of population in 1950;

3.4 per 1,000,000 head of population in 1960.

Tan (1967)[165] has recorded information from the Commonwealth Bureau of Consus and Statistics in Canberra, which indicates that over 5 years from 1959, 996 persons in Australia died from oral cancer, giving a mortality rate of 1.6 per 100,000 population per year.

Prognosis

The prognosis for patients with oral cancer depends on a number of factors such as, extent of the lesion, anatomical site, presence of metastasis or multiple primary lesions, age and general health of patient. Obviously, the more advanced the lesion, the greater the difficulty of adequate treatment, the greater chance of metastasis and the poorer the prognosis. Since the advent of the vaginal smear in Greece, there has been an 13 per cent drop in mortality rate from uterine cancer, but during the same period a 16 per cent increase in mortality rate from oral cancer (Spengas, 1967)[151]. A number of studies (Amies, 1967)[2], (Gardner, Hamburger and Love, 1963)[41], (Sandler, 1962)[123], have recorded

the incidence of malignancy in different regions of the mouth, which have generally different prognoses, depending on the richness of lymphatic supply and hence the likelihood of metastasis. Frazell and Lucas (1962)[39], in a series of 1,554 cases of cancer of tongue, stressed the bearing on prognosis of anatomical situation of the lesion, whether it is on the anterior third or posterior third of the tongue. The incidence of metastasis from squamous cell carcinoma in the floor of the mouth has been recorded as 30-35 per cent on first examination (Sandler, et al., 1960)[121]. Metastases were found in 54.4 per cent of cases of oral cancer in Gardner's study (1965)[43]. More recently, Gobbel, Adkins and Sawyer (1967)[47] reported that 53 per cent of cases of carcinoma of the tongue had palpable cervical lymphadenopathy at the time of admission, and of these 70 per cent were confirmed metastases histologically. It is a widely held view that a person having developed one oral carcinoma, is more susceptible to the development of another, than a person who has never been so affected. The effect of this fact on prognosis was pointed out in a Swedish study recently (Einhorn and Jakobsson, 1964)[36], where it was suggested that the reasons for this could be attributed to;-

(a) the action of a carcinogenic agent in the patient causing the multiple tumours;

(b) an increased susceptibility of the patient to malignant disease before the first lesion;

(c) an increased susceptibility as a result of the first lesion.

Of the cases in the Frazell and Lucas (1962)[39] study, 11 per cent developed multiple primary tumours, of which two thirds were found in the upper respiratory or alimentary tracts. Satellite tumours, which may be inconspicuous and easily missed, are sometimes found adjacent to the main mass in oral pharyngeal or laryngeal tumours, as a result of the multicentric nature of the disease (Strong, Vaughan and Ineze, 1968)[159]. Cahn (1963)[21] refers to the same phenomenon as 'field cancerisation'. The clinical features of oral carcinoma are in the early stages often misleading, thus preliminary clinical diagnosis is only moderately reliable. In the study of 208 oral cancer cases, Sandler (1962)[123] recorded induration present in only 35.5 per cent, 30.8 per cent were firm, the rest being either soft, fluctuant, rubbery, or normal to palpation. Inconspicuous clinical appearance and absence of pain or discomfort in many cases has been

suggested as the reason that the number of oral carcinomas of less than 1 centimeter detected is extremely small (Sandler et al., 1960)[121]. Whilst it is generally true that this disease is most usually found in people of the age group 45-75 years (Amios, 1967)[2], with peak incidence in the age of 60-69 (Tan, 1967)[165], it is certainly not limited to people in this age bracket. For example, Gardner et al., (1963)[41] in a group of 189 patients, found the youngest patient to be 19 years old, and the oldest to be 86. It is interesting that a case of squamous cell carcinoma of the lower lip of a 16-year-old boy has been treated fairly recently in Sydney.

HISTOLOGICAL BASIS AND MECHANISMS OF CELLULAR EXFOLIATION

The stratified squamous epithelium lining the oral cavity is similar to that found in other regions of the body not covered with specialised epithelium. The epitholial cells of oral mucous membrane are organised into four layers from the basement membrane outwards:-

stratum basalis - (basal or germinal cell layers)
stratum spinosum - (prickle cell layers)
stratum granulosum - (granular cell layers)
stratum corneum - (superficial or keratinised cell layers).

The stratum lucidum seen in skin is usually absent in oral epithelium (Sicher and Orban, 1962, p.222)[130]. The basal layer cells have a large nucleus with little or no visible cytoplasm, but as the cell is pushed into the adjacent layer, it acquires progressively more cytoplasm, appearing cytologically as the inner layer basal cell, then the outer layer basal cell (Graham, 1964, p.1)[52]. Further progression through the epithelium is associated with cell flattening and loss of roundness in shape, to appear as an angular intermediate cell. Decrease in nuclear size, which is noticeable in the intermediate cell, is more marked in the keratinised superficial cell. Basal cells are much less frequently found in smears from the mouth or upper respiratory tract, than in smears from the female genital tract, where desquamation of cells is more prolific (Graham, 1964, p.197)[52].

Since exfoliative cytology is intimately concerned with cell maturation and exfoliation, a brief review of present knowledge follows.

Cell Maturation

The keratinised squamous epithelium

The basal cells in all stratified squamous epithelium

usually form a single layer of high cuboidal cells attached by protoplasmic processes (hemidesmosomes) to the basement membrane. As the cells mature and move outwards, this attachment is lost and the cell assumed a polyhedral shape and increased size, being larger than in keratinised epitholium and appears almost devoid of "prickles" or inter-cellular bridges (Stoner and Orban, 1962, p.221)[139]. This change in shape which occurs in all stratified squamous epitholium is caused by a progressive flattening and widening, and develops such that the basal cells form an apex with their progony, the base being the superficial cells (Bowcott, 1964)[35]. In non-keratinised epithelium, the surface cells are simply flattened squames. This is the epitholium of the cheek, lips and floor of the mouth in health.

Keratinised squamous epitholium

The basal cell layer is the same here as in non-keratinised epitholium, but the intercellular bridges of prickle layer cells are much more conspicuous because the cells are more widely spaced (Stoner and Orban, 1962, p. 221)[138] As the cells further mature and move outward, basophilic keratohyaline granules appear in their cytoplasm and the cells comprise the granular layer not recognisable in non-keratinised epitholium (Chafer, Bino and Levy, 1963, p.57)[132]. Finally, the cells reach the keratinised or

horny layer at which stage their nucleus is either lost or pyknotic. In the superficial layers, the attachment between cells becomes less firm so that they are readily exfoliated as they reach the surface. Interdigitation between adjoining cell borders in these outer layers may be quite deep and extensive as a result of the increased cellular size and flattened morphology (Fleming and Grams, 1968)[38]. As the cytoplasm becomes more transparent, the nucleus contracts to a dense pyknotic chromatin mass (Silverman, Becks and Farber, 1958)[139].

The areas of the soft tissue in the mouth subject to functional trauma have been termed "masticatory mucosa", and it is these areas — gingivae, palate, dorsum of the tongue — that are normally keratinised to a greater or lessor degree in health (Sicher and Orban, 1962, p.224)[138].

Mitosis and basal cell maturation

Mitotic figures in normal tissue are characteristically restricted to the basal cell layers as is DNA synthesis in interphase nuclei (Greulich, 1964)[53]. Cell division, therefore, occurs only in this zone, hence the reason why spinous or granular layer cells are never seen to divide in normal tissue. Transitional cells have been described as occurring occasionally as a result of basal cells being

pushed out of the basal layer into a more superficial strata (Leblond et al., 1964)[72]. Mitosis of a basal cell gives rise to two daughter cells, both with some basal morphological characteristics, but only one of these migrates superficially into a higher strata where, after a greater or lesser period, it loses its basal morphology and proliferative capacity and differentiates into a more mature epithelial cell. Which of the two daughter cells migrates and which remains in the basal layer appears to be a random event (Greulich, 1964)[53]. The abnormal substitution of one type of epithelium for another not normally found in that particular location is referred to as metaplasia, and seems to indicate the pluripotency of basal epithelial cells (Bertalanffy, 1963)[12]. Under conditions of continued stress and stimulation, the basal cells may give rise to abnormal forms, rather than daughter cells of their own kind, presumably in an attempt to lay down an epithelium better suited to the particular conditions. This differentiation of basal cells into abnormal or atypical forms ceases when the stimulus is removed and the epithelium returns to normal after desquamation of the abnormal cells.

Intercellular Bridges (Desmosomes) and Keratohyaline Granules.

The structures connecting cells in the stratum spinosum recognised as prickles or intercellular bridges with the light microscope, have been identified with the electron microscope as "desmosomes" (Fleming and Orems (1968)[38]. These structures are actually present in all strata of stratified squamous epithelium serving as points of cohesion between cells and areas of insertion of tonofilaments within the cells (Wilgram, Caulfield and Hadgic (1964)[187]. In some disorders of the epithelium, e.g. lichen planus, acanthosis (thickening of the spinous layer) with intracellular oedema (Shafer, Hine and Levy, 1964, p. 87)[192], may be seen causing apparent elongation of the intercellular bridges. Acantholysis, however, results when intracellular oedema is such that the desmosomes are lost, resulting in free acantholytic cells, such as may be seen histologically in the bulla of pemphigus.

The function of the tonofilaments is not clear, but it has been suggested that they are closely associated with the keratinisation process (Wilgram, Caulfield and Hadgic (1964)[187]. In the granular layer of the epithelium the tonofilaments increase in size and appear to be

intimately related to the kerato-hyaline granules which appear in the cytoplasm of cells in this strata. Loss of desmosomes produces disorientation of the tonofilaments spatially through loss of attachment, and subsequently prevents keratinization of the cell (Wilgram, Caulfield and Madgic, 1964) [187].

Kerato-hyaline granules are basophilic structures in the cytoplasm of cells in the stratum granulosum. Their chemistry and function is not fully understood and in fact their name, given by Waldeyer in 1882, is misleading as they are neither keratin nor hyaline but probably conglomerates of tonofibrils (Rothman, 1964)[114]. Kerato-hyaline granules are found to increase markedly in hyperkeratotic tissue; and when present in large numbers, the overlying stratum corneum is usually either anucleate or contains only very degenerate nuclei, thus suggesting the granules may have a role in keratin synthesis (Silverman, 1967)[146].

Stratum Corneum.

The cells of the outer layers of keratinised squamous epitholium are comprised of an exo skeleton of rugged cell membrane and an endo skeleton of fibrous protein (Wilgman, 1964)[70], (connected by modified desmosomes (Greulich, 1964)[53]. Rothman, (1964)[114] notes that since 1880, it has been thought that the horny layer of skin is dependant on its content of lipids. He cites Swanbeck (1959) [162] who

found that a layer of lipid soft thick must surround the protein fibril in the horny cell for skin to remain normal, and that this lipid behaves abnormally in pathological keratinization (Gumbel, 1962) [163]. Under normal conditions, skin and sebaceous gland enzymes act on acetic acid to form squalene, which is converted to cholesterol in skin surface fat, or passing as squalene in sebaceous glands. (Hoffman, 1964)[114]. Between the cells is a cementing substance.

It is interesting that the protection from the effects of ultra violet light in dark skinned races was attributed, early in the century, to a greater thickness of stratum corneum. However it is now realised that there is no significant difference in thickness of this layer between light and dark skinned people in general, and the protective effect in the result simply of greatly increased melanin content (Milgam 1964)[70].

Oral keratins have not been fully identified biochemically or physically, although it is recognised that there is variation in type and degree of keratinisation from one part of the mouth to another (Sicher, 1964, p.222) [138]. (Silverman, 1967) [146]. Unfortunately, there appears to be some confusion in terms associated with keratinisation.

The following basis of terminology is used in this thesis.

Koratinised epitholium - squamous epitholium with a superficial layer of koratin that consists of anucloate koratinised cells. This is often called ortho - keratosis (Sicher, 1962, p.226)[138].

Cornified epitholium - Normal squamous epitholium with a superficial layer of nucleated fully keratinised cells. Also known as Parakeratosis, (Shafer, 1963)[132]. (Sicher, 1962)[138]; although in this thesis this term is usually used when superficial cells in hyper keratotic lesions are nucleated. Hyper keratosis refers to abnormal increase in thickness of the keratin layer, with or without parakeratosis.

The functional significance of the structual differences in normal and leukoplakic epithelium, or for that matter of the different degrees of keratinization in various parts of the mouth, is not fully understood (Silverman, 1967)[146].

It has been hypothesized (Van Scott,1964)[176] that basal cell carcinoma represents cells with a deletion of the keratinisation factor present in normal squamous cells, making the cells less antigenically active, hence the absence of inflammatory cell infiltrate around early basal cell carcinoma in histological sections. In contrast, squamous carcinoma cells constitute an increased potential for keratinisation, making the cell antigenically more active than normal tissue, thus explaining the frequent histological finding of inflammatory reaction around early squamous cell carcinoma.

Whereas normal basal cell division gives rise to cells which differentiate into non-dividing higher cell forms, mitotic division of malignant basal cells results in similar cells which retain the ability to divide and may be differentiated to a greater or less extent. (Bertalanffy,1963)[12].

Cellular Exfoliation.

As cells in the stratum basale are continually multiplying and differentiating, exfoliation of superficial cells is occurring simultaneously, thus maintaining constant thickness of epithelium. Bullough, (1962)[19] produced evidence for the existence in health, of a regulatory mechanism in the form of a mitosis - inhibiting local tissue hormone which

controls this process of cell renewal and exfoliation in skin. The level of this hormone influences whether a newly formed cell will undergo mitosis or differentiate into a keratinised cell. Systemic control of epidermal mitosis and exfoliation occurs to some extent through the action of thyroid hormones which accelerate, and adrenal cortical steroids which inhibit the process. (Rothman, 1964)[114].

The fact that exfoliative specimens frequently contain greatly increased numbers of cells suggests that at times the desquamation rate is increased, and it is probable that under adverse conditions the mitotic activity is increased. (Bertalanffy, 1963)[12].

The rate of cell desquamation is difficult to calculate accurately due to the enormous numbers of cells that are daily being shed. However it is generally assumed that for each new cell formed another cell is exfoliated in normal adult tissue (that is, where growth has stopped), thus maintaining equilibrium. (Bertalanffy, 1963)[12]. Since renewal time also gives an indication of the average life span of the cells, various methods of investigating cell renewal have been developed. <u>The Colchicine Technique</u> is used in animal studies where the drug is injected into the animals a certain time before sacrifice. The effect of the drug is to arrest mitotic activity at the metaphase. After sacrifice, counting of metaphases in a specimen of tissue

with low mitotic activity is carried out in various animals sacrificed at different times throughout a 24 hour period. From this, percentage numbers of multiplying cells and hence turnover Times and cellular life spans can be calculated. Radioactive tracers in methionine incorporated into cellular DNA prior to mitosis can be followed during its course from basal to superficial layers with radioautographs. (Bertalanffy, 1963)[12].

The turnover times in oral tissues is rapid, being 4.3 days renewal time in buccal mucosa and 4.9 days in superior surface of the tongue, these figures having been calculated from estimated daily cellular division rates of 24 percent in buccal epitholium and 20 percent in tongue epitholium, that is percentage number of cells that divide daily. (Bertalanffy, 1963)[12].

Since oral smears usually represent direct scraping, they do not only contain truly exfoliated cells, although for all intents and purposes the cells can be considered as exfoliating. Tissue that normally sheds its cells readily would be expected to yield abundant cells on gentle abrasion (Walker et al,1959)[172].

In most malignant tissue, except for some highly differentiated squamous cell carcinoma the ability to divide is maintained by the cells at all levels and the

daily mitotic rate is greater than in normal tissue, thus producing tumour proliferation. (Bertalanffy, 1963)[12]. However loss of tumour cells occurs not only by desquamation at the surface, but by tissue necrosis, particularly in rapidly growing lesions, and by deep shedding into the lymph vessels and blood stream, although this haphazard cell loss is always less than the rate of cell formation. (Bertalanffy, 1963)[12]. There is evidence that a direct correlation exists between rate of exfoliation of cells from a carcinoma and incidence of lymph node metastases. (Umiker, 1959)[172].

It would seem probable that the purposes of cell renewal are associated with resistance of the epithelium to destruction by chemical or physical or other agents. The cells are shed before they are badly damaged by external agents. Degenerative processes and pyknosis are probably a result of gradual removal of the cell from the nutrient source (blood or tissue fluid). This possibility is supported by the fact that in simple (as distinct from stratified epithelium) the cells retain their nuclei. (Bertalanffy, 1963)[12].

MORPHOLOGICAL CHARACTERISTICS OF EXFOLIATED CELLS.

The characteristics of cells found in oral smears can be discussed under four broad categories, Normal, Abnormal, Atypical and Malignant. From the point of view of simplicity, "Normal" as applied here relates to cells from healthy (normal) oral mucosa, which show no abnormalities in nuclear and cytoplasmic morphology.

This is to draw a distinction from the more commonly used connotation in cytology of "normal" being neither malignant nor atypical, (Shapiro, Gorlin and Jordan, 1964)[134]. even when these cells show abnormalities such as virus induced changes. Graham (p.324, 1964)[52] defined an atypical cell as one with neither all characteristics of a benign cell nor all the characteristics of a malignant cell.

Normal oral cytology

Cells exfoliated form normal stratified squamous epitholium of the oral mucosa, as in other regions of the body, show considerable morphological variation. Although there are greater relative numbers of keratinised cells in oral smears than gynaecological smears, normal oral exfoliated cells are similar to normal vaginal exfoliated cells. (Von Haam, 1966)[56].Montgomery in 1951[93] found it most practical to classify oral cell types in terms of

their cytoplasmic staining affinities, as basically blue, red or yellow cells. This colour differentiation roughly corresponds to the stages in the keratinisation of the squamous epithelial cell, as shown by the Papanicolaou staining method. The fully keratinised cells stain yellow, the non-keratinised cells stain blue. The maturing process of the cell is associated with an upward movement through the epithelial strata, hence classification of the exfoliated cells is also made as basal, precornified and cornified cells (Sandler et al, 1960) [121], or basal, intermediate and superficial cells (Graham, 1964)[52], which is the classification adopted in this thesis. Bowsett, 1964 [35] in a study on normal buccal cell types devised six categories, based on site of origin and staining reaction of the cells, which seems valuable for comparative studies using counting techniques for different areas of squamous epithelium or under varying conditions. However, for normal purposes in cytology laboratories classification of cells as basal, intermediate and superficial seems most usually accepted.

Nuclear degeneration may be seen in exfoliated squamous cells from all layers and produces variations in cellular morphology from that usually seen. The two forms of degeneration of the nucleus that occur are karyorrhexis and pyknosis.

In the former, the individual chromatin particles merge into amorphous blobs, of approximately equal size (Graham 1964 p.151)[52], which in the "incipient" stage may be confused with malignant chromatin clumping. Pyknosis is perhaps the more commonly seen nuclear degeneration. It is a gradual contraction in nuclear size (sometimes leaving a perinuclear vacuole (Graham 1964 p.13)[52]) and with condensation of chromatin to form a small dense hyperchromatic structureless nucleus, which may entirely disappear when the cell reaches the surface layers (Scheman, Altchuler and Wilson, 1967)[129], thus forming the "anucleate squame". (Dowsett, 1964)[35]. Nuclear degeneration, however, is a progressive phenomenon, with karyorrhexis and pyknosis being the final stages. (Graham, 1964 p.13)[52].

Basal or Parabasal (Scheman, Altchuler and Wilson, 1967)[129] Epithelial Cells are quite small compared with intermediate and superficial cells and are characterised by high nuclear/cytoplasm ratio, definite rounded cell borders small nucleoli and a centrally placed nucleus, the chromatin and nuclear background of which is fine and regular, (Graham, 1964 p.2)[52], or it may show a reticular chromatin pattern (Scheman, Altchuler and Wilson, 1967)[129]. Graham ,1964 p.1 [52] distinguishes two types of basal cell — inner and outer layer basals — on relative size of nucleus and cytoplasm. Cytoplasm is cyanophilic and stains blue.

Intermediate Cells are distinguished from basal cells by their angular cell outlines or polyhedral shape. Size varies from 25-55u according to Peters (cited by Williamson and Shapiro 1964)[188]. They are flatter and have a smaller nucleus/cytoplasm ratio, varying from 1/6 to 1/50 (Sandler et al 1960)[121]. With the Papanicolaou preparation, the cytoplasm shows differential colour tones, generally staining pink or pale blue green the basophilic intermediate cells, and orange or yellow the superficial cells which are fully mature and keratinised.

Superficial cells appear more flat and have a less dense cytoplasm than the intermediate cells, with a more pyknotic nucleus when it is present. Average size of the larger cells is 60-75u (Williamson and Shapiro 1964)[188].

The water content of superficial cells rarely exceeds 10 percent according to Schaman et al 1967[129], as the keratinisation process is accompanied by cellular dehydration. The effect of this on the degree of eosinophilia of the cells is perhaps shown by the fact that the fully keratinised superficial cells do not appear to take up eosin, and invariably stain yellow or orange with the Papanicolaou staining method. Writers have stressed that the morphological characteristics of the cells, rather than the staining reaction, should be the

basis for identification of cells (Graham 1964 Ch.28)[52] or criterion of maturity (Silverman, Becks and Farber 1958)[139] but a great value of the Papanicolaou stain is the ease with which highly keratinised elements can be instantly identified during scanning a smear.

Montgomery 1951[83] reported that differential cell counts from various parts of the mouth showed statistically significant cytological patterns. Masticatory mucosa (areas subject to functional trauma) such as the tongue, gingiva and hard palate; had cells showing acidophilia and cornification in comparison to other parts of the mouth where the cells exfoliated tend to be more basophilic (Silverman and Hare, 1960)[141]; Ageing and nutritional environment does not appear to affect this constant pattern (Miller, Soberman and Stahl, 1952)[81]. Suad-Al-Ani et al (1966)[160] studied the effects of full maxillary dentures on the cytology of the hard palate and reported that although there are differing degrees of cornification of the palate in health, in both denture wearers and non-denture wearers, in general there is a decrease in cornification under a denture. In patients with palatal irritation or hyperplasia, a lesser number of anucleate cells are found in palatal smears. It should perhaps be noted here that, although this pattern of the types of cells

exfoliate is a variation from the 'norm', the individual cells appear quite normal morphologically.

Abnormal Oral Cytology

In addition to squamous epithelial cells, smears from oral mucosa which is not clinically normal contain various numbers of non-epithelial cells — polymorphs, histiocyts, lymphocytes, plasma cells and red blood cells. Usually these cells are readily identified and are simply an indication of an infective or inflammatory process which is generally obvious clinically. Graham, 1964[52] points out that leucocytes, which always remain of constant size, are valuable in smears as a point of reference for comparing the relative size of epithelial cells whose size can be so variable. She provides considerable data for the differential recognition of the one non-epithelial cell which can cause trouble in cyto-diagnosis — the histiocyte.

The histiocyte, because it is a cell which is active, mobile and continually changing shape, appears to "mimic" epithelial cells by its variability in nuclear position, size and apparent activity. However, it is stressed (Graham, 1964, Ch.3)[52] that strict attention to morphological details makes accurate identification possible. The cellular border of the histiocyte is indistinct and may show large cytoplasmic vacuoles indicative of the cells

phagocytic properties. Large or giant histiocytes are typically multinucleated with bean-shaped or oval nuclei peripherally placed and are fairly readily identified. Small histiocytes can be differentiated from basal epithelial cells by, in most cases, an eccentrically placed nucleus and indistinct nuclear and cytoplasmic borders which contrast strongly from the sharp borders of the basal cells. Although the nucleus may appear "active" in some histiocytes by virtue of some chromatin strands, the nuclear background is always fine and evenly dispersed.

The effect of infection or inflammation in general on squamous epithelial cells in oral smears is to increase their apparent degree of keratinisation and provide many more degenerative cell forms. Smears from areas of deep ulceration naturally contain increased numbers of basal cells and lesions due to chronic irritation produce mature squamous cells; moderate hyperchromasia may sometimes be seen (Silverman, Eecks and Farber, 1958)[139]. In most cases, these changes are non-specific. However certain conditions do produce recognisable or pathognomonic cellular changes (Weathers and Griffin 1963)[183] which make oral smears valuable diagnostic aids, apart from detection of malignancy.

Virus Induced Cellular Changes

Herpetic Smear

Local epithelial degeneration and intracellular

oedoma caused by the herpes virus results in vesicle formation. Blank et al 1951 [15] and Cooke,1958[29] have confirmed Tzanck's [170] original finding (1949) that epithelial smears taken from the base of freshly broken herpetic vesicles consistently contained varying numbers of giant epithelial cells of characteristic bizarre appearance. The large swollen cells are described as undergoing "ballooning degeneration"; their nuclei may be swollen or the cells may contain many nuclei which are often clumped together, the individual lobes being structureless amorphous blobs. This typical "virus type" multinucleated giant cell is the result of amitotic division of the nuclei (Blank et al 1951)[15].

Intranuclear Lipschutz bodies are rarely seen in smears according to Cooke,1958[29] although Shafer, Hine and Levy, 1963 p.277)[132] consider these ovoid, eosinophilic homogenous bodies which peripherally displace nucleolus and chromatin, to be somewhat characteristic. Lipschutz bodies are probably the final stage of a dynamic intranuclear process of inclusion body formation, preceded by stages of nuclear swelling, chromatin margination and chromatin nucleolus replacement with a basophilic amorphous mass (Blank et al , 1951)[15].

These characteristic cytological features are common

to primary herpes simplex, herpes zoster and varicella lesions (Blank et al 1951)[15], but are not found in recurrent herpetiform eruptions or any other oral condition (Cooke, 1958)[29].

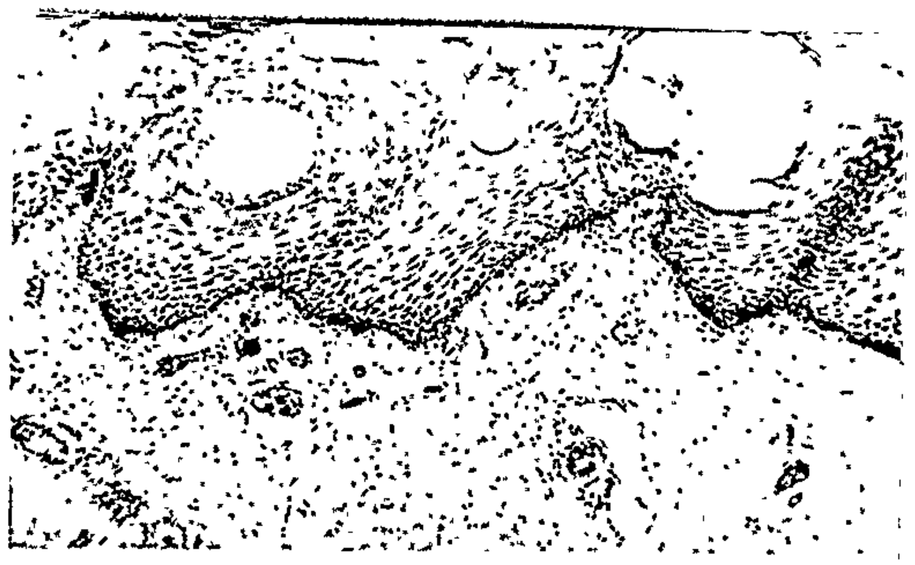
Epithelial smears from erythema multiforme lesions contain only inflammatory cells and normal epithelial cells. (Cooke, 1960)[32].

Aphthous Vesicles and Ulcerations apparently shed no cells of pathognomonic value. Shafer, Hine and Levy, 1963 p.282[132] indicate that these lesions may become secondarily infected with Vincent's organisms, making for confusion with Vincent's infection, but the basis for differentiation is purely clinical.

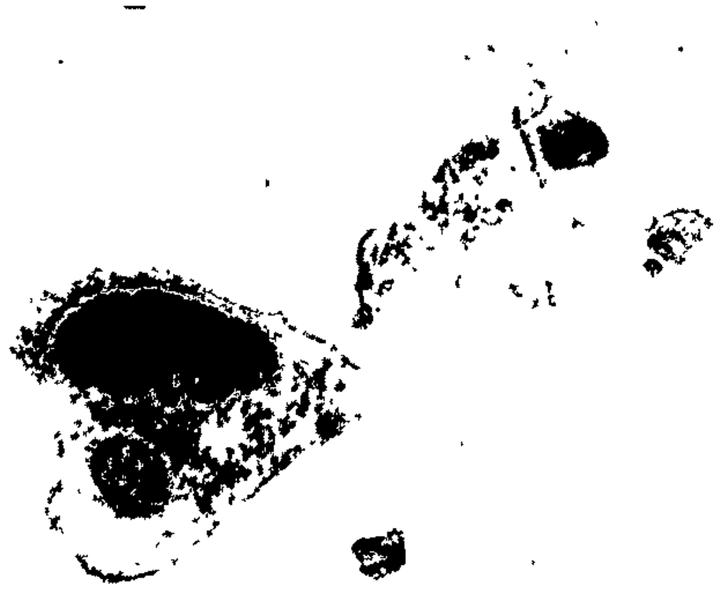
Pemphigus-like lesions

Pemphigus vulgaris.

Pemphigus is basically a dermatological disorder characterised by bullous eruptions but with oral manifestations which may appear first (Cooke, 1960)[31], (Shafer, Hine and Levy, 1963)[132]. The character of oral lesions, however, is modified by moisture, heat, trauma and secondary infection, so it is never as distinct as that of skin lesions, and may last for a much shorter time. (Cooke 1960) [31]. Cytological aids to diagnosis of intra-oral

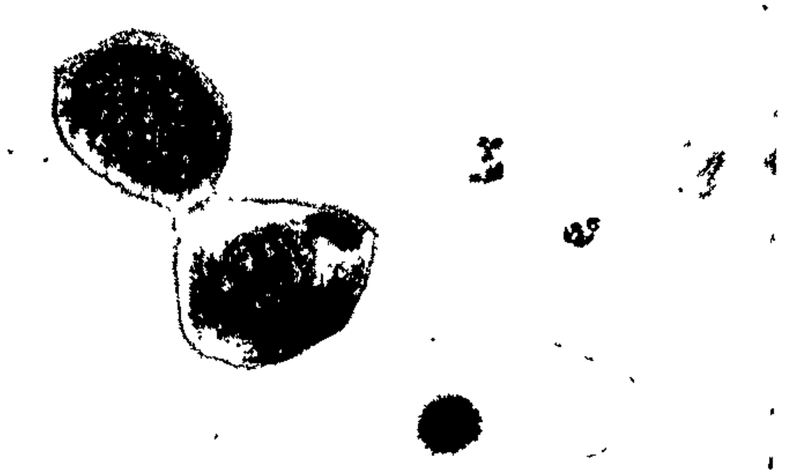


—Primary herpes simplex. Buccal mucosa. Intra-epithelial vesicle and reticular degeneration of prickle cells. H. and E. × 45.



—The lobes of the nuclei in this multinucleated balloon cell lie so close together as to give a mulberry effect. H. and E. × 548. (*Brit. dent. J.*, 1958, 104, 97.)

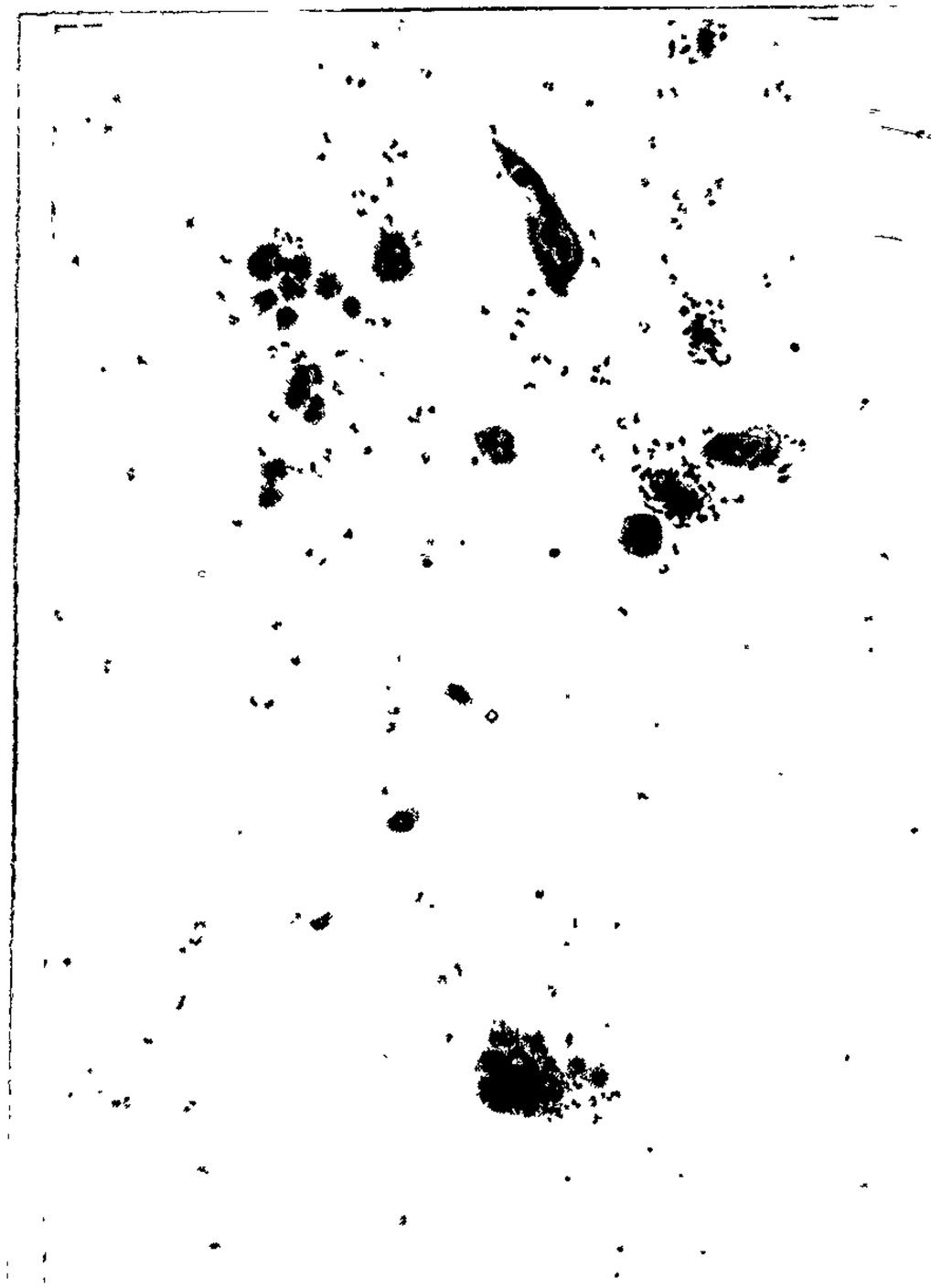
Fig. 1.
From Cooke, B.E.D., 1958.



—Pemphigus vegetans. Two acantholytic cells. H. and E. × 520.

—Pemphigus vegetans. A multinucleated epithelial cell. H. and E. × 690.

Fig. 2.
From Cooke, B.E.D., 1960.



—Pemphigus vegetans. Acantholytic epithelial cells lying singly and in clusters. H. and E. × 155.

Fig. 3.
From Cooke, B.E.D., 1960.

bullae, therefore, would seem to be most valuable. Smears made from the base of freshly opened bullae in search of diagnostic cells constitute the "Tzanck test". In the absence of secondary infection, the bullous or vesicular fluid and the underlying connective tissue has an almost characteristic scarcity of inflammatory cells in pemphigus (Shafer, Hine and Levy, 1963 p.703)[132].

Degeneration and liquefaction of epidermal prickle cells with acantholysis results in the pemphigus bulla. Characteristic acantholytic cells, which are round, contain spherical nucleus and have clear cytoplasm with an outer definite ring, can be readily seen in scrapings from the floor of the bulla (Solomon, 1965)[150]. These isolated, floating cells, in the pemphigus bulla, first described by Tzanck et al in 1949, are the result of detachment through loss of intracellular bridges between the epithelial cells in acantholysis (Cooke, 1960)[31]. Electron microscopy has shown that the loss of intercellular bridges or desmosomes in acantholysis is associated with a disorder of the keratinisation of the cell (dyskeratosis) (Wilgram, Caulfield and Madgic, 1964)[187]. As desmosomes are normally present in all parts of the stratified squamous epithelium, absence of them in any one part constitutes an abnormality. Wilgram et al [187] whose work was carried out on skin, state that, as a result of this, there are

many pathological acantholytic disorders which can be specific or non-specific according to standardised features. However, they point out, as does Cooke [31], that in pemphigus, acantholysis is strictly limited to a certain intra-epithelial zone, and that the acantholytic cells undergo such complete degeneration after destruction of the tono-filament desmosome complex that death prevents further keratinisation of the cells (Hilgram, Caulfield and Hadgic, 1964)[187].

The acantholytic cells are characteristic of pemphigus vulgaris and pemphigus vegetans. Other oral pemphigus lesions, that is pemphigus foliaceous and pemphigus erythematosus are very rare (Cooke, 1960)[31], (Shafer, Hine and Levy, 1963)[132], and therefore not subject to intensive investigation.

Familial Benign Chronic Pemphigus

Smears taken from these recurrent bullae or vesicular lesions show only partially acantholytic cells with some remnants of intercellular bridges, but no typical Tzanck cells (Cooke, 1960)[31].

Benign Mucous Membrane Pemphigus

This is a sub-epithelial lesion formed by separation of the basal epithelial layer from the corium. Smears

contain only normal epithelial cells and inflammatory cells (Cooke, 1960)[32], (Harris, 1967)[58], making cytological differentiation from the more serious pemphigus vulgaris possible.

Apart from the above, no other oral bullae or vesicular lesions produce pathognomonic cells in epithelial smears from their bases. There are, however, two other conditions in which typical abnormal squamous epithelial cells may be obtained in oral smears, these being from patients with pernicious anaemia or irradiated mucosa. Also fairly recently three hereditary diseases, all of which result in white lesions of the oral mucosa, have been reported as showing relatively characteristic cytological changes, although similar changes may be seen in smears from patients after radiotherapy or chemotherapy (Fleke and Blozis, 1966)[169].

Pernicious Anaemia

Graham, (1964 Ch. 7)[52] states that the erythrocytic cellular changes resulting from a vitamin B_{12} deficiency are accompanied by changes in squamous epithelial cells. The most marked cellular change is macrocytosis, with either a concomitant increase in nuclear size, or with many nuclei arising from amitotic division.

Nuclear chromatin distribution may also be abnormal, but the cellular changes disappear with specific therapy. (Stahl and Ulmon, 1962)[152].

Radiation induced changes.

The changes seen in pernicious anaemia are similar to those seen in cells from irradiated stratified squamous epithelium (Graham, 1964)[52]. The effects of radiotherapy on oral cytology are dealt with more fully later. Briefly, the principal changes are macrocytosis, increased multinucleation and vacuolisation of the cytoplasm.

Hereditary Benign Intra-epithelial Dyskeratosis

This hereditary condition may produce oral lesions (in addition to conjunctival lesions) which consist of a soft white mucosa of variable thickness and which may appear to fold in on itself (Tieke and Bloxis, 1966)[168]. Hilton et al 1960-61)[189, 190] have done considerable work correlating the clinical, histological and cytological findings of this Darrier White's disease and white sponge naevus, also benign hereditary conditions.

Cytology

Quantitative cell counts showed an abnormal maturation pattern, predominately red-orange staining cells.

"Tobacco cells" are characteristically found in the smear, in addition to "cell within cell" arrangements. The tobacco cells have a red-orange staining cytoplasm, with

Nuclear/cytoplasm size ratio varying from 1/2.5 to that of a cell with more nuclear fragments or even anucleate. These cells are distinct from parakeratotic cells and frequently appear as cells with oval hyperchromatic nuclei. The cell-within-cell structures appear as a rounded body resembling a precornified or cornified cell, those sickle-shaped nucleus is displaced to one side, wrapped around a tobacco cell.

Histology

The sections show hyperplasia and acanthosis, with vacuolisation of the prickle cells and intra-epithelial dyskeratosis characterised by waxy eosinophilic cells and a "cell-within-cell" pattern (Witkop and Gorlin, 1961)[190].

Darrier-White Disease

This condition, which is characterised by an external eruption of crusted papules on the skin, may also affect the oral mucosa, usually that of the hard palate (Fiske and Blozis, 1966)[168]. Shafer, Hine and Levy (p. 709)[132] indicate that mucosal lesions are generally apparent only when there are extensive skin lesions and appear as rough, minute white papules on tongue gingivae or palate.

Cytology

The quantitative cell counts in the smears showed variation from normal in types of cells present. Frequent small

.—Familial benign chronic pemphigus. Epithelial smears showing a few partially acantholytic cells loosely adherent to one another. The cell outlines are ragged suggesting remnants of prickles. H. and E. × 700.

Fig. 4.
From Cooke, B.E.D.

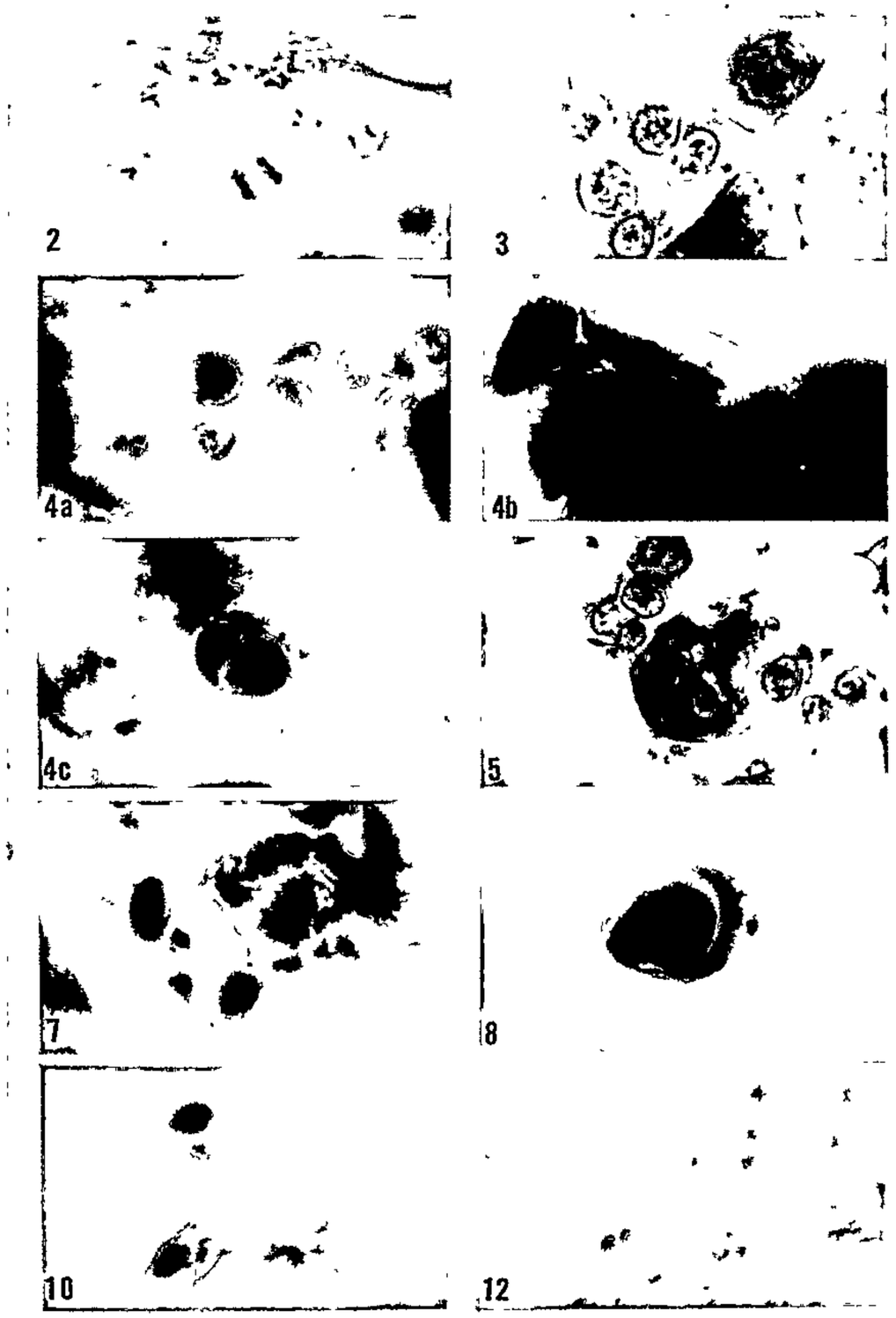


Fig. 5.
From Witkop & Gorlin, 1961

Fig. 5.
Sections 2-5 represent smears from Darier - White's Disease.

Sections 7,8 represent smears from hereditary benign intraepithelial dyskeratosis.

Section 10 represents smear from white sponge naevus.

Section12 represents smear from pachyonychia congenita.

Papanicolaou stain.

UNIVERSITY OF SYDNEY DENTAL LIBRARY

dyskaryotic-appearing cells may be seen, with blue cytoplasm, and hyperchromatic nucleus, sometimes with nuclear or paranuclear acidophilic degeneration. Increased parabasal cells are also seen. The grains seen in tissue sections are represented in smears by orange staining cells with elongated nuclei, which gave the cell a Nuclear/cytoplasm size ratio of 1:1 or 1:2. Large round bodies containing several nuclei and resembling minute epithelial pearls were seen, which consisted of a central orange cell surrounded by an inner or outer layer squamous cell more closely normal in appearance, the nucleus of which was indented and depressed to one side (Witkop and Gorlin, 1961)[190].

Histology

The epithelium shows marked acanthosis and supra-basilar lacunae lined by small epithelial cells and a benign dyskeratosis characterised by corps ronds (in the stratum spinosum) and grains (in the stratum corneum). Corps ronds are larger than normal squamous cells and are characterised by round basophilic nuclei and dark eosinophilic cytoplasm, while the grains are small, frequently elongated, parakeratotic cells (Witkop and Gorlin, 1961)[190].

White Sponge Naevus of Cannon

The oral lesions of this condition are similar to those of hereditary benign intra-epithelial dyskeratosis but are

sometimes accompanied by lesions in other mucosal surfaces, such as in the vagina (Hicke and Blozis, 1966)[168]. The oral lesions, which may be widespread, appear as thickened, folded or corrugated mucosa with a spongy texture and a peculiar white opalescent hue (Shafer, Hine and Levy, 1963 p.19)[132].

Cytology

Again, differential counts showed variation from normal buccal smears. Numerous cells showed an acidophilic or orange staining circumscribed condensation of the cytoplasm, with hyperchromatic nucleus, similar to the acidophilic dyskeratotic cells seen in tissue section. (Witkop and Gorlin, 1961)[190].

Histology

The epitholium appears thickened and acanthotic. The basement membrane was intact and there are no lacunae. Large spongy cells showing intra-cellular oedema can be seen in the epithelium of the spinous layer and above. There is little cornification of the superficial layer. Dyskeratotic cells with rigid appearing eosinophilic cytoplasm can be demonstrated throughout the epithelium. (Witkop and Gorlin, 1961)[190].

The histological picture is characteristic, though not always pathognomonic in these three lesions (Shafer, Hine and Levy, 1963 p.20)[132]. Witkop *et al* [190] indicates that the cytological features of them are distinctive and hence

can be of considerable assistance in the differential diagnosis of these conditions.

Other epithelial cells in oral smears

Occasionally, columnar or cuboidal epithelial cells may be found in oral smears. These may be cells from ectopic sebaceous glands or may originate as contaminants of nasal or pulmonary secretions (Silverman, Becks and Farber, 1958)[139], but identification is not difficult and in the case of respiratory tract cells, the presence of cilia distinguish them from adenocarcinoma cells (Graham, 1964 p.324)[52].

Atypical Oral Cytology

An atypical exfoliated cell has been authoritatively defined as one with neither all the characteristics of a benign cell nor all the characteristics of a malignant cell. (Graham, 1964 p.324)[52]. Such cells are termed dyskaryotic cells (Graham, Ch. 4)[52] when there exists a typically malignant nucleus surrounded by a normal amount of cytoplasm. This represents the extreme stage of cellular atypia, and there are numerous atypical forms with recognisable but less severe changes. King et al (1966)[69] distinguished three types of atypia, beginning with smears consistent with benign hyperkeratosis, based on the percentage number of keratinised cells present above normal limits; smears containing cells consistent with epithelial atypicality or

marked epithelial atypicality (carcinoma *in situ* — malignant change limited to epithelium). The cellular characteristics of the second group are refractile cytoplasm, nuclear enlargement and border irregularity, with some chromatin abnormality and minimum background clearing. When these changes become more definite the cells are graded in the third group, (King, Coleman and Pierce, 1966)[69] and would seem to represent typical dyskaryotic cells. In oral smears, nucleolus prominence and cytoplasm refractility are the primary feature of such cells, which tend to be seen in clumps (King, Coleman and Pierce, 1966)[69], although some (Peters and Rijsinghani, 1956)[101] claim they are usually isolated.

In addition to the above "direct" features of an atypical smear, there are cytological features, often associated with malignancy, that caused Papanicolaou (1954)[97] to call them "indirect criteria". The presence of lymphocytes and histiocytes in large numbers are often found in malignant cervical smears and not usually found in benign lesions. Those smears which contain numerous small histiocytes, in particular, should be screened with great care, as these cells are commonly seen in smears from malignant lesions, but no definite relationship has been found associated with their presence in any benign lesion (Graham, 1964)[52].

The significance of cellular atypia to the examiner, having been recognised, must be translated to the clinician in diagnostic terms with which he is familiar. It is at this point that experience, as well as familiarity with criteria, plays a dominant role, as interpretation of atypia must be associated with clinical findings. It could then be asked, what range of oral conditions produce atypical cells? Using the abovementioned grading (Ming, Coleman and Pierce, 1966)[69] it has been found that most white lesions, including some clinical leukoplakia, yield smears whose atypia is consistent with benign hyperkeratosis. Some oral inflammatory conditions produce cells with highly refractile cytoplasm and moderate nuclear hyperchromatism (Silverman, Becks and Farber, 1958)[139]. Usually, these degrees of atypia can be recognised as associated with benign lesions. In a study of the cytology of the stratified squamous epithelium of the oesophagus, it was pointed out (Stahl and Ulman, 1962)[152] that differentiation cytologically between long-standing oesophagitis and carcinoma is often extremely difficult, a fact that would seem to apply in some cases to the closely related oral epithelium. Cytology has failed to demonstrate consistently any particular atypical cell from a "premalignant lesion" that will ultimately become malignant, although such lesions as histological leukoplakia

and epithelial dysplasia do yield dyskaryotic cells. The frequent finding of dyskaryotic cells associated with malignant cells in smears from carcinoma has been stressed, (Graham, 1964, p.72)[52] and smears containing these very atypical cells are very carefully searched for typical malignant cells.

Experience after many years with the cytology of the vagina and cervix has led to a high degree of accuracy in interpreting these smears with respect to their clinical significance. Cervical smears containing only dyskaryotic cells represent epithelial dysplasia which in only 10 percent of cases will become intra-epithelial carcinoma, the others returning to normal (Graham, 1964 p.72)[52]. Bodington, Cowdell and Spriggs, 1960)[17] state that only one third of cases of "carcinoma <u>in situ</u>" of cervix will progress to invasive carcinoma, but that this third is not evident cytologically beforehand. There is a different set of influences in force in the mouth, where the mucosa is subject to oral bacterial, physical, chemical and systemic factors so that the diversity of reactions and conditions is considerably wider. For this reason, the experience gained from gynaecological cytology is not directly applicable to oral cytology, even though basic principles still apply.

Very little assistance can be gained from histology in sorting out the significance of various degrees of atypia. Shafer, Hine & Levy,(1963 p.87)[132] indicate that dyskeratosis forms the basis for deciding that an oral lesion is premalignant, but that distinction between benign and malignant dyskeratosis must be made although in some cases distinction between carcinoma in situ and dyskeratosis cannot be made. Here again the relative percentage of lesions showing dyskeratosis that will go on to invasive carcinoma is not known.

In attempting to summarise the relevance of this subject of cytological atypia then, to the clinician, it could be said that atypia can be recognised fairly accurately as benign, doubtful or suspicious, roughly corresponding to Papanicolaou's [97] classes I and III. Lesions which are dysplastic or dyskeratotic will probably shed dyskaryotic cells but are still reversible and clinical healing may take place in the face of a suspicious cytology report. A doubtful report may be given where a smear shows some feature directly or indirectly, suggesting an association with smears from malignant lesions, though obvious malignant or dyskaryotic cells are absent. This attempts to cover a smear that may have no representative cells. Doubtful or suspicious cytology is an indication for repeat smears and biopsy, or if this is not possible, at least clinical observation with repeated smears.

Cytological Changes in Malignancy.

Criteria for malignancy in exfoliated cells as been established for many years following the work of Papanicolaou (1954)[97]. It is apparent also that these criteria are applicable to all stratified squamous epithelia, (Graham 1964)[52], (Montgomery and Von Haam, 1951)[85] and are therefore the basis of recognition of malignancy in oral smears. (Sandler et al 1960)[121], (Silverman, Becks and Farber, 1958)[139]. The following is an attempt to summarize the criteria for malignancy as originally set down, (Papanicolaou, 1954)[97], and as since modified by others working chiefly with oral cytology.

Morphological characteristics of individual malignant cells

Nucleus

1. Grossly enlarged 'Nuclear/cytoplasm size ratio'. This would seem to be the most consistent cellular aberration found in oral smears.[85, 121, 139] Graham, 1964, Ch.28[52] stresses the importance in recognising the "free nucleus", one in which the cytoplasm is so small as to appear through the light microscope as non-existent.
2. Increased nuclear chromatin producing hyperchromasia. Not all malignant cells are hyperchromatic (Graham, 1964)[52] and in fact there may be as few as 9 out of 15

in oral smears, although Von Haam (1954)[54] considers this a reliable feature in cervical specimens.

3. Irregularity in chromatin distribution to form clumps or strands. Although this feature is apparently overlooked in some papers, it is repeatedly emphasized as being of great importance by Graham 1964 (Ch.3)[52], who differentiates some benign from malignant cells on the prominence of chromatin clumping and irregular dispersion of the background chromatin. Sandler et al[121] place this as the most prominent feature of malignancy in oral cytological specimens. Work presented in this thesis, lead to the conclusion that irregularity in nuclear outline is a factor which appears to be important and associated with chromatin irregularity. This has been referred to by other workers with oral cytology. [69, 121, 139].

4. Enlarged or multiple nucleoli, often of abnormal shape, may take a prominent place according to Montgomery and Von Haam. [54, 35].

5. Multinucleation associated with nuclear atypia does not receive much significance in the literature, but overlapping of nuclei has been reported as being a common cytological feature (Cawson, 1960)[26].

6. Mitotic activity is rarely seen, as smears consist primarily of superficial cells, (Montgomery and Von

Haam, 1951[85] but, especially in specimens from healing erosions mitotic figures could possibly be found. (Papanicolaou, 1954)[97].

7. Thickened nuclear membrane tends to be unreliable (Montgomery and Von Haam, 1951)[85] as it is common in cells from inflammatory as well as malignant lesions. (Papanicolaou, 1954)[97].

8. Nuclear degeneration and inclusion formation may be often seen (Montgomery and Von Haam, 1951)[85] but is certainly not a characteristic of malignant cells.

Cytoplasm

1. Staining reaction. Papanicolaou [97] notes the importance of acidophilia in some epidermoid neoplasms where these strongly orangephilic cells are quite prominent, a fact which tends to be supported by others [69, 85, 121] dealing with oral smears. Graham, 1964 p. 324 [52] however tends to "play down" this aspect in general. It has been the experience of this study that refractile, highly orangeophilic cells associated with a somewhat atypical nucleus, are not infrequently seen in benign oral conditions, as well as in malignancy. Therefore in attempting to establish the significance of fine distinctions of cellular atypia in oral cytology

this particular feature, it would seem, should not be over-emphasized.

2. Cytoplasmic inclusions may be significant or pathognomonic in certain forms of malignancy and in certain parts of the body; e.g. melanin pigment in melanoma cells and leucocytes in adenocarcinoma cells, (Papanicolaou, 1954)[97] but they tend to be of less importance in oral smears where the great majority of malignancies are squamous carcinomas. Conventional thought used to be that vacuolisation was a form of degeneration, but it is likely that this is in fact a sign of cell vitality, evidence of phagocytosis and pinocytosis of nutritive material (Graham, 1964 p. 324)[52]. It is of interest to note that Graham claims there is only one cytoplasmic feature that identifies a cell positively as benign - the presence of cilia.

Alterations in cellular size and shape

1. The "tadpole" and "fibre cells" seen frequently in gynaecological smears from invasive squamous cell carcinoma ,[52, 97] are uncommon in malignant oral smears (Cowen, 1960) [26]. Of greater significance is the tendency for keratinised cells to assume a spherical shape, (Sandler et al, 1960)[121] although Silverman et al [139] disagree with this and it would

seen from my study that such cells are not peculiar to malignant or indeed premalignant lesions, as far as could be ascertained.

2. Enlargement of oral mucosal cells occurs under numerous conditions, such as radiation effect, anaemia and viral infection and therefore has no diagnostic importance with respect to malignant changes.

Inter-relationship of cells.

1. Anisokaryosis and aniscocytosis, according to Papanicolaou [97] are important diagnostic criteria. This lack of uniformity in size of the nuclei throughout the smear, has been found very significant in my project as other studies on oral cytology. (Sandler et al, 1960)[121], (Silverman, Decks and Forbes, 1958) [139].

2. Apparent loss of cell outlines in clumped exfoliated cells may be seen in smears from well keratinised oral squamous carcinomas, associated with hyperchromatic nuclei (Sandler et al, 1960)[121], and to some extent in benign hyperkeratotic lesions.

3. Crowding of cells and nuclei in clumps can be evaluated satisfactorily in cytological specimens, (Papanicolaou, 1954)[97], but as parakeratosis is a fairly common finding histologically in oral white

lesions, nuclear morphology seems to be of greater significance than the amount of cytoplasm visible between nuclei, although this factor may be of no little significance.

Inflammatory cells, as noted by Papanicolaou,[97] the presence of leucocytes, histiocytes, and lymphocytes seems to be of little value in oral smears where they are so commonly encountered in simple inflammatory lesions, although histiocytes are often prominent in malignant oral smears and may be mistaken for malignant cells.[52, 57].

Usher et al (1960)[173] claim that the sudden appearance of pus cells in smears from irradiated oral mucosa, which previously yielded negative smears, is an indication of recurrent neoplasm or radionecrosis. This appears somewhat meaningless if the aim of post-irradiation smears is to detect recurrent or residual tumour as distinct from radionecrosis.

The above seems indicates that, to some extent, overall general features of a smear, as well as morphological details of individual cells, often give an impression of malignant or grossly atypical changes which influence the cytology report. It is obvious that if a smear perchance fails to contain definite representative malignant cells, or they are so few as to be overlooked, an examiner will be unlikely to give an unequivocal negative report.

It is suggested that at this stage, it would be useful to note the comparative relationship between the cytological and histopathological pictures in various epithelial states that are found in oral epithelium. Such a relationship for stratified squamous epithelium generally has been shown by Graham (Ch. 4-6) [52] and could be briefly summarised as follows:-

Dysplasia:	Histology Loss of cellular polarity, abnormal chromatin distribution in nuclei but adequate cytoplasm in each cell, particularly in the superficial strata. Cytology Typical cell is dyskaryotic cell - malignant nucleus but adequate cytoplasm.
Carcinoma-in-situ	Histology Further loss of cellular polarity, irregular nuclear chromatin, nuclei crowded and lack of differentiation of strata within the epithelium beyond, which the cells do not penetrate. Cytology Most typical cell is a third type differentiated squamous cancer cell -

Carcinoma-in-situ: (Continued) malignant nucleus, round or oval cell with distance from nuclear border to cell border being less than maximum diameter of nucleus.

Invasive Carcinoma: Histology

Malignant cells invading sub-epithelial tissue.

Cytology

Superficial invasive oral carcinoma [43]

(1) Large cell with refractile orangeophylla cytoplasm and malignant nucleus.

(2) Cell similar to third type differentiated cancer cell.

Invasive carcinoma generally

(1) Undifferentiated cancer cell - malignant nucleus with no visible cytoplasm.

(2) Fibre or tadpole cell. (uncommon in oesophageal (Graham, 1964)[52] and oral (Cawson, 1960)[26] smears.

Cytologically, the amount of cytoplasm around the nucleus of a malignant cell indicates to some extent the degree of differentiation. (Graham, 1964 Ch. 5, 28)[52].

Malignant cells in well differentiated squamous cell carcinoma contain hyperkeratinised cytoplasm (Silverman, Focks and Farber, 1958)[139] in contrast to the undifferentiated cancer cell. It has been suggested that cytological evaluation of the degree of differentiation compares well with that of histopathology (Umiker, 1966)[174] and therefore may supplement biopsy, especially where the specimen is inadequate or there is a massive inflammatory cell infiltrate. However, one does doubt whether it is reliable enough to be of clinical value.

CARCINOGENIC CHANGES.

In attempts to achieve a greater insight into the nature of carcinogenic changes, considerable work has been done in the form of animal experiments. One form of study employed the use of a carcinogen such as 9 - 10 Dimethyl, 1 - 2 Benzanthracene, in various vehicles (ec-carcinogens) (Ullverman and Shklar, 1963)[143] which was painted on the buccal mucosa of Syrian hamsters two or three times a week for about four months. It has been pointed out (Diezis, 1965)[16] that such experiments do not show a direct causal relationship between the agent used and carcinogenesis, but rather that the incidence of carcinoma is significantly higher in treated, than untreated animals. Cytology has been found an accurate and convenient method of assessing the epithelial changes that take place during the experiments [14, 23, 32, 75, 153] as an adjunct to clinical and histological findings. The chronological sequence of changes that have been noted by various workers [14, 21, 23, 32.25, 116, 153, 154, 155] in these animal experiments can be set out as follows.

(1) Inflammatory Stage. (approximately two to four weeks after start of experiment).

Gross Appearance. Inflammatory reaction with or without ulceration of the painted mucosa.

Cytology. Normal epithelial cells with numerous leucocytes.

Histology. Inflammatory cells infiltration beneath the mucosa.

(2) Stable or Recovery Stage. (approximately 5th - 8th week.)

Gross Appearance. Diminished inflammatory response with some initial superficial necrosis, which is soon lost, leaving almost normal appearing mucosa.

Cytology. Normal, with some minor atypia.

Histology. Epithelium shows some thickening with acanthosis, parakeratosis, but inflammatory reaction reduced.

(3) Secondary Response - Papillomatous Stage.

Gross Appearance. Mucosa shows increased erythema, some white hyperkeratosis, and the formation of papillomatous outgrowth in the involved region.

Cytology. The cells show increased Nuclear/ Cytoplasm size ratio, are at first clumped, but later isolated, nuclear hyperchromatism and chromatin clumping becoming more severe with time during this stage.

Cytology. (Continued) Nucleoli may be prominent in many cells, and cornified 'pearls' may be seen. The cells, at this stage, could be described as dyskaryotic.

Histology. Epithelium at first is simply hyperplastic, then dyskeratosis becomes progressively more severe, with marked mitotic activity in the basal cell layer, alteration in cell polarity and hyperchromatic nuclei.

(4) Pre-Malignant to frank carcinoma stage.

Gross Appearance. Further growth of papillomatous lesions into malignant tumours. Multiple lesions occur in each animal in the treated area.

Cytology. Presence of malignant cells in smears.

Histology. Histological picture of carcinoma-in-situ - precede the development of frank invasive squamous cell carcinoma.

It would appear reasonable that malignant transformation should take place in the basal epithelial cells rather than the superficial cells which are shed within a few days. (Bertalanffy, 1963)[12]. The genetic structures of the basal

cells are possibly altered by the chemicals, so that, on division, progressively more atypical cells are formed, with malignancy being the final outcome. Salley (1961)[117] has been impressed with the apparent importance of accessory structures such as sebaceous glands and hair follicles as a means of conveying the agent to basal layers in studies of epidermal carcinogenesis, but notes that their role is still open to conjecture, as oral experiments with the hamster buccal pouch produce consistent results where these structures, or any other obvious portal of entry for the carcinogenic agent to the deeper epithelial layers, are absent. Goldhaber (1957)[48] commented on the fact that although tumour formation is easily provoked in hamster cheek pouches, it is difficult to induce on the oral mucosa of mice. The hamster cheek pouch may not be representative of true oral smears, as it is not constantly bathed in saliva. Goldhaber then found that tumours could be induced in de-salivated mice after 10- 12 months application of the carcinogen, which he claims was able to breach the 'physiological barrier' (saliva and normal mucosal resistence) by ulcerations in the mucous membrane produced by food and hair abrading the abnormal 'dry' tissue. He later found (1958)[49] that the carcinogen becomes localised in high concentration in mucosal ulcers in animal experiments despite adequate salivary flow.

UNIVERSITY OF TORONTO DENTAL LIBRARY

These findings have been supported by Levy (1963)[76], who records that a carcinogen injected beneath epithelium in the tongue of mice produced carcinoma, but when applied topically, did not. Others (Ronstrup, Saulov and Glickman, 1962)[112] have also found support for the belief that mechanical irritation hastens carcinogenesis in animals.

It is well recognised that species differences and the effects of many known and unknown variables prevent the direct application of the findings of animal experiments to the human situation. Morris (1961)[87], for example, reported the effect of numerous variables such as age, sex, concentration of agents on carcinogenesis in animal experiments. However, a number of points emerge from these studies which could appear to be of direct clinical significance. The effect of chronic irritation of oral tissues in an individual in a susceptible situation could be particularly deleterious. (Pindberg, 1965)[105]. In 1953, Promeranz and Stahl, [110] suggested that harmful effects could result from repeated biopsies in suspicious areas of tissue. Goldhaber(1957)[48] has noted the findings of some investigations with betel nut chewers, where carcinoma was found not to be produced by betel chewing per se, but that it was produced if chronic irritation and ulceration existed as a result of, say, a sharp tooth. Furthermore, the mucosal atrophy which occurs in Plummer-

Vinson syndrome and syphilitic glossitis, (Pindborg, 1963)[102] may be associated with increased incidence of oral carcinoma in these patients. Pindborg, (1965)[105] has indicated a definite relationship between the oral mucosal atrophy that characterises submucus fibrosis and a high predisposition to carcinoma in tobacco chewers. Cahn (1963)[21] pointed out that experiments have demonstrated the reversibility of the carcinogenic process at the papillomatous stage, but that if, at a later time, a physical irritant, such as a suture, is applied to the healed site, a neoplasm may develop. He suggests that this is akin to the situation in which a patient with an oral pre-malignant lesion associated with smoking stops the habit with resultant healing of the lesion, but that blatant cancer develops at the site years later when he resumes smoking again. Von Haam (1954)[54] reported a similar finding with vaginal smears from untreated carcinoma in-situ, which repeatedly showed atypical and malignant looking cells, over a two to six year follow-up. He suggests the lesion could be reversible, or remain 'arrested' for sometime.

The susceptability of an individual as a whole to malignancy has been hinted at by Levy (1963)[76] who records that a study has shown that there are characteristic nuclear changes in buccal smears of over 70 percent of patients with malignant tumours at sites other than the oral cavity.

Moctel and Foss (1958)[82] note that the concept of carcinoma originating from a single minute focus has been seriously challenged in recent years. They found that 3.7 percent of 732 patients with proven oral cancer, had two or more discrete oral neoplasms, and leukoplakia was found associated with the multiple lesions in 75 percent of these patients. This tendancy for multicentricity shown by patients with oral cancer, is not limited to the oral cavity, but extends to the contiguous squamous epithelia of the pharynx, oesophagus, and larynx. These authors cite the work of Slaughter (1946)[149] as providing the most substantial histological proof of multicentricity. The possibility of demonstrating cellular changes in oral smears taken some distance from a localised neoplasm, for the determination of 'field cancerisation', has been raised (Cahn, 1963)[21], but no studies in support of this contention have been encountered.

Generalisations have been made with regard to the accuracy of cytology in detecting subtle malignant changes in animal experiments, but the question could be raised as to how accurate is the cytological smear in detecting malignant change in a chronic lesion over a period of time, in the clinical situation. Boddington, Cowdell and Spriggs (1960)[17] state that it is unjustifiable to expect any

cytological warning before invasion supervenes, as in about 50 percent of cases of 'cervical pre-cancerosis', the lesion disappeared within one year, but of those that persisted, 50 percent developed carcinoma within eight years. They found that, on review of the smears from a series of persistently positive cases (carcinoma-in-situ), the abnormal cell type remained remarkedly constant over a period of years, and in cases where invasion supervened, no alteration in cell type was observed. Johnson et al (1960)[65] recognise cytological and histological changes consistent with dysplasia, which always precedes carcinoma-in-situ, and which itself has all the morphological properties of carcinoma, except stromal invasion. This would seem to be in aggreement with Graham (1964) p. 72 [52] who indicates that, as lesions progress through the stages of dysplasia, carcinoma-in-situ to invasive carcinoma, the smears show respectively dyskaryotic cells, then dyskaryotic and third type differentiated cells, then frank carcinoma cells with probably some of the previous cell types being also present. Bertalanffy, Nasin and Masin (1958)[14] indicate that during carcinogenesis, cytoplasmic changes, evident in smears, precedes the nuclear changes. These cytoplasmic changes are increased basophilia and RNA content (detected with fluorescent microscopy) while altered nuclear DNA

content appears only in certain types of malignant tumours in advanced stages of carcinogenosis.

In oral smears, the finding of dyskaryotic cells can be correlated with histologically evident dyskeratosis, and should be regarded as arising from a potentially malignant lesion. (Stahl, Sandler and Cahn (1964)[155]. Cahn (1961)[20] has described histological changes in oral 'disquiet' epithelium, and since he points out that the abnormal cells may be found throughout the entire thickness of the epithelium, it is feasible to expect them to appear in exfoliative smears. Such cells show variation in shape and size, some being very large with multiple nuclei, while others have hyperchromatic plump nuclei, frequently irregular in shape, which may occupy most of the cell leaving a thin cytoplasmic rim. We cites Anderson (1960)[3] who stated that when a macronucleolus is seen in a cell, even though it does not exhibit other malignant characteristics, or when several nucleoli are found in a single cell, malignancy must be considered.

Examination oral lesions from the cytology viewpoint.

While most oral lesions are thought never to undergo malignant transformation, a small percentage of some lesions have been reported as consistently associated with malignancy, and have therefore been classified as "premalignant". However, since the true nature of this transformation is generally poorly understood, there appears to be some danger in the usage of this generalisation based on the clinical appearance of the lesion if one excludes the possibility of malignancy in all other lesions. Numerous cytology studies have shown that malignancy is occasionally detected in apparantly completely innocuous lesions. A false sense of security may be engendered in clinician and patient if the latter is assured that the lesion, on the basis of clinical diagnosis, is absolutely benign. The rationale for oral diagnosis is to use cytology in clinical practice and to consider every oral lesion as pre-malignent or malignant until proven otherwise. The cytological evidence should however be supported at biopsy.

Leukoplakia.

As the name infers, leukoplakia is a white patch, occurring on mucosal surfaces in the uterine cervix, urinary bladder, renal pelvis, upper respiratory tract

and oral cavity. (Shafer, Hine and Levy 1963 p. 80)[132]
The term is clinically appropriate for many lesions which simply have the features of a white plaque, where in differential diagnosis other specific conditions, such as lichen planus syphilitic mucous patches, have been excluded (Swan, 1965)[161] The conventional histological connotation of leukoplakia is more specifically pre-malignant, as it is used to indicate the presence of dyskeratosis, in addition usually to hyperkeratosis, parakeratosis and acanthosis. As Swan[161] has pointed out, dyskeratosis is not limited specifically to lesions presenting clinically as a white patch. Ogoke (1967)[34] suggests that since the term leukoplakia is well established as a clinical entity, it should be maintained, but that histological usage should be prefixed with either "dyskeratotic" or "non-dyskeratotic" leukoplakia. This would be of distinct advantage to the clinician, upon receiving a biopsy report, in that it would remove uncertainty about the pathologist's usage of the term leukoplakia. The term as used by me applies to a clinically evident white patch of oral mucosa which, as Lindberg et al (1968)[108] define, cannot be removed by rubbing and which could not be classified as any other diagnosable disease.

The percentage number of leukoplakia cases that undergo

malignant transformation as indicated in the literature is not clear. (Svan, 1965)[161]. One recent study (Pindborg, et-al 1968)[108] indicates that over one to five years, 6 per cent of leukoplakia lesions undergo malignant change; in another (Silverman and Rozen, 1968)[147] of 248 patients prevalence for malignant transformation over a period was put at 4.4 per cent; and Cook (1967)[34] indicates that 12 per cent of cases become malignant over ten years and 30 per cent become either malignant or dyskeratotic over this period. He feels that acute leukoplakia, a white patch of short duration, appears far more dangerous than chronic white plaque lesions.

Silverman, Beck and Farber (1958)[139] stated that leukoplakia remains a diagnostic enigma prior to biopsy. The clinical features of the condition do not indicate its biologic potential (Silverman and Rozen (1963)[147]. However, it is generally recognised that the problem of obtaining a specimen of tissue from an area of leukoplakia is the selection of the most active site, otherwise a false negative report through an error in sampling may occur. (Silverman and Hare (1960)[141]. The reported value of cytology in this situation is somewhat conflicting. Silverman et al [139] and others (Cooke, 1963)[33], (Umiker et al, 1960)[173] claim that prior to fissuring or ulceration, leukoplakic lesions yield simply superficial

cornified cells which give no indication of malignant transformation occurring in the deeper layers. Unkar et al (1960)[173] attribute this to the fact that histologically most of the nuclear abnormality is found in the lower layers of the epithelium and it becomes less and less obvious as the cells migrate superficially, so that the superficial cells show little or no abnormality. This, he feels, indicates the need for improved methods of abrasion of such lesions, as suggested by Sandler et al (1960)[121], Sandler (1964)[124], prior to taking the epithelial smear, although King, Coleman and Pierce (1966)[69] feel that this is not necessary to obtain representative smears. It has been reported (Silverman, 1965)[144] that cytology will yield representative cells prior to ulceration as a result of four facts:-

(1) Oral mucosa has a fast renewal time, hence abnormal nuclear characteristics are not completely lost in superficial cells.

(2) Leukoplakias that appear histologically as hyperkeratosis, hyperorthokeratotic (Silverman, 1965)[144] in effect contain intact tightly packed superficial cells, some of which contain nuclei, which presumably are visible cytologically.

(3) Malignancy never develops under hyperorthokeratosis (hyperkeratosis) but may after the surface has

become hyporparakeratotic (parakeratotic).

(4) Malignancy in leukoplakia occurs usually not in regions of greatest hyperplasia and cornification but in areas of atrophy.

Cooke (1958)[30] appears to be in agreement with this latter point in his remarks concerning selection of a biopsy site in an area of leukoplakia. He states that since a prickle cell can either divide or keratinise, and cannot do both at the same time (presumably a non-malignant cell), the part that is likely to show the most advanced pre-malignant change is that which resembles granulation tissue or may only be flecked with keratin. Pindborg et al (1963)[103] refer to the speckled type of leukoplakia as probably the most dangerous. It was found by biopsy of 35 such lesions, that 5 (14per cent) had become malignant and 18 (51 per cent) showed epithelial atypia.

It is virtually impossible to assess the accuracy with which the cytological smear reflects the true nature of an extensive leukoplakic lesion at any one point in time. As early as 1951, Montgomery and Von Haam (1951)[84] found in a series of benign leukoplakias that no typical "leukoplakia" cell was evident cytologically and even detailed cytological study failed to reveal any abnormality in the nucleus or nucleolus suggestive of change in cell morphology.

Naturally, the smears from leukoplakia show an increased percentage of cornified cells compared with normal smears from that region, and many of the cells show perinuclear vacuolisation. (Montgomery and Von Haam, 1951)[84], (Umiker, 1960)[174]. Peters and Rijsinghani (1956)[101] found that cases of early leukoplakia yielded a uniform smear type while advanced cases yielded smears with great variation in cell morphology. Umiker (1960)[174] seems to be in agreement with this in that he found unusual abnormality in most leukoplakia smears, but in some the atypia was so severe as to be difficult to differentiate from carcinoma, although he feels that atypical benign cells tend to remain in sheets or large clusters, while malignant cells are solitary or in small groups.

Four false positive cytology results were encountered by Ingram et al (1963)[64] in their oral cytology project of 422 lesions as a result of misinterpretation of an atypical large hyperchromatic glassy cell with blurred details shed from some leukoplakias. These false positives all occurred early in their series as later less significance was attached to this cell.

The presence of parabasal or intermediate cells in significant numbers in smears from leukoplakia requires careful scrutiny and biopsy is indicated if cells are found with nuclear pleomorphism or hyperchromasia. (Hughes and Dodds, 1968 p. 125)[62]

Mehl, Path and Kohar (1966)[173] report that exfoliated cells from leukoplakia show marked atypical keratinisation. The anucleate squames and intermediate cells are smaller than normal, and the chromatin pattern, while predominantly finely granular, may appear actively associated with prominent or multiple nucleoli.

King et al (1966)[69] found some lack of correlation between cytology and clinical findings in leukoplakia patients but good correlation between the cytology and histopathology of the lesions.

There is evidence (Pindborg et al, 1964)[104] to suggest that while many oral lesions are clinically similar and on this basis are classified as leukoplakia, they may differ markedly when examined microscopically, depending on aetiology and location within the oral cavity. In a study of leukoplakia in industrial workers (Krychalska-Karwan, 1966)[66], cytology was found to be a valuable method in determination of the advancement of the condition over a period of eight years.

In summary, it would appear that malignant change in leukoplakic oral mucosa is most likely to manifest clinically as atrophy, erythema or ulceration. In such cases cytological smears are reliable. Rarely, malignant change may occur under a thick unbroken white plaque in which cases cytology will be less reliable, for the same

reason that smears for tumours invading deep tissue with no surface involvement are unreliable. In this project, cytological smears from areas of leukoplakia appear to have accurately represented the nature of the lesion.

Lichen Planus

This is classically described as a dermatological disorder with oral manifestations, although in many cases the oral lesions may occur without skin lesions. Although Shafer, Hine and Levy, 1963 p. 696[132] claim that the condition is benign and not pre-malignant, Cooke (1967)[34] indicates that it is of increasing prevalence and may come to be regarded as a precancerous state affecting oral mucosa. Pindborg (1968)[109] seems to be in aggreement with this. Cahn (1961)[20] has reported a case which was diagnosed as lichen planus, but became malignant subsequently, and a similar observation has been made by me. Neoplastic change is preceded, in Cahn's belief, by a "prediscernible" or "prediagnostic" stage. Wahi, Path and Kehar (1966)[178] have included lichen planus in their study of 275 cases of precancerous lesions. These writers have described the cytology as being characterised by abundant exfoliation of inflammatory and epithelial cells (predominantly nucleated superficial cells with vacuolated cytoplasm and fragmented nuclei).

The finding of cases diagnosed clinically as lichen planus (particularly the erosive form) that have become malignant does not necessarily indicate that the condition is, in fact, premalignant or indeed is even part of a carcinogenic process. Shafer, Hine and Levy,1963 (p.695)[132] state that the microscopic appearance of lichen planus is a characteristic and pathognomonic one. The implication seems to be that before a lesion is diagnosed as lichen planus, it must satisfy the histological criteria as well as the clinical, thus making lichen planus a specific definite clinical entity, rather than a somewhat vague label to be conveniently attached to puzzling erosive lesions of doubtful aetiology. In such cases then, biopsy is clearly indicated to establish a definite diagnosis.

Leukoedema is an oral white lesion closely related to early leukoplakia but which Shafer, Hine and Levy (1963) p.91[132] feel is a distinct clinical entity to be included as a premalignant oral condition. It is a chronic lesion in which the oral mucosa is covered by a grey film. (Pindborg, 1968)[109]. It is unlikely that the exfoliative cytology of the lesion would differ markedly from that of cornified or parakeratotic mucosa.

Erythroplasia of Queyrat or Erythroplakia

Well defined velvety erythematous plaques with little evidence of induration constitute the clinical features of this oral mucosal premalignant condition [20, 109, 132]. The histological appearance is of epithelial atrophy associated with anaplasia and dyskeratosis.

The cytology of the lesion seems to be similar to that recognised as dysplasia in cervical mucosa.

Sub-Mucous Fibrosis

This oral soft tissue condition which is prevalent among the chilli eating population in India, has come to be recognised as a premalignant lesion. (Pindborg et al, 1965)[106]. Clinically the condition is characterised by blanching and stiffness of the mucosa, while histologically marked epithelial atrophy is evident (Pindborg, 1967)[107], which results in a high susceptability to the action of carcinogenic agents, such as tobacco extracts.

Carcinoma-in-situ (Bowen's disease) (Intra-epithelial carcinoma)

This condition is generally regarded as a 'malignant' lesion limited to the epithelial tissues, preceding active invasion and development into frank carcinoma, but it is a disease state which has been the centre of much controversy in cancer diagnosis and treatment for many years. (Hartman, 1951)[57]. It is a condition which arises frequently on the

skin but occurs also on mucous membranes, including those of the oral cavity. (Shafer, Hine and Levy, 1963, p.92)[132]. Carcinoma-in-situ is characterised histologically by dyskeratosis, crowding of nuclei and lack of differentiation, so that the cells in the upper regions are as immature as those in the basal regions, but the cells are all contained within the epithelium. (Graham, 1964, p.75)[52]. It is uncertain whether a less severe condition, known gynaecologically as dysplasia always progresses to carcinoma-in-situ. (Johnson et al, 1960)[65]. Histologically the appearance of dysplasia differs from that of carcinoma-in-situ in that there is no nuclear crowding, indicating that the malignant appearing nuclei found at all levels of the epithelium are surrounded by adequate cytoplasm. (Graham, 1964)[52]. Some confusion has developed as a result of the free usage of such terms as carcinoma-in-situ, intra-epithelial carcinoma or stage 0 carcinoma, as their histological definition is difficult and may differ from one pathologist to another. (Eddington, Cordell and Spriggs, 1960)[17]. 'Epithelial instability' has been recommended as an alternative. It has been pointed out (Eddington, Cordell and Spriggs, 1960)[17] that, although only one third of such cases progress to invasive carcinoma, it is of utmost importance to ensure that each case of epithelial instability (of the cervix) is not, in fact, an early invasive carcinoma that has been missed.

In the uterine cervix, where these two conditions, dysplasia and carcinoma-in-situ are recognised entities, the cytology is fairly well defined, being characterised by dyskaryotic cells (cells with nuclear characteristics of malignancy but containing an acceptable amount of cytoplasm) only in dysplasia; and dyskaryotic and third type differentiated malignant cells (round or oval cells with malignant nuclei, the diameter of which exceeds the distance from the nuclear border to the cell border) in carcinoma-in-situ (Graham, 1964, p.75)[52]. Obviously, varying numbers of normal epithelial cells will be found in all smears. Bizarre malignant cells are uncommon in smears from carcinoma-in-situ (Hughes and Dodds, 1968 p.52)[62].

While the cytology of dysplasia and carcinoma-in-situ appears to be well defined in gynaecological work, there is less certainty with early malignant change in oral mucosa, (King, Coleman and Pierce, 1966)[69], (Medak et al, 1967)[79] although Hughes and Dodds, (1968, p. 132)[62] report that the characteristic features of malignant cells from squamous cell carcinoma are essentially the same. It has been reported that the carcinoma-in-situ period preceding frank malignancy is considerably longer in cervical mucosa than it is in oral mucosa where infiltration occurs at an earlier stage. (Selbach and Von Haam, 1963)[131].

Sandler, (1961)[122] confidently notes that intraepithelial carcinoma is detectable by oral cytology, and Stahl, Sandler and Cahn, (1964)[155] found dyskaryotic cells in addition to cancer cells in smears from these lesions. Certainly, in animal experiments with oral carcinogenesis, it has been found that pre-invasive dyskeratotic changes in oral epithelium are accompanied by dyskaryosis in the cytological specimens.

It should be noted here that all the above-mentioned lesions are pre-malignant and therefore are not yet irreversible. Graham, (1964, p. 83)[52] has noted the finding by other workers that only 20 -30 percent of cervical carcinoma-in-situ may be seen to progress to invasive carcinoma if followed for a long enough period.

CYTOLOGICAL ASPECTS OF ORAL CARCINOMA.

Basal Cell Carcinoma (B.C.C.).

This is an epidermal tumour with locally destructive tendencies, but it rarely metastasises. It never arises in mucous membrane, although it may penetrate to a mucosal surface, such as in the mouth after invasion from the skin (Shafer, Hine and Levy, 1964, p. 93)[132]. The histological picture of basal cell carcinoma is one of nests, sheets or islands of basal epidermal cells, with large, deeply staining nuclei, mitotic figures and indistinct cell membranes. These tumour cells show no tendancy to keratinise, due possibly to a genetic defect in the ability of the cells to synthesize keratin. (Van Scott, 1964)[176]. That varying degrees of keratinisation may be seen in histological sections from some basal cell carcinoma could result from any of three possibilities; (a) that the defect in the keratinisation mechanism is incomplete, (b) there is an admixture of normal and tumour cells, (c) lesions showing this may represent another tumour type. Because of the clinical nature and situations of these lesions, exfoliative cytology is of, virtually, no value, and as a result has received no attention in the literature in this regard.

Squamous Cell Carcinoma. (S.C.C.)

Squamous cell carcinoma is the most frequently

found oral soft tissue malignant neoplasm, and may occur in any site within the mouth, or oropharynx. The clinical feature of advanced lesions are often diagnostic, presenting induration, ulceration, fungation, fixation and elevation, but in early stage may be very misleading.

The histological picture of squamous cell carcinoma varies from lesion to lesion, although in general they tend to be moderately well differentiated neoplasms with some evidence of keratinisation (Shafer, Hine and Levy, 1963, p. 98)[132]. The squamous carcinoma cells retain the ability to divide irrespective of the distance from the supporting connective tissue, in sharp contrast to normal stratified squamous epithelium which divides in the basal layer only (Van Scott, 1964).[176] Whereas the normal cell is involved in either cytoplasmic protein synthesis (keratin) or mitotic division, the squamous tumour cell population is involved in both. As there is considerable histological variation, so is there variation in the exfoliative cytology of squamous cell carcinoma, depending on the degree of differentiation, and to some extent, degree of invasion of the lesion. Hughes and Dodds (1968, p. 132)[62] indicate that the characteristic features of squamous cell carcinoma of oral mucosa are essentially similar to those of squamous cell carcinoma

of the female genital tract. However, the literature and the findings of this study do indicate some differences in the cytology of this neoplasm in the oral cavity to elsewhere, and those are dealt with more fully in the section under 'Malignant Oral Cytology'.

A number of poorly differentiated malignancies occur in oral mucosa and contiguous tissues, and these, (lympho epithelioma, transitional cell carcinoma, and undifferentiated squamous cell carcinoma) have been described by Shafer, Hine and Levy,(1963, p. 113)[132]. The individual cells in these lesions show no keratinisation, are moderately large, round or polyhedral with eosinphilic or basophilic cytoplasm, the border of which is indistinct the nucleus is large, round and may show mitotic activity, or in the case of lympho epithelioma, is oval vesicular and characteristically contains one or two large prominent nucleoli. That the cells are obviously malignant and tend not to be keratinised, is evident in oral smears.

Malignant Melanoma.

This is an uncommon oral neoplasm which usually occurs as a deeply pigmented area, is frequently ulcerated and bleeding, and which tends to increase progressively in size (Shafer, Hine, and Levy, 1963, p.114)[132]. No reference specifically to the exfoliative cytology of this

lesion has been encountered, but it is presumed that, particularly as it is often seen as an ulcerated oral lesion, the desquamated cells would be similar to those seen histologically. Graham, (1964, p.138)[52] notes that melanoma of the cervix does not readily dosquamate cells into vaginal secretions, but of those cells that are shed a high proportion are quite often not pigmented at all. However, the chromatin structure of the individual cells is typical of any malignant nucleus. The cells in oral malignant melanoma are cuboidal or fusiform in shape and may not show prominent mitotic activity. (Shafer, Hine and Levy, 1963)[132].

ORAL CYTOLOGICAL TECHNIQUES.

In most cases where the lesion is accessible, the specimen is usually obtained by scraping the surface with a spatula. Where it is not accessible alternative methods are needed. Koss and Durfee, 1961)[71] have pointed out that the character of the tissue, accessibility and possible hazards are factors which influence the method of obtaining cytological specimens.

Preparation.

Although it has been stated that most lesions require no preparation and therefore need not be wiped or dried (Sandler, 1964)[124], isolation of the field with gauze facilitates a more satisfactory smear. Elimination of excess saliva and removal of debris, blood and slough prior to scraping the area, is advantageous (Sandler et al, 1960)[121]. It is generally accepted that the presence of necrosis greatly increases the possibility of obtaining a false negative smear, so that removal of superficial debris could be regarded as essential. Another possible advantage of first drying a lesion has been pointed out, (Silverman, Beeks, and Farber, 1958)[139], namely, that it minimises contamination by cells shed from other areas, which could be the cause of considerable confusion.

Hyperkeratotic lesions pose some problems when obtaining a specimen for cytological examination, as their superficial keratin layers may be covering a more sinister process. (Sandler et al, 1960)[121], (Sandler, 1964)[124] have suggested removal of this thick keratin layer prior to taking the smear by scraping with a curette or grinding with a mounted wheel stone until pink tissue is visible. This is considered unnecessary by others (King, Coleman and Pierce, 1966)[69] who feel that these superficial cells are diagnostically important, and should be removed onto a slide with a cotton swab, rather than a spatula which obtains so many anucleate squames that the true cytological picture of the surface cells is lost. Diagnostic deeper layer cells can be obtained from fissures and cracks in hyperkeratotic lesions (Williamson and Shapiro, 1964)[188], so that it has been suggested that these may be spread open for better access. (Sandler et al, 1960)[121]. There is normally no discomfort associated with obtaining a smear but topical anaesthetic on a very tender lesion may be helpful, (Sandler, 1964)[124], although presumably those in ointment form would be contra-indicated because of their interference with transfer of adequate cells and fixation of the cells to the glass slide.

Procedure. (Normal)

Following preparation of the site, exfoliating

cells are removed and transferred to a glass slide. Some workers have recommended using saline moistened cotton tipped applicators [69, 139, 152], but this has been criticised (Walker, 1960)[174] on the grounds that many cells are absorbed into the cotton and lost; the most widely accepted means of obtaining oral cytological material is with a spatula [1, 35, 121], usually plastic or wood, and this has been found most satisfactory in this project.

Collection of secretions.

As Graham, (p.337, 1964)[52] has pointed out, direct spatulation of the tissue obtains more cells from that particular area, and although cervical scrapings are widely used for diagnostic purposes in gynaecology, vaginal smears prepared from a collection of secretions are preferable as the cells obtained are truly exfoliated and are representative of much wider areas of the female genital tract. The value of this principle was demonstrated in this present study where saliva and mucous secretions were occasionally collected from a particular region. In one case, this was done from the area of an oro-antral opening which was showing continual clinical improvement and no visible residual or recurrent neoplasm, following radiotherapy for a bucco-alveolar carcinoma. The

secretions repeatedly contained malignant cells, but it was not until six months later that recurrent malignancy showed itself clinically by rapid invasion of orbit and maxilla. Aspiration of secretions has been found particularly useful in D.N.T. cytology as have sputum specimens. (Stahl and Ulman, 1962)[152].

Rinsing and gargling techniques have been used for both screening and diagnostic purposes (Helsper and Sharp, 1964)[59], (Watanabe and Shiraishi, 1961)[181], and are considered particularly valuable in assessing post-treatment progress in oral or pharyngeal cancer patients and as an aid in detecting early malignant change in extensive oral lesions (Watanabe and Shiraishi, 1961)[181]. The resultant smears are thought to be representative of the entire oral cavity . (Helsper and Sharp, 1964)[59]. The specimen is obtained by having the patient rinse the mouth and gargle with saline or Geys balanced salt solution (which is reported to produce less cellular distortion, (Helsper and Sharp, 1964)[59]) after which the fluid is collected, centrifuged or multipore filtered in the laboratory and the residue of cells smeared onto slides and placed in fixative prior to normal staining. The Veterans' Administration Study in the U.S.A. (Sandler, 1964)[124] has rejected this proceedure together with suction

and saliva aspiration methods, as unsatisfactory as a normal routine for oral cytological smears, although these alternate methods may be indicated in specific circumstances.

Irrigation is a similar means of obtaining cytological material using a saline or Coy's salt solution as a vehicle, being chiefly indicated in dentistry for collection of cells from post-extraction sockets, or antral lavages (Whitten, 1968)[186] in patients with oro-antral fistulae.

Aspiration of body fluids has been a recognised method of obtaining diagnostic cells since the nineteenth century (McGrew, 1961)[70], and is widely used today in medicine [52, 73, 97], applied to peritoneal, pleural, cerebro-spinal fluids and blood, and fluids in pathological situations. A comprehensive study (Whitten, 1968)[186], has been carried out on the cytology of fluid aspirated from cysts and cyst-like lesions of the jaws. Such fluid was found to contain:-

(a) normal mature epithelial cells in dentigerous cysts (large numbers of mature squames); developmental cysts, antral mucous cysts and ameloblastoma. (There are no tumour cells diagnostic of ameloblastoma as cells do not desquamate).

(b) Histiocytes, inflammatory cells and multi-nucleated giant cells in addition to epithelial cells in dermoid cysts.

(c) No epithelial cells in traumatic cysts.

(d) Epithelial cells with irregular outline and morphology, similar to pemphigus-acantholytic cells in apical periodontal cysts.

Needle aspiration lends itself to examination of tissue in lymph nodes and areas in bone suspected of containing metastatic neoplasm (Graham, 1964, Ch. 26)[52] as distinct from chronic inflammation. Differentiation must be made between undifferentiated malignant cells and histiocytes. Needle aspiration of osseous lesions may often contain multi-nucleated benign cells - osteoclasts and megakaryocytes, therefore, diagnosis is best made on cells which are not multi-nucleated (Graham, 1964)[52].

The method of needle aspiration is particularly applicable to oral tumour masses that cannot be reached by a simple incision, although in such cases a small incision should be made prior to insertion of the needle to avoid incorporation of surface epithelium in the specimen. (Schoman, 1965)[128].

Touch preparations.

Scheman, (1965)[126] suggests that all soft tissue removed from patients during oral surgical procedures should be subjected to some sort of microscopic examination. For this purpose the touch preparation has been recommended whereby pieces of tissue are gently abraded on to a glass slide after removal from the patient and before placing in formal saline. In the absence of any suspicious cells in this preparation, histological sectioning and staining of the tissue is probably not necessary.

Sponge biopsy.

Gladstone described in 1950[45] a sponge biopsy technique as an alternative to conventional biopsy in the diagnosis of ulcerated oral lesions. The procedure here involves absorbing superficial tissue on to a sponge which is immediately placed in standard formalin fixative and sent to a pathology laboratory where it is embedded in paraffin and sectioned using routine histological techniques. The report is made on cellular material contained within the trabeculae of the sponge.

Smears from oral bullae or vesicles.

The value of smears made from the floor of freshly opened oral bullae or vesicles had been documented [29, 31, 132, 150, 170], as a means of establishing diagnosis of pemphigus and herpetic lesions, or differential diagnosis

from those conditions. Cocko (1958)[29] has suggested that a platinum wire loop is the best instrument with which to remove cells from the floor of the vesicle, the cells then being mixed with a drop of saline on a glass slide and dried. Haematoxylin-eosin or Papanicolaou stain can be used to demonstrate these cells.

Fixation.

The specimen is taken from the patient and smeared on to a glass slide, which must be placed immediately in fixative. Marked cellular distortion and loss of nuclear detail results if there is any delay in placing slides in fixative, due to rapid drying [78, 139, 174]. Dislodging of the cells from the slides when placing in fixative solution should be avoided as much as possible (Silverman, Books, and Farber, 1958)[139] by careful handling.

Numerous fixative solutions may be used for cytological smears. Methylated spirits or 95 percent alcohol (Graham, 1964)[52], (King, Coleman and Pierce, 1966)[69] is generally satisfactory for oral smears if other fixative solution is not immediately on hand. Equal parts 95 per cent alcohol and ether has come to be regarded as the most satisfactory (Dorsett, 1964)[35], (Shapiro, Gorlin and Jordan, 1964)[134] but the value of this over plain 95 percent alcohol has been disputed and, in fact,

criticised as constituting a fire hazard in the laboratory because of the volatility of the other (Graham, 1964, p. 344)[52]. Graham, (1964)[52] has stressed, however, that if alcohol alone is to be used, concentrations of less than 95 percent are not satisfactory. It has been claimed (Sandler et al, 1960)[121] that, providing cells are not distorted through drying, and the surface of the lesion was moist at the time of taking the smear, the presence of fresh blood does not detract from the staining or reading of the smear. In this study, however, it was found that large numbers of red blood cells presented major difficulties in interpretation of smears by obscuring many of the epithelial cells. To overcome this, Carnoys fixative was used as it has the ability to lyse the red blood cells and provides satisfactory fixation of smears without significant cellular distortion.

The minimum time required for fixation of smears is 20 - 30 minutes, but slides may stay in the solution indefinitely without deterioration. (Silverman, Deeks and Barber, 1950)[139].

To avoid cross contamination of specimens the fixative solution should be filtered after removal of the slides and placed in cleaned bottles before re-using. (Graham, 1964) (p. 344) [52].

Drying and mailing.

Following fixation, slides may be immediately stained or allowed to dry, to enable packaging and transport to a cytology laboratory. Air drying following adequate fixation does not significantly affect cellular detail. (Silverman, Decks and Farber, 1956)[139]. A method has been suggested (Papanicolaou and Bridges, 1957)[90] to eliminate the possibility of cellular distortion during mailing by covering the smear with a small amount of Diaphane solution (resin in alcohol) which dries on the slide in 20 - 30 minutes to a hard protective coat. This coat is removed by the laboratory when the slide is received, by soaking in alcohol-ether prior to staining.

It is essential for the person examining a cytological smear to know the history and clinical data concerning the patient so that no smear is examined out of context. (Cahn, 1963)[22]. This information should be forwarded with the slide, together with a provisional diagnosis. (Mathers and Griffin, 1963)[103]. Williamson and Shapiro (1964)[188] specify that the following information should accompany the smears to the cytology laboratory:-

(1) Location of lesion.

(2) History.

(3) Clinical description.

(4) Provisional diagnosis.

Staining.

The staining procedure for routine exfoliative cytological smears described by Papanicolaou, 1954)[97] is now almost universally used, with or without slight modifications. I have used Papanicolaou's method for the material on which this study is based.

Other staining techniques such as Fluorescence; May-grunwald Giemsa have been suggested and will be discussed later. These are generally supplementary methods to the Papanicolaou technique.

Additional aids.

Techniques have been used in attempts to determine directly *in vivo* the nature of the lesion, and have been found valuable in delineating areas of tissue for biopsy or cytological examination. These are as follows:-

Schiller Iodine Test - Supravital Staining.

This test, first described in 1933 by Schiller,[130] is based on observations of the absence of intracellular glycogen in the epitholium in invasive cancer, carcinoma-in-situ and in keratinised lesions. (Cahn, 1961)[20]. The majority of parakeratotic lesions contain glycogen. Although this finding was not absolute, an iodine staining test based on it was found useful in gynaecology. In the mouth, however, it was found unreliable because some

benign tumours fail to take the brown stain (an indication of potential malignancy) and some keratinised cancers contain sufficient glycogen to stain faintly. (Melbel and Chomet, 1964)[92].

Toluidine Blue Supravital Staining.

This procedure has received more attention (Strong, Vaughan and Inczo, 1968)[159], as an aid to diagnosis of oral lesions. It was described by Richart (1963)[113] for delineation of dysplasia and carcinoma - in - situ in the cervix.

The supravital staining technique as suggested by Melbel and Chomet, (1964) [92] is as follows:-

Firstly, the lesion is cleaned with a swab soaked in 1 percent aqueous acetic acid. The mouth is then rinsed with water, the region to be stained is subsequently dried and then swabbed with 1 percent aqueous toluidine blue. After several minutes, the mouth is again rinsed and acetic acid is lightly and uniformly applied. The acetic acid acts as a decolouriser to remove stain from non-neoplastic tissue. Neoplastic areas retain the blue stain and the intensity is related to the nuclear density and severity of the malignant process, (Richart, 1963)[113] presumably the stain has an affinity for DNA. As has been pointed out (Melbel and Chomet 1964)[92], the accuracy of

biopsy or cytological smear depends on whether or not the material is representative of the lesion, so that a method that delineates *in vivo* malignant or potentially malignant areas is of great value. Frozen section preparations of stained tissue have shown that toluidine blue penetrates the surface epithelium to a depth of three - four cells. (Strong, Vaughan and Ineze, 1968)[159]. This explains the finding that invaded corium or sub-mucosal lateral extensions of malignancy are not delineated by the technique, but only the margins of the mucosal changes. (Neibol and Chenet, 1964)[92], (Strong, Vaughan and Ineze, 1968)[159]. There is, unfortunately, no absolute diagnostic staining test, and toluidine blue supravital staining is inaccurate in certain cases, such as traumatic or herpetic ulcerations, necrotic lesions, leukoplakia, debris coated tongues where there is sufficient nuclear density or mechanical factors for the stain to persist, though the tissue is not malignant.

In a recent paper (Shedd *et al*, 1967)[136] a toluidine blue mouth wash technique was reported as being successful in indicating malignant and pre-malignant (dysplasic and carcinoma-in-situ) areas of oral mucosa.

Other Cytological Techniques.

Although the Papanicolaou cytological method has been widely adopted for diagnostic purposes, various other techniques have been developed in attempts to provide greater ease and rapidity of scanning and diagnosis. The most attention in the literature has been given to Acridine-Orange Fluorescent Microscopy of exfoliative smears.

Acridine-Orange (A-O) Fluorescent Cytology

Acridine-Orange (3-6 Tetramethyl-diamino acridine) is a dye which when carefully applied to cellular material within a certain pH range is highly specific for nucleic acids. (Bertalanffy and Nagy, 1962)[11]. Further, because of the dye's polychromatic properties, it distinguishes DNA from RNA by causing them each to fluoresce with a different colour (Von Bertalanffy and Bickis, 1956)[13] when illuminated with ultraviolet light. Ultraviolet illumination was first used by Kohler (cited by Caulder, 1967)[25] for microscopic study in 1904; but its use was not of clinical value till the development of the fluorescent antibody technique in the 1940's. (Caulder, 1967)[25].

The application of A-O fluorescence to cytology

promised to offer greater caps of scanning smears, as not only did the nucleus DNA stain differently (yellowish-green) to the cytoplasmic RNA (orange-pink) but the intensity of fluorescence depended on the concentrations of the nucleic acids present (Liu, 1961)[77]. Thus, in proliferating cells where there is increased protein synthesis and increased amounts of DNA in the nucleus and RNA in the nucleus and cytoplasm, (Stahl and Mmen, 1962)[152] there is increased intensity of fluorescence, such that the RNA (cytoplasm and nucleus) may in atypical and malignant cells assume a brilliant flaming red colour. (Liu, 1961)[77].

The Acridine-Orange fluorescent cytology technique, as developed by L. Von Bertalanffy[13] for rapid identification of malignant cells, requires as apparatus any standard microscope and a fluorescent lamp (that emits ultraviolet light) with exciter and barrier filters. (Caulder, 1967)[25] Smears are taken and fixed by the same methods as in conventional cytology but stained differently. (Caulder, 1967)[25] A reliable staining procedure has been recommended by F.D. Bertalanffy [11] who, however, stresses the importance of using an A-O solution with the correct optimum specificity of fluorescence. The smears are examined unmounted and wet, with or without a cover slip, under the microscope in a similar fashion to conventional cytology

specimens. However, dotting of the cover slip to mark significant cells is somewhat pointless as the fluorescence lasts only four to five hours. (Stahl and Ulman, 1962)[152]. Removal of the stain is achieved by placing the slide in 50 percent alcohol for two minutes, after which the smear can be restained by conventional methods. (Caulder, 1967)[25].

Although A-O fluorescence differentially stains nucleus and cytoplasm, it is important to note that morphological detail is lost and, therefore, various cells produce diagnostic problems with this method. Histiocytes in fluorescent smears cannot be recognised by their characteristic foamy cytoplasm as in conventional smears and it may be very difficult to distinguish the difference in fluorescence between undifferentiated malignant cells, parabasal cells and histiocytes (as well as endometrial and endocervical cells in vaginal smears) (Liu, 1961)[77]. Further, well differentiated squamous carcinoma cells may not give increased fluorescent due to comparatively little cytoplasmic RNA. (Stahl and Ulman, 1962)[152].

The diagnostic disadvantages of this method are, therefore, obvious. False positive results are not infrequent, particularly in rapidly proliferating benign lesions or acute conditions, such as traumatic ulcers, or herpetic infections. (Caulder, 1967)[25]. False negative

results also occur when the malignant cells are sparse and of the poorly fluorescing type, (Uhler, 1961)[175], or are relatively mature cells from carcinoma-in-situ. (Liu, 1961)[77]. The literature contains conflicting reports on the accuracy of A-O technique as compared with the Papanicolaou technique and biopsy, which may be partly due to lack of standardization of the fluorescent method. (Caulder, 1967)[25]. However, from studies that have been done on large numbers of patients,[4, 77, 157] it appears that this method is somewhat less accurate than conventional cytology.

Uhler[175] has suggested that a combined method of A-O fluorescence and Papanicolaou cytology gives 11.7 percent greater accuracy in oral cytology than the Papnicolaou method alone, and 20.2 percent greater accuracy than fluorescent method alone, excluding suspicious reports. However, as suspicious reports are subject to the greatest error in cytology, these figures seem of little meaning. It has been suggested that since the Papanicolaou method is time-consuming and requires highly trained personnel, (Conroy, 1961)[28] the fluorescent technique is more advantageous as a screening device as it is faster and requires relatively untrained personnel. (Caulder, 1967)[25]. However, it is doubtful whether untrained scanners could more rapidly cover fluorescent smears than conventional smears with reasonable reliability, particularly when there

is obviously considerable equivocation with respect to the fluorescence of certain cells. Other workers have found that this method is, in fact, no faster. (Liu, 1961)[77], (Von Haam, 1966)[56].

Further disadvantages to the adoption of the A-O technique have been pointed out. Firstly, since morphological detail is not brought out by the stain, before a diagnosis can be made of the smear, it has to be restained and examined by conventional methods. (Bertalanffy and Nagy, 1962)[11], (Liu, 1961)[77].

Secondly, the fluorescence lasts only a short period and, therefore, does not permit reviewing of smears at a later time. (Von Haam, 1966)[56].

Thirdly, ultraviolet radiation can be harmful to the eyes and skin (Liu, 1961)[77], and is therefore a hazard to the scanners. Wellman, McDermott and Gray (1963)[184] have concluded that the method is neither safe nor economical as a routine diagnostic screening or pre-screening device, but suggest, as do others, [11, 77, 157], that the best application of this technique is as a research tool, such as for the study of cell proliferation and metabolism, (Bertalanffy, 1962)[11], rather than for routine diagnosis.

Sulfhydryl activity in exfoliated cells.

Sulfhydryl (SH-) groups occur naturally in tissue cells in a free form or as part of an amino acid, such as cysteine,

glutathionine or methionine in a protein molecule. (Stahl and Ulman, 1962)[152]. Since protein synthesis is an integral part of mitotis and growth activity in cells, interest developed in the concentration of sulfhydryl groups in normal and malignant cells with the result that several workers [7, 8, 177] produced evidence of markedly increased sulfhydryl activity in malignant cells. (Stahl and Ulman, 1962)[152].

A reliable method of demonstrating histochemically protein-bound sulfhydryl groups was described by Barnett and Seligman (1952)[9] who also showed the reaction to be specific. A deep blue colour of intensity directly proportional to the number of protein-bound sulfhydryl groups is retained by the cells. [7, 8, 152]. Although, in general, whilst normal epithelial cells are pale staining, malignant cells are readily identified by their deep blue or violet stain, however the reaction is not specific for malignant cells only but for any cell with increased protein synthesis. (Stahl and Ulman, 1962)[152]. Morphological detail of the cells also is not as well defined as with conventional cytological techniques, making this method somewhat inferior as an aid to diagnosis, which can only indicate malignancy on the basis of cellular morphology. (Bertalanffy and Nagy, 1962)[11].

May-Grunwald Giemsa stain, which demonstrates cellular morphology well and differentiates DNA from RNA by different colours, has been described as a complementary method to the Papanicolaou stain in cytological material. (Stahl and Wiman, 1962) [152].

GRADING ORAL CYTOLOGICAL MATERIAL.

The exfoliative cytology classification system laid down by Papanicolaou (1954)[97], is widely accepted as the basis of grading cytology specimens with respect to normality, atypia or malignancy.

Class I - Absence of atypical or abnormal cells.

II - Atypical cytology but no evidence of malignancy.

III - Cytology suggestive of, but not conclusive for, malignancy.

IV - Cytology strongly suggestive of malignancy.

V - Cytology conclusive for malignancy.

Papanicolaou (1954)[97] points out that Class V is actually the only conclusive group and that conservative grading can result in 100 percent accuracy in this group. Errors in interpretation of varying degrees can be expected in the other four groups. Graham, (p.331,1964)[52] agrees with this in principle but claims that in practice distinction between Class IV and Class V in most instances

is hazy and so reports simply as positive, negative, doubtful or unsatisfactory. "Unsatisfactory" is used where the smear (1) contains inadequate cells; (2) the cells have been allowed to dry out thus distorting nuclear detail; (3) the smear is too thick or unevenly spread to allow proper study of cell detail; or (4) is thought not to consist of cells representative of the lesion.

The modified system advocated by King, Coleman and Pierce (1966)[69] for oral cytology, though obviously aimed at specificity, tends to be over-complicated and confusing by its overall similarity to, but individual differences in meaning from, the Papanicolaou system. This modified system is as follows:-

Negative - No significant abnormality present.

$II^{b.b}$ - Consistent with benign hyperkeratosis. Numerical variation of cell types from normal as reported by other workers, e.g. Montgomery and Von Haam (1951)[85].

II - Cytological abnormality not felt to be suspicious of malignancy.

This included minimum dyskaryosis, cells with "active" nuclei and cells which could otherwise not be classified as negative.

Repeat recommended.

III - Presence of atypical cells.

IIIA - Cells strongly suggestive of epithelial atypicality.

IIIB - Cells consistent with marked epithelial atypicality.

IIIC - Cells strongly suggestive of superficial invasive carcinoma.

IV - Atypical cells consistent with malignancy, few cells present.

V - Atypical cells consistent with malignancy, many cells present.

There are doubts about the practicability of this system with regard to its specificity and accuracy in view of the marked variation in clinical behaviour and characteristics of oral malignant neoplasms. Firstly, as I have pointed out elsewhere, necrosis on the surface of an advanced lesion may result in smears containing little evidence of malignancy. Secondly, of the approximately 45 carcinomas involved in this project, there was considerable variation in the cytological picture and number of malignant cells present in smears from each lesion and even variation with time in smears from the same lesion. Finally, the degree of differentiation and the anaplastic tendencies of the lesion must be considerations, with

respect to type and number of malignant cells seen. It appears as if this system is advocated primarily to determine the type and state of development of a lesion, rather than to indicate the nature of the cells with respect to malignancy, atypia or benignity.

LaGrev (1961)[78] has pointed out that considerable experience is necessary in order to differentiate between malignant cells and those rendered atypical morphologically by inflammation, radiation, or other effects, and in fact it is impossible to draw a definite line between benign variation and malignant change. Graham, (1964, p.238)[52], commenting on smears from the oesophagus indicates that cells very similar to metaplastic cells seen in sputum ("active" appearing nucleus, high "nuclear/cytoplasm size ratio", round to oval shape) may be found in smears from cases of oesophagitis. It seems quite probable that a similar effect of denuding the epithelium of its upper layers allowing exfoliation of deeper cells, occurs in some oral inflammatory conditions, making interpretation equally difficult to that in the oesophagus. Figs. 24 and 25 show atypia found in completely benign erosive lesions of buccal mucosa, in this study.

Certainly with the King, Coleman and Pierce (1966)[69] system, unlike Papanicolaou's, there are only quantitative differences between classes IV and V, and Class V

does*not* seem to be intended as a conclusive grading. Generally, it could be argued, a conclusive grading is not necessary with oral cytology, as biopsies in the mouth are so easily taken. (Shapiro, Gorlin and Jordan, 1964)[134]. However, occasionally, situations arise where cytology may be the only diagnostic aid available, so that reliability is essential and a conclusive report of much greater value than a "suspicious but not conclusive report". Such situations may occur in patients who have received radiotherapy for a previous oral neoplasm. Owing to the endarteritis and increased tissue fragility, biopsies are not undertaken lightly in the irradiated region, so that cytology can be invaluable in determining the presence of absence of residual, recurrent or new primary neoplasm.

Two cases in this project serve to illustrate the point. Case I. Patient appeared at a routine follow-up visit, having had no recurrence following treatment for an squamous cell carcinoma of parotid gland and numerous small squamous cell carcinoma and basal cell carcinoma of face, over past six years, with a fluctuant swelling of the side of the nose. X-rays showed no bone destruction. No tumour could be seen through the nose or in the mouth, though swelling was obvious to palpation in the upper

labial sulcus. Aspiration of the swelling with an 18 gauge syringe produced pus and reduced the size of the swelling somewhat. Cytological smears made from the aspirated material, showed malignant cells. Subsequently, malignancy was confirmed by surgical exploration and histopathological examination of tissue.

Case II. Patient developed a painful ulceration in retromolar region adjacent to a radon needle implant for carcinoma on the lateral surface of the tongue. Cytology indicated malignant change, although clinically the lesion resembled a radionecrotic ulcer. Surgery was undertaken to remove the ulcer. Histopathology of the tissue showed no malignancy and radionecrosis developed more seriously than before.

In this case where biopsy proceedures are undesirable if not contra-indicated the guide for diagnosis rests heavily on cytology. The unfortunate outcome illustrates a point made by Graham (1964, Ch.29)[52] that an unexpected positive report should always be repeated before being acted on, and in fact extensive surgery should never be undertaken on cytological diagnosis alone.

Oral cytology then, if implemented as a diagnostic aid, may be an influencing factor directly or indirectly in management of patients. The suggestion made by Papanicolaou (1954)[97], therefore, that it is essential

to distinguish between results of a conclusive nature from those where there is an element of doubt certainly applies here.

A six-unit probability system, based on the Papanicolaou classification has been adopted by Sandler, (1962)[123] as follows:-

Normal

Atypical

Doubtful

Possible carcinoma

Probably "

Positive "

The introduction of this extra "doubtful" grading into the usual system is aimed at "minimising subjectivity but retaining sensitivity of cytological screening" by deferring classification of borderline cases until repeat smears can be obtained and presumably more information is available about the nature and behaviour of the lesion. It has been noted elsewhere, Graham, (1964, Ch.30)[52], that cytology loses much of its specificity and clinical applicability if too many cases are allowed to remain in doubtful or suspicious categories, and efforts should be made to regrade such smears more precisely when further information or smears are made available.

With any system of classifying cytological material there should be agreement in reading between cytology and histology, to avoid any confusion clinically. (Graham, 1964, p.71)[52]. If the histological picture of carcinoma-in-situ is one of crowded nuclei with little cytoplasm visible, and that of dysplasia as one where abnormal nuclei are separated by abundant cytoplasm, the cytology of the latter (where only normal and dyskaryotic cells will be present) should not be called positive (indicating the presence of definite malignant cells) but rather, "doubtful dyskaryotic cells present". (Graham, 1964, p.71)[52]. Since dyskaryotic cells are often seen in smears as well as malignant cells from carcinoma-in-situ, it is essential to ensure that no actual malignant cells can be found before labelling a smear "doubtful" or "atypical".

Similarly, when reporting on smears containing malignant cells, it is helpful to indicate the nature of the cells as "consistent with squamous carcinoma" or "consistent with adenocarcinoma" or "undifferentiated cancer cells present" in addition to labelling the report as "positive" (Graham, 1964, p.331)[52].

Since the method of classification adopted in different places does vary to greater or lesser extents, it is essential for the dentist or any person using oral

cytological smears as diagnostic aids, to be familiar with the system adopted by the laboratory to which he sends his specimens (Alling and Secord, 1968)[1]. The system used by Papanicolaou tends to be widely accepted in Australia and has therefore been used in this study with only little modification.

"False Positive" and "False Negative" Results.

Since cytology is not absolutely accurate, it is inevitable that "false" reports will occur from time to time, which incidentally means that a "negative" or "suspicious" report does not free the clinician of the obligation to obtain a definite diagnosis. (Shapiro, Gorlin and Jordan, 1964)[134]. Various workers have approached this subject of "false" reports from different angles and their results tend to indicate their subjectivity. (Shapiro and Gorlin, 1964)[133].

If oral cytology is introduced to an institution as a routine diagnostic aid, situations will almost certainly arise where its accuracy is of paramount importance, as has been shown earlier, and a positive result may be the basis of a surgical procedure. Obviously, then a "false" positive report in such a situation could be very detrimental to the patient. If, on the other hand, grading of cytological material is approached very

conservatively in order to avoid false positive reports, the number of carcinomas missed will increase and the sensitivity of the technique in detecting 'silent" or early malignancies will be lost, thus depriving patients of the opportunity for early successful treatment. (McGrew, 1961)[78].

<u>False Positive Reports.</u> Although no definite borderline can be drawn cytologically or histologically between malignancy and non-malignancy, the cytological grading systems in general are such that borderline cases are graded neither negative nor definitely positive. Thus, this cellular variation should not be a major cause of false positive results, so when one does occur it is probably the result of an error in microscopic interpretation. (Sandler, 1966)[125, 126]. Laboratory errors, though rare, could account for a false positive report, by failing to recognise that malignant cells on a smear are in fact contaminant, from another smear, or a mix-up of specimens or names. (Graham, 1964, P.326)[52]. It is suggested therefore that in the event of an unexpected positive cytology report, a repeat smear should always be taken and in the mouth, where it is relatively simple, a biopsy should be done to confirm the diagnosis.

<u>False Negative Reports.</u>

These may result from a number of factors. Firstly a

conservative interpretation of the field may overlook significant but not clearly obvious cells or if diagnostic cells are not plentiful on the smear, representative cells may be missed during scanning. However, these factors are not so important as other reasons for the occurrence of false negative specimens, such as the nature of the lesion, (Rovin, 1967)[115], and errors in sampling. (Sandler, 1966) [125, 126]. Encrusted lip lesions yield very little exfoliative material of diagnostic value, and oral lesions covered by necrosis may not yield representative cells. (Rovin, 1967) [115]. Smears which are not representative of the lesion or contain inadequate cells will, of course, result in "misleading" cytological reports and may produce a false sense of security in the clinician. (Sandler, 1966)[126]

One feels that explanation of the basis by which a report is declared positive or negative should be always made. Usually, biopsy is the standard against which the cytology report is held but it has been readily conceded that biopsy reports are not absolute. Since spontaneous healing of neoplasms without treatment is extremely rare, a cytology report indicating that a lesion is positive prior to the lesion completely regressing, could be regarded as falsely positive. However, as Cohn (1963)[21] has pointed out, epithelium can be in a "restless state"

with malignant potential (and presumably will exfoliate doubtful cells) but under favourable conditions will heal completely. Thus it seems quite possible that cytology may demonstrate truly representative cells from a "reversible" dysplasia, which, after removal of irritating factors will alter sufficiently in a period of weeks to present a benign histological appearance on biopsy, and will make the cytology report appear falsely suspicious or positive. Obviously, this problem will exist with routine oral cytology, but should be borne in mind when researchers attempt to assess the accuracy of the technique under control conditions.

Oral cytology is essentially an aid to oral diagnosis and the reports made on the specimens should have optimal reliability to be of greatest value to the clinician. It is equally unacceptable to the clinician to have no false negative reports but many false positives (as occurs with fluorescent cyto-diagnosis (Caulder, 1967)[25] as to have no false positives by sacrificing the main purpose of cytology in detecting early or subtle malignant changes. Either situation will produce frustration and eventually complete rejection of this valuable technique by the clinician. It is necessary therefore that cytological grading be approached objectively with the aim of

minimising the incidence of both false positive and false negative reports, but that both pathologists and clinicians should be prepared to accept the consequences of an occasional false positive (McGrew, 1961)[78], or false negative report.

The consistency of reporting on histopathological specimens was the basis of a recent study (Cocker, Fox and Langley, 1968)[27], where it was found that considerable variation occurred between different pathologists or with the same pathologist over a period of 18 months in applying criteria to diagnose cervical epithelial abnormalities. The greatest variation occurred in categories of carcinoma-in-situ and borderline carcinoma-in-situ. Naturally, grading in oral cytology is prone to the same subjectivity and shift in diagnostic criteria, and the suggestion that standard photographs are valuable as a reference guide certainly seems applicable.

THE VALUE OF ORAL EXFOLIATIVE CYTOLOGY.

The inadequacies of the conventional diagnostic proceedure of history, clinical examination, observation and biopsy have been clearly shown in other projects [1, 20, 120], and have become evident in this project, as early oral carcinoma may often appear innocuous and therefore escape biopsy. Furthermore, there appears to be some danger in the attitude adopted by many clinicians with regard to a doubtful lesion, of treating it initially as a simple lesion, with the view of biopsying it if there is no improvement next visit. (Cooke 1958)[30]. Not only may a false sense of security be engendered in the patient, whom the clinician has been careful not to alarm unduly, but a clinical impression is often rather subjective, and therefore may not be consistent from one week to another.

Silverman et al (1958)[139] have pointed out that the clinician is not asked to choose between cytology or biopsy, but between cytology or nothing. Oral surgical proceedures and biopsy techniques, indications and contra-indications, are clearly set out in standard textbooks of oral surgery. (Archer, 1966)[6] (Castigliano, 1965, p.569)[24]. Cooke,(1958)[30] has summarized various biopsy procedures and claims agreement with Boyle, that in the presence of clinical suspicion of intra-oral squmous carcinoma, the biopsy should be taken by

the specialist who will assume responsibility for the treatment. Obviously, small non-suspicious lesions are best examined by biopsy when first seen in general dental or medical practice. Sinclair et al (1968)[137] claim a 100 percent accuracy with biopsy, but note that false negative biopsies may result if inadequate tissue is taken (or presumably, tissue from a non-representative part of the lesion) or the histological patterns are indefinite. It is interesting that, although Cooke (1958)[30] does not agree with cytological smears as a basis for diagnosis, he suggests that they may be of value in the assessment of leukoplakic lesions, a point strongly supported by Pindborg et al (1963)[103].

ADVANTAGES OF CYTOLOGY.

Simplicity. The oral smear technique is extremely simple as a clinical routine and requires no elaborate instruments or equipment (Sandler et al, 1960)[121] to be carried out satisfactorily in very little time. The laboratory procedures of staining and seaming are also relatively simple.

Patient Acceptance of oral cytological smears is without problems as the technique causes neither inconvenience , (Sandler et al, 1960)[21] nor trauma, nor pain (Sandler, 1964)[124] and is "bloodless". (Carsen, 1960)[26].

As a result smears can be repeated as often as necessary. Numerous specimens can be taken from various parts of the mouth at the same time, thus providing a great amount of material for examination when necessary as a supplement to biopsy. (Schenan, Altchuler and Wilson, 1967)[129].

Because of the minimal amount of damage caused to tissue, (Carson, 1960)[26], the technique allows microscopic examination of tissue following irradiation. Oral cytology in relation to post-irradiation cases is to be dealt with more thoroughly elsewhere in this thesis.

ACCURACY AND RELIABILITY OF ORAL CYTOLOGY AS A CANCER DETECTION METHOD.

The detection of carcinoma-in-situ or intraepithelial carcinoma by cytology, even in the face of negative biopsy, (Morrissey, 1961)[89], has been shown by numerous workers (Sandler et al, 1960)[121], (Usher et al, 1960)[173]. Cahn (1963)[21] has noted the discovery of early signs of restless epithelium in oral cytological smears, and the finding of dyskaryotic cells in oral smears correlates with dyskeratosis in the tissue section taken from the lesion at the same time. Furthermore, cytology has been found invaluable in assessing changes during follow-up of chronic oral lesions such as leukoplakia. (Sandler and Cahn, 1958)[119]. Cooke (1958)[30], (1962)[33] a strong critic

of routine oral cytological methods as a means of cancer detection, states that no investigation has evaluated exfoliative cytology in the diagnosis of non-ulcerative early oral carcinoma, and further notes the failure of a number of studies to find diagnostic epithelial cells from suspicious areas of leukoplakia prior to fissuring and ulceration. However, in discussing selection of a site for biopsy in areas of leukoplakia, he points out (Cooke, 1958)[30] that the most representative part of a leukoplakic lesion is not the margin or most heavily keratinised part, but an area of ulceration or granulation, where the deeper cells are dividing rather than keratinising.

The literature contains numerous studies, which show a high degree of accuracy and reliability of oral cytology as a general application (Williamson and Shapiro, 1964)[188] to detect oral carcinoma in groups of people (Von Haam, 1966)[56] (King, 1963)[68]. In terms of individual situations the accuracy varies, having maximal accuracy with ulcerative (King, 1962)[67] and erythematous lesions, but minimal accuracy and hence, unreliability, in at least three specific situations, namely:-

(1) encrusted lip squamous cell carcinoma (Rovin, 1967)[115] (Umiker et al, 1959)[172].

(2) advanced squamous cell carcinoma covered by a necrotic slough. (Rovin, 1967)[115].

(3) tumours producing no break or growth through overlying mucosa but presenting simply as a lump or swelling. (Umiker et al, 1959)[172], (Williamson and Shapiro, 1964)[188].

Furthermore, any assessment of the accuracy of cytology must assume that consistently representative smears are taken in each case. False results in many cases may represent sampling errors. (Tieke and Blozis, 1966).[168]

Correlative figures have been given in a number of studies to indicate accuracy of oral cytology. Sandler (1958)[119], (1961)[122] indicates an accuracy correlation of .91 - .92; Umiker et al (1960)[173] found a .96 correlation of cytology with biopsy and clinical finding, Von Haam, (1966)[56] claims an accuracy of .86 and Cawson (1960)[26] found .81 accuracy. In a survey of literature, Selbach and Von Haam (1963)[131] found a correlation of .925 between positive cytology and patients with confirmed oral cancer. (Blozis, 1965)[16].

The studies recorded can be basically divided into three types, depending on the nature of lesions examined - that is, whether they represent a random selection of lesions, or consist of suspicious or provon malignant lesions, or consist only of benign lesions. It is felt that objectivity in examining cytological smears, may vary depending on the orientation of the study.

STUDIES ON A RANDOM SELECTION OF ORAL LESIONS.

The most impressive oral cytology study to date (The Veteran's Administration Study directed by H.C. Sandler,[121, 122, 123, 124, 125, 126.]) can be included in this category. In a series of 2,758 patients, cytology was positive in 307 cases of which seven were falsely positive, all others being confirmed by biopsy. The cytology was negative in eight cases which were subsequently shown to be malignant. In 22 cases, multiple biopsies were necessary to confirm a cytological indication of malignancy. Strong criticism has been levelled at this study by Shapiro and Gorlin (1964)[133] on the grounds that for a large group of the subjects (2,166) where the clinical picture was not suspicious and the cytology was negative, no biopsy was obtained, thus giving no indication of the accuracy of the negative cytology report. These authors (Shapiro and Gorlin, 1964)[133], in retrospect, felt that the same criticism applied to their other investigation of 404 intra-oral lesions. In an earlier study of 51 patients (38 had oral cancer) Sandler and Stahl (1958)[119] reported four false negative smears.

Meke, Calandra and Kendrick (1960)[166] examined 150 patients and obtained 43 positive smears from 50 confirmed neoplasms. The seven false negatives occured early in the series, and there were no false positives from normal tissue.

In a later project (Tieke, Hendrick and Calandra, 1961)[167], 75 oral lesions (including 53 cancers) were examined cytologically and histologically, resulting in eight false negatives, but no false positive smears. In a Chicago project in 1965, smears were obtained from 90 lesions (62 with cancer), and two false positives and 11 false negative smears were obtained. (Tieke and Blozis, 1966)[168].

Alling and Secord (1968)[1] correlated 177 smears with biopsy and clinical findings. Thirty four smears were positive, all being confirmed. No false results were noted.

Relatively high numbers of false negative results were obtained in three other similar studies. Cawson (1960)[26] in Britain, obtained three from 29 confirmed malignancies in a series of 40 patients, Rovin (1967)[115] found 17 from 48 confirmed malignancies in a series of 372 patients, Shklar et al. (1968)[137] found 12 from 70 confirmed malignancies in a series of 2,052 patients. It is worth noting here that in all of these studies, no false positive results were obtained, suggesting that grading of smears was carried out very conservatively, which, while eliminating the incidence of false positives, also decreases the sensitivity of the technique to detect subtle malignant change. (McGrew, 1961)[78].

Gaither (1967)[40] examined 75 visible oral lesions, of which 34 were confirmed neoplastic, and obtained ten false negative smears and one false positive.

Some earlier cytological studies resulted in no false positive or false negative reports. In 1949, Nordson, Hopp and Hu,[88] used the smear technique to examine 22 patients with naso-pharyngeal lesions of which ten were malignant. Gladstone's (1950)[45] sponge biopsy technique was used successfully on ten squamous cell carcinomas on a series of 38 patients, and Wahi and Gupta (1954)[179] carried out a study on 50 patients, 41 of which had oral cancer.

King (1962)[67], in a series of 179 patients, (52 with malignancy), experienced one false negative, but no false positive cytology results. Silverman (1959)[140] reported no false cytology results in a series of 100 patients, seven of these had confirmed oral cancer. Ingram et al (1963)[64] experienced four false positives as a result of repeated misinterpretations early in the project of an atypical cell found in smears from leukoplakia. Subsequent examination of their material removed this difficulty. Their study included 422 lesions of the oral cavity of varying types, 17 being overt carcinomas, but were unable to state an accurate incidence rate of false negative smears.

Studies on Malignant Oral Lesions.

A number of studies have been made, using cytology correlated with biopsy, on lesions known to be malignant or clinically diagnosed as malignant. Presumably these studies were, in most cases, aimed at assessing the incidence of false negative cytology reports. The validity of such reasoning can perhaps be questioned on the basis that since most of the lesions were clinically obvious or suspicious (and therefore probably not early lesions) the incidence of false negatives occurring in situations where cytology is of most value, namely clinically innocuous lesions, is not really being assessed. The (below) table outlines the results of these studies or sections of studies associated solely with malignant lesions, therefore, negative results constitutes false negatives +

Ref.	Year	No. of Patients	Positive Cytology	Negative Cytology
Montgomery & Von Bern.	05	1951	35	13	2
Pendegrass and Stehl.	110	1953	9	?	1
Peters.	100	1956	263	252	11
Rapp.	62	1956	25	24	1
Silverman.	139	1958	15	14	1
Endlow	173	1960	80	77	3
Hirsch and Von Bern.	131	1963	93	90	3

(Positive includes suspicious)

TABLE I

The converse of the above, namely a cytological comparison with biopsy in a large series of clinically non-suspicious cases, while possible, is unlikely to be meaningful, especially in situations such as leukoplakia where biopsies may not represent the most active centre. Montgomery and Von Haam in 1951[84] noted qualitative and quantitative exfoliated cell changes on ten patients with leukoplakia, but found no significant atypia with this condition. Walker (1960)[173] attempted to correlate cytological findings in 45 cases of leukoplakia, but as biopsies were not obtained at all in 17 of these, the number of false results (negative or positive) cannot be assessed accurately. Similarly, Peters (1956)[100] in his study of 893 patients included 58 with clinical leukoplakia, but found that long term clinical follow-up and biopsies would be necessary to determine the accuracy of the smear.

Solbach and Von Haam (1963)[131] correlated cytology and biopsy in 30 cases of leukoplakia and 167 cases of inflamatory or otherwise benign oral lesions. In the leukoplakia group, 26 smears were negative, six suspicious, but none were graded definitely positive. In the other group, only six lesions yielded suspicious smears, which was considered a relatively low level of false suspicions, justifying the conclusion that benign oral lesions do not simulate cancer cytologically.

The finding of unexpected malignancies in studies has been reported [16, 64, 100, 122, 131.] as has the occurrence of 'false negative' biopsies by various authors [16, 100, 122, 139].

DISCUSSION ARISING FROM THESE PUBLISHED RESULTS.

The accurate establishment of the incidence of false negative reports in oral cytology is notoriously difficult (Ingram et al, 1963)[64], (Shapiro and Gorlin, 1964)[133], as biopsy reports are not absolute nor are clinical features. Sandler (1966)[125] noted the occurrence of oral malignancy on follow-up in lesions which were originally considered clinically innocuous and yielded negative or atypical smears, and thus were not biopsied. This implies clinical and cytological error if, at the time of the original appointment, the lesion was indeed malignant, or in the process of malignant change, but, and this can never be ascertained, if malignancy had not actually begun at that stage, the smears cannot, in retrospect, be called falsely negative.

The percentage number of false negatives has been incorrectly recorded in some studies, according to Rovin (1967)[115], by giving the percentage of false negative smears for total number of lesions rather than percentage of false negative smears for total number of malignant lesions, which would obviously give a much higher figure.

In his study, he found that the nature of the lesion, that is encrusted or necrotic surfaces was the main cause of false negative smears, although this explanation did not apply to all cases.

Ellis (1965)[16] has stressed that the chief point of the Veteran's Administration study, is that smears were made from all lesions whether clinically suspect or not, and, that as a direct result 62 unsuspected cases of oral cancer were detected that would otherwise have been passed over at that stage.

With regard to false positive oral cytology results, which Sandler (1966)[125] claims are always a source of embarrassment, it can be seen that few recorded studies experienced any at all. Graham (1964)[52] P.111 notes that a false positive figure of 5 percent is not regarded as excessive in gynaecological cytology, which would appear to be in agreement with Nature's (1961)[78] statement that both clinicians and pathologists must be prepared to accept the consequences of an occasional false positive report.

The Advantability of the cytological method to the clinical conditions is a valuable factor. Firstly, where a lesion is extensive and defies complete biopsy or selection of the most suitable biopsy site, direct smears allow screening of the entire surface (Cooke, 1958)[30] and may

indicate the most representative area from which to take a tissue specimen. (Umiker, 1960)[174]. A similar situation applies with multiple lesions. Secondly, diffuse widespread lesions may be best screened by smears made from mouth-washings or gargings, which have been found reliable. (Molspor and Sharp, 1964)[59], (Watanabe and Shiraishi, 1961)[181] Collection of secretions or irrigation fluids and needle aspirations have all been shown as reliable means of obtaining cytological specimens in special situations.

LIMITATIONS OF ORAL CYTOLOGY.

The oral cytological smear, while a valuable aid to diagnosis, is not a substitute for biopsy, which is the diagnostic procedure of choice. (Sandler et al, 1960)[121]. Although the smear technique has numerous advantages and obviates some of the undesirable features of biopsy, (Von Haam, 1966)[56], (Silverman et al, 1958)[139], it has a restricted application. (Selbach and Von Haam, 1963)[131], (Williamson and Shapiro, 1964)[188]. Positive cyto-diagnosis must always be confirmed by biopsy. (Graham, 1964)[52], (McGrew, 1961)[78]. Tumour type and degree of differentiation is less reliably recognised from smears than from biopsy specimens (King, 1962)[67], (Ehrlich et al ,1960)[173], a disadvantage resulting from the fact that the cells are isolated (Shapiro, Gorlin and Jordan, 1964)[134] and growth patterns (and gross tissue architecture (King, 1962)[67] cannot

be studied as in tissue sections (McGrew, 1961)[70]. Cocio, (1962)[33] has emphatically pointed out that biopsy can be the only basis of diagnosis of malignancy, as loss of polority, dyskeratosis and disorientation of cells can only be observed in tissue section and not amongst isolated cells of smears. This criticism must apply not only to oral cytology, but to exfoliative cytology in general. The results of the present study do not support the suggestion that exfoliative cytology is of value solely in ulcerative oral malignancies, although smears from encrusted lip squamous cell carcinoma (Rovin, 1967)[115], (Shapiro, Gorlin and Jordan, 1964)[134], and from necrosis covered oral neoplasms [79, 115, 172.] are somewhat unreliable. Obviously, if a cancer does not involve the surface, malignant cells cannot be exfoliated, and a cytological smear of a squamous cell carcinoma presenting simply as a subaucoal lump or swelling in the oral tissues, is virtually useless (Mulder, 1967)[25], (Williamson and Shapiro, 1964)[183], Shapiro et al (1964)[134] stress that it is absurd to imply that a scraping from a lesion could reveal malignancy more readily than a biopsy, where, not only are there more cells obtained, but in situ tissue relationships are preserved. The best application of exfoliative cytology is for erythematous and ulcerative surfaces (Cahn, 1961)[20],

(King, 1962)[67] rather than, for example, simple keratosis, although King et al (1966)[69] suggest that the superficial cells may be diagnostically more valuable than deeper layer cells.

INDICATIONS AND CONTRA-INDICATIONS FOR ORAL CYTOLOGICAL SMEARS.

In all situations where the clinical findings suggest carcinoma, biopsy is clearly indicated [30, 33, 64, 110, 121, 164], and a cytological smear may serve as a supplementary aid, (Blozis, 1965)[16], or be used for correlation. Ingram et al, (1963)[64] were not impressed with the reliability of smears from overt carcinomas. Delay in first taking smears when a biopsy obviously should have been taken may lead to loss of valuable time before treatment is instituted. (Shapiro, Gorlin and Jordan, 1964)[134]. However, as Blozis, (1965)[16] pointed out, citing results of the Veteran's Administration Study, where in 22 patients more than one biopsy was necessary to confirm a cyto-diagnosis of malignancy, cytology should be used in conjunction with biopsy, as errors in biopsy through inadequate tissue sampling, do occur. The cytological technique is most valuable in detecting malignant changes in small innocuous appearing lesions (Alling and Secord, 1968)[1] or in cases where there are clinical suspicions although the biopsy was negative. (Silverman, Doers, and Farber, 1958)[139]. The consistent

finding of suspicious cells in smears obligates the clinician to take repeated biopsies until the histological diagnosis is definite. (Sandler, Freund and Stahl, 1959)[120]. Candler (1961)[122], (1962)[123] reports seven instances in a series of 139 patients whose cytology suggested malignancy, but repeated biopsies were necessary to confirm it. An explanation offered for this sort of situation is that the smear contains cells from a much wider area of tissue than biopsy. (Cooke, 1959)[30], (Sandler and Stahl, 1959)[119]. Hendershott (1968)[60] notes that the national body set up for the control of chronic diseases in U.S.A. has emphasised the value of oral cytology and, while admitting that false negatives do occur, has pointed out that there is a danger in the clinician regarding the biopsy technique as foolproof. It has been stressed (Cahn, 1961)[20], (Sandler, Freund and Stahl, 1959)[120], that a negative cytology or biopsy report is not conclusive, but the clinician can accept a positive report as highly significant. After cytological examination of 40,373 oral smears, Colbach and Von Haam (1963)[131] state emphatically that cytology is not indicated as a mass screening for oral cancer, as, firstly, the incidence of the disease is fairly low (at least in most European countries, in contrast to India where oral cancer represents 36 percent of all cancers seen) (Sirsat and Doctor, 1967)[148] and, secondly, they found that the

utilisation of 'untrained' personnel to take smears was unsatisfactory. However, they, with others, (Sandler et al, 1960)[121], (Sandler and Stahl, 1958)[119], feel that cytological examination of all visible oral lesions is valuable. Oral cytology is well suited as a clinical aid to the follow up of long standing chronic lesions such as leukoplakia, (Sandler and Stahl, 1958)[119], denture irritation, erythema, or lichenplanus. Although Shafer, Hine and Levy, (1963, p.696)[132], indicate the essentially benign nature of lichenplanus, Pohl, Path, and Rohan, (1966)[178] include it in their study as a pre-malignant lesion, and in the present project a lesion clinically diagnosed as erosive lichenplanus was subsequently shown by oral cytology to be, in fact, malignant. It is interesting that in one study of leukoplakia patients (Silverman and Rosen, 1969)[147] the decision not to perform a biopsy was made if (a) the lesion was not clinically suggestive of carcinoma, (b) underwent no change between initial visit and follow up appointments, and (c) the exfoliative cytology was normal. Cancers of the soft palate may assume the appearance of an inflammatory process, with no palpable induration, a situation in which cytology can be particularly valuable. (Sandler, Freund and Stahl, 1959)[120]. Cooke, (1963)[33] has suggested that the greatest value of oral exfoliative cytology is in differential diagnosis of bullous or vesicular lesions, herpes, pemphigus, or other conditions

which produce oral hullae. He sets out (Cocho, 1963)[33] three situations where cytology could be of value in oral cancer detection -

(1) for screening large areas of abnormal mucosa as an aid in selection of biopsy site.

(2) as a preliminary investigation in a patient too nervous to allow biopsy.

(3) Finally, oral smears are indicated for post-irradiation follow up on patients with a previous oral cancer. Residual or recurrent neoplasm can be detected cytologically after treatment, (Sandler et al, 1960)[121], and indeed, in this project, residual recurrent tumour in a number of patients was indicated by the exfoliative smears some months prior to obvious clinical changes.

EFFECT OF RADIOTHERAPY ON ORAL EXFOLIATIVE CYTOLOGY.

General Considerations.

Treatment of oral cancer in this country is by one of several methods - irradiation; surgery; chemotherapy or combinations of these. Various factors influence the choice of treatment methods such as anatomical position of the primary lesion, its size, degree of invasion into adjacent tissues, and the presence or absence of probable lymph node metastases. (Frazell and Lucas, 1962)[39]. In the U.S.A. in recent years, radical surgical procedures have gained wide

acceptance in the management of oral cancer. (Gobbel, Adkins and Sawyer, 1967)[47]. However, in treatment of lip carcinoma (the most common oral malignancy) and malignancy in various other oral regions, radiotherapy, with it's effectiveness and ability to minimise deformity appears to be the treatment of choice.

In 1945, Gluksmann and Spear (1945)[46] reported that maturation of tumour cells, rather than destruction, was the important factor in 'radiocurability'. Later, however, others (Watanabe et al, 1962)[182], found no correlation between maturation and tumour sensitivity, radiation dose or clinical results. Impaired respiration and increased glycolysis in tumour cells has been accepted since the 1920's, and it is thought that the mitochondria, which regulate these cellular functions, may be deficient in structual protein (Gause, 1967)[44]. Andrews (1968, p.129)[5] places great importance on the oxygen effect in tumours influencing the response to radiotherapy, and thus altering apparent radio sensitivity. Oral cytological smears have been found to provide a painless atraumatic method for the study of radiation changes in tumours. (Umiker, 1960)[174].

Radiation Changes in Exfoliated Cells.

In 1951, Graham[50] described the effects of radiation on exfoliated cells (malignant or benign) which had earlier resulted in false positive cytological diagnoses.

UNIVERSITY OF SYDNEY DENTAL LIBRARY

The four main changes are:-

(1) Vacuolisation of the cytoplasm.

(2) Grossly increased size of the cell (giant cells).

(3) Multiple nuclei (rarely seen in non-irradiated cells).

(4) Pronounced nuclear changes.

The same radiation changes (radiation response) occur in malignant and normal cells, although they are seen to take place much more rapidly in the former. A more detailed presentation of these changes has been made recently by the same author (Graham, 1964, Ch.13)[52], and can be summerised as follows.

Vacuolisation of the Cytoplasm.

This change occurs first and is originally limited to basal cells, though later it is evident in intermediate cells, but rarely is seen in superficial cells. The vacuolisation in some cells may be fine and give the appearance of fine fibrillation. Vacuolisation is not a specific radiation effect, and may be seen in basal cells in pre-treatment smears.

Increase in Cell Size.

Squamous epithelial cells may react to irradiation (especially at about 3,000 rads.) by increase in overall size of cell and nucleus. The 'Nuclear/Cytoplasm size ratio' in normal cells is maintained as is the fine nuclear chromatin pattern. Like all the other changes, this is not specific,

and it may occur as a result of any interference to cell division. After radiation, the nuclei appear to divide, but the cells, as a whole, do not, hence resulting in multiple nuclei.

Multinucleation.

This is relatively common and produces no diagnostic problems unless the nuclei are atypical.

Wrinkling of the Surface of the Nucleus is related to the increase in size of the cell, or more specifically the nucleus, which appears to outgrow the space allowed in the cell. Nuclear wrinkling must be differentiated from chromatin irregularity.

Bizarre cellular shape is much less frequently seen in post-radiation smears but should be recognised and differentiated on the basis of nuclear morphology from bizarre malignant cells. It is likely that radiation changes persist in the exfoliated normal cells for some time, even years after treatment. (Stahl and Wiman, 1962)[152]. Graham,1964)[52] notes that radiation produces one of two reactions on exfoliated cells. Firstly, some degenerative changes are caused by the direct effect of the radiation producing destruction of cellular structures. Such cells are dying cells, and exhibit either broken cellular borders, or nuclear chromatin which has merged into amorphous blobs,

and the cell borders are indistinct. In contrast, however, cells showing radiation response are active cells, and are probably still viable when exfoliated. The giant cells are probably the result of suddenly increased growth and protein synthesis, as their cytoplasm is deeply staining, and has an intact border and the nuclear chromatin is well preserved. Vacuolisation indicates pinocytosis and metabolic activity rather than degeneration. Histiocytes and polymorpho-nuclear leucocytes form the typical background of an irradiation smear. It is of utmost importance that radiation effects be recognised to help assess the reaction to treatment, and, in post treatment smears, to differentiate effected cells from malignant cells in the diagnosis of residual or recurrent tumour.

Value of Smears during Radiotherapy.

Much of the original work on the prognostic value of exfoliative cytology was also done by the Grahams (1951)[50], (1955)[51]. Cell counts were performed on smears taken from patients during radiotherapy and plotted on graphs. The number of benign cells showing radiation response per 100 benign epithelial cells, and also the percentage number of malignant cells present were recorded. It was found that two distinct types of graph resulted - those showing a high percentage of radiation response (above 75 percent, called good response) and those showing a low percentage of radiation change (poor response group). With respect to the

presence or absence of malignant cells in smears taken during radiotherapy, it is interesting that Graham (1964, P.170)[52] has found different situations to exist with different forms of radiotherapeutic treatment. Originally, it was felt that the presence or absence of malignant cells in smears was of no prognostic significance, but later, with smears from patients treated by a different radiotherapeutic method, the presence of malignant cells in post treatment smears was found to be unfavorably significant. It was concluded that the presence of malignant cells in cervical smears taken from patients at the end of radium therapy is associated with a poor prognosis, but the prognosis is not indicated by the presence of such cells in smears from patients who have received mainly external irradiation, such as cobalt.

Oral carcinomata receive either external irradiation or radon implantation, if they are to be treated by radiotherapy. Unger et al (1959-1960) [172, 174] have noted that in smears from oral cancers, as in cervical cases, (Graham, 1964, P.170)[52], the desquamation of malignant cells during the first one to two weeks of treatment, appears to increase, after which, they decrease in number and disappear if the tumour responds to the radiotherapy, or in more resistant cases, the malignant cells persist throughout the entire treatment. Even so, the presence of malignant cells in smears taken at the end of

treatment is not necessarily an indication of failure of treatment, but should such cells be persistently present in smears taken several weeks after treatment, residual or recurrent neoplasm is generally the cause. Negative smears, at the end of treatment, on the other hand, are of little value, as they do not rule out the possibility of residual tumour. Radio-sensitivity and disappearance of malignant cells from smears was found to have no relationship to the initial size of the tumour. (Umiker et al, 1959)[172].

Silverman and Sheline, (1961)[142] reported on the application of Graham's methods to assess the outcome of treatment of oral carcinoma and found that in this series the radiation response in nearly all was below 50 percent, possibly due to poor clinical prognosis in each case, and different criteria to that which apply in cervical smears. They claim that the morphological features previously described appear to be of little prognostic value, with vacuolisation and multinucleation not being significantly increased,and nuclear enlargement and wrinkling frequently seen. However, the persistence of malignant cells in post treatment smears was, in all cases, seen to be associated sooner or later with recurrent neoplasm. Similar results have been reported by Hopp (1958)[61], and have also been found by me. In a recent paper Silverman, Sheline and Gilooly (1967)[145] have confirmed their early findings;

that morphological changes in normal exfoliated oral cells after radiation reflect neither radio sensitivity nor the local controlling effect of the treatment, which they claim is consistent with the findings of Umiker; but that the persistence or recurrence of malignant cells in smears appears to be a useful supplement to clinical assessment.

THE CLINICAL VALUE OF POST-TREATMENT ORAL SMEARS.

Oral cytology is of effective assistance in the follow-up of patients treated for oral cancer. (Sandler et al, 1960)[121], (Shapiro, Gorlin and Jordan, 1964)[134]. Following treatment, there frequently developes fistulae, ulcerations, granulation tissue, some as a result of residual or recurrent neoplasm, others from radionecrosis or infection. In such situations the poor healing ability of the tissues in many cases rules out biopsy and the clinical features are equivocal. Cytological smears in such situations constitute a convenient diagnostic aid which, providing radiation changes in cells is recognised as such and not interpreted as malignancy, is reliable. (Shapiro, Gorlin and Jordan, 1964)[134]. Umiker et al, (1960)[173] have noted that the sudden appearance of pus cells in post treatment smears from patients where previous smears contained none, indicated either recurrent neoplasm or radionecrosis. The value of such indirect criteria is small

In cases where differential diagnosis between the two conditions is required, and conventional morphological criteria of the epithelial cells must be closely applied. One study which Umiker et al, (1960)[173] carried out indicates that cytology had a degree of reliability overall, in diagnosis of post treatment lesions. Although it is accepted that the persistence of malignant cells in post treatment smears is almost certainly an indication of recurrence, Silverman et al, (1967)[145] indicate that negative post-treatment smears are of virtually no significance, as in his project in 43 percent of cases which were not at any stage considered controlled clinically, negative smears were obtained. It is, however, admitted that in many of these false negative cases, the residual tumour showed deep induration, but no ulceration, or the lesions had undergone surface changes, presumably necrosis or crusting. In such situations, exfoliative cytology by normal sampling techniques (such as scraping) is not indicated. Gardner (1964)[42] has indicated that very few malignant cells are present on the surface of residual oral carcinoma, and hence, few are found in smears. To help overcome this, it has been recommended that more than one smear be taken and examined.

Morphological changes in exfoliated oral epithelial cells have been noted (Monto, Fino and Mizok, 1963)[86] in patients receiving chemotherapy for oral cancer. As with radiation changes, the most common effect is variation in size and shape of cells. It was found useful to observe these cell changes as an indicator of early toxicity in the patient, which might not be obvious clinically.

EVALUATION OF EXFOLIATIVE CYTOLOGY AS AN AID IN ORAL DIAGNOSIS

Experimental Work

Introduction

The aim of the investigation was primarily to determine the practicability and clinical reliability of oral cytology in various situations associated with oral disease. In addition a number of other factors arising during the course of the investigation have been examined. The most important of these was an attempt to determine the significance of atypia in exfoliated cells from non-malignant oral lesions, with regard to premalignancy and an assessment of the diagnostic value of exfoliated cells from benign oral conditions which have been reported as pathognomonic, such as bullous and vesicular lesions.

The application of exfoliative cytology in oral radiotherapy also has importance. There are two issues involved here; firstly to determine the reliability of the technique in the detection of residual, recurrent or new primary oral malignancy in patients who had been irradiated already for an oral cancer; and secondly, to evaluate the extent to which cytological smears reflected the clinical progress of the lesion throughout treatment and the prognostic significance of the cellular changes

observed.

Materials and Methods.

The material used consisted of epithelial smears obtained mainly from oral lesions of two groups of patients. The largest group were patients attending the surgical dressings department associated with the Department of Oral Surgery at the United Dental Hospital of Sydney. The other group consisted of patients attending the Cobalt Therapy Unit of Royal Prince Alfred Hospital, Sydney.

In some cases, patients seen in the Dental Hospital were again seen at Royal Prince Alfred Hospital later. A small number of smears (twelve) were received from private dental practitioners.

The 240 dental patients from whom smears were prepared were selected on the basis of the clinical features of their oral lesions. Smears were generally taken routinely from soft tissue lesions, excluding those where a definitive diagnosis of a simple inflammatory process was obvious, such as pericoronitis, aphthous ulcers, alveolar abscess. Some smears were also obtained which provided material for a study of the morphological changes in aphthous ulcers, herpetic, and bullous lesions.

The radiotherapy patients included were selected on the basis of the clinical condition of their oral neoplasms.

Smears were generally taken from all neoplasms, either when first seen in the Clinic, or early in their course of treatment, excluding the majority of lesions which were covered by a necrotic slough or were encrusted, such as on the lip. Follow-up smears were also taken from a number of patients - usually those with possible residual or recurrent neoplasm.

The selection of patients was not influenced by age and sex.

Repeated smears were taken of seven patients during and after radiotherapy.

Equipment.

1. Standard microscopic glass slides (7.6 x 2.5 x 1cms) were used. These were cleaned by thoroughly wiping with a clean towel, after removing from a jar of absolute alcohol in which they were stored for at least 12 hours prior to use. The slides were mounted with 2.2 x 3.7cms cover slips.
2. Small wooden spatulas (10.7 x 1 cm) were sterilised and kept in 6 x 1 in. sterile test tubes containing sufficient normal saline to thoroughly moisten one end of the spatulas. Gauze swabs 7 cm square were used in preparation of the field.

3. Fixative.

The following solution was used and kept in small jars sufficiently large to contain the glass slides immersed -

Carnoy's Solution.

70 ml. 90% Ethyl alcohol.
25 ml. Chloroform.
5 ml. Glacial acetic acid.
per 100 mls. solution.

4. Staining solutions.

Absolute ethanol.

95% Absolute ethanol (95 parts ethanol, 5 parts distilled water
80% " " (80 " " 20 " " "
70% " " (70 " " 30 " " "
50% " " (50 " " 50 " " "

Distilled water.

Harris Haemotoxlyn smear stain -

OG 6 smear stain -

EA 50 smear stain -

As prepared by Ortho Pharmaceutical Company, Sydney.

Scott's Blueing Solution.

Sodium Bicarbonate 3.5 gm.
Magnesium Sulphate 20 gm.
2 - 3 small crystals of thymol.
1,000 cc distilled water

Xylol.

The solutions were kept in containers sufficiently large to allow immersion of the slide into solution.

5. Clinical record and report form.

6. Leitz Wetzlar microscope with -

low power magnification of 100x;

High power magnification of 450x;

Oil immersion magnification of 1,000x.

Additional materials occasionally used when altornate collecting procedures were employed.

Normal saline (0.85% NaCl in distilled water)

M.S.E. Centrifuge.

Standard platinum wire loop - 24 gauge; approx. 5cm. long.

Cotton-tippod applicator sticks.

10 cm. long metal irrigating syringe.

20 ml. glass barrel aspirating syringe with 16 gauge needle.

Clinical Procedure.

The patient's oral cavity was examined and soft tissue lesions assessed for suitability on the basis of:-

(a) Access.

(b) Extent and nature of the lesion.

(c) Provisional diagnosis and, to some extent,

(d) Clinical history.

Cellular material was collected by firmly stroking the lesion a number of times with the spatula then wiping the material collected on to a glass slide. Preparation of the tissue prior to scraping varied with the situation, nature and location of the lesion. Necrotic slough covering a lesion was always wiped away. Where the field was surrounded by saliva, the tissue to be smeared was isolated with a gauze swab. Some lesions, particularly those on the lips, were too dry to obtain smears from initially, in which case, the patient was asked to moisten it with his tongue.

An attempt was made to get cells from cracks and fissures in those leukoplakic or hyperkeratotic lesions that were fissured. In some cases, a number of smears were taken, firstly of the superficial cells, and then of deeper cells after the thick keratinised cover had been partially removed.

Where the lesion was small and access satisfactory, only one smear was taken generally. If the first smear seemed inadequate, or if the lesion was large or its surface not firm, or was in the presence of excess secretions, more than one smear was taken.

In a number of cases, 1 percent toluidine blue supravital stain was applied to the lesion after the technique

suggested by Keibel and Chomet (1964)[92], to help delineate areas of tissue most likely to yield diagnostic cells.

A brief relevant history and clinical description of the lesion was noted on a form with the patient's name, age, sex and hospital registration and file number to assist in the grading of smears and the subsequent follow-up.

Immediately the material was smeared on to the glass slide, the slide was placed in a bottle of Carnoys fixative. If more than one smear was made, the slides were placed 'back to back' to avoid disturbing the cells. A minimum time of fixation was 30 minutes.

Staining.

The slides, following fixation, were removed from the fixative, marked with the patient's name by a diamond pencil, and stained as suggested by Papanicolaou [96].

1. After fixation, slides were transferred, without drying, directly into 80 percent alcohol and run through 70 percent and 50 percent alcohols to distilled water.
2. Stained in Harris Haematoxylin for 1 minute.
3. Rinsed three times in distilled water, using three separate containers. (All rinsing should be gentle to prevent smears being washed off slides.)

4. Rinsed with 50 percent alcohol.

5. Placed in a solution of 1.5 percent ammonium hydroxide in 70 percent alcohol (or Scott's Bluing solution) for one minute.

6. Rinsed in 70 percent alcohol and run through 80 percent and 95 percent alcohols.

7. Stained in OG6 for 1½ minutes.

8. Rinsed in three jars of 95 percent alcohol.

9. Stained in EA50 for three minutes.

10. Rinsed three times in 95 percent alcohol, using three separate containers.

11. Dehydrated and cleared by rinsing in two containers of absolute alcohol followed by a mixture of equal parts of absolute alcohol and xylol and finally two containers of xylol.

The glass coverslip was mounted with Eukitt. The slide was then labelled with the patient's name, the date, and reference number. The latter was also noted on the record form.

Scanning.

Smears were scanned completely using low magnification (x 100) and progressively moving field by field over the slide, in descending rows. Higher magnification was brought into focus for cells requiring more attention.

Occasionally, oil immersion was used to study nuclear detail. Fields containing cells of particular interest or those suggesting some change from normal were marked with a dot of indian ink applied to the coverslip while viewing on low power.

Classification of smears.

Smears were graded according to the accepted classification basically, with a small modification being the insertion of a class for questionable or doubtful atypia. Smears were graded thus if they contained atypical cells that could probably not be suspected as malignant, but at the same time whose morphological characteristics were outside the understood range of atypia associated with benign lesions.

The classification used was as follows:-

Class I. Normal cells only present.

Class II. Atypia present but within acceptable normal range.

Class? II. Indeterminate.

Class III. Atypia suggestive of malignancy but not conclusive.

Class IV. Highly suspicious of malignancy, though not conclusive.

Class V. Conclusive for malignancy.

Classes I and II are considered negative.

Classes ?II and III doubtful - suspicious.

Classes IV and V positive.

The report based on the examination and grading of the smear(s) was noted on the record form, and a copy sent back to the clinician. In some cases, it was recommended that repeat smears be made, as a result of indefinite grading, inadequate numbers of cells present, or unsatisfactory preparation. Where doubt existed about cellular atypia observed in a smear, consultation was made with an experienced general cytologist. (Dr. E. Way)

Variation in Procedure.

1. To obtain cells from the base of a bullous or vesicular lesion, a standard platinum wire loop was used. The cells thus obtained were mixed with a small drop of normal saline on a glass slide, then when the excess moisture had dried, the preparation was treated as a normal smear.

2. In situations where access to a particular area was poor, such as the posterior third of the tongue or oro-pharynx, or the involved mucosa was very extensive, or cells of unknown origin had been found in a previous smear, mouthwashings or gargles were collected. The patient was asked to rinse the mouth thoroughly and gargle

with some normal saline which was then expectorated into a stainless steel kidney dish. In the laboratory, the fluid was distributed into centrifuge tubes and spun at 270 G. in a centrifuge for five minutes. Subsequently, the supernatant was drawn off, and the sediment collected on a swab stick and smeared on to glass slides, which were then placed in fixative and treated as normal smears.

3. In cases of oro-antral fistulae or tissue cavities such as surgery (eg. extraction sockets) sites, exfoliating cells were obtained by irrigating the wound with normal saline, and the lavage was subsequently collected in a stainless steel dish, and handled in a similar method to mouthwashings.

4. Aspiration of fluid from swellings or tissue cavities was sampled with a swab stick and prepared into smears.

5. Secretions were collected occasionally with a wooden spatula if viscous, or a swab stick if watery, and then smeared on to glass slides, prior to the usual fixation, staining and microscopic examination.

Discussion of foregoing techniques.

1. It is essential that the spatula is moistened with normal saline prior to taking a smear or scraping so that transfer of cells from tissue to glass slide is satisfactory. A dry spatula withdraws moisture from the cells making them adhere to the instrument and the diminished number of cells which can be wiped on to the slide are dried out

and distorted. Normal saline is preferable to water, as it causes less cellular distortion.

The urethra is sterilized not so much to avoid contaminating the patients' tissue by using a sterile technique, but simply because growth of fungus developed after a short time in saline on spatulas that were not sterilized.

2. Benedict's diuretic solution was used in this project because its haemolytic effect on obscuring red blood cells in smears was found advantageous. It should be noted that whenever diuretic solution is to be re-used, it should be mixed through filter paper, and placed in freshly cleaned jars. Many cellular material may often be seen floating around slides in the jar during fixation, the necessity for this precaution is obvious.

3. It became clear that the interpretation of various degrees of atypia as seen in sore oral smears made it desirable initially to have the additional grading of questionable atypia, as a modification of the conventional cytological grading system. However, with increasing experience of the degree of cytological atypia in oral smears, there must follow greater accuracy of grading, and in fact, elimination of the intermediate group 'questionable atypia' with such smears being graded either two or three.

4. Marking of slides during scanning with dots of Indian ink enables rapid review of various features or individual cells in smears. This proved to be not only a valuable time saving device, but aided consistency and accuracy in evaluating a smear.

5. The criteria for malignancy as outlined in the review were the basis of assessing the cell's status. However, a few general comments could be made of the morphological features of malignant and atypical cells observed in this study, in the light of their apparent significance to the writer.

Naturally, nuclear detail was the prime consideration, but no one feature was consistently found in all malignant cells. High Nuclear/Cytoplasm size ratios, irregular chromatin distribution, hyperchromasia, and border irregularity were all noted in varying combinations in malignant cells. Prominent nucleoli may be often seen in malignant cells from oral lesions in contrast to their comparatively rare occurence in vaginal/cervical smears. Thin type cells were not commonly seen and unlike gynaecological cytology, are just as likely to be found in invasive oral squmous cell carcinoma as carcinoma-in-situ. Malignant 'tadpole' and fibre cells are occasionally seen in smears from invasive squamus cell carcinoma. Simple ulcerative inflammatory lesions, particularly of

buccal mucosa may shed squamous cells with large hyperchromatic nuclei and highly keratinised cytoplasm suggestive of dyskaryotic cells, but such cells don't necessarily indicate suspicion in oral cytology. Cells with highly orangeophylic cytoplasm may be found in many benign oral conditions as well as in malignant lesions. In smears containing thick masses of superficial cells, marked hyperchromasia associated with variation in nuclear size appears to justify suspicion. It is apparent also, that smears made from the most superficial cells of a lesion are very important diagnostically, and in fact as implied by King et al, (1966)[69] may be of greater value than smears consisting of deeper layer cells, after the superficial cells have been scraped away. Although there is sufficient evidence on both to indicate malignancy, the superficial smear's cells are much more informative and the number of diagnostic cells is greater. It would seem desirable, therefore, that for most oral lesions, two smears should be taken, one of the superficial cells and one of deeper cells. Obviously this applies only to lesions not covered by a necrotic slough, which must be removed prior to taking any smears.

6. Where an unusual situation exists clinically and smears are not obtained by the usual spatula technique, the clinical history is of utmost importance. If a

rinsing or gargling technique is used to obtain cells, the reasons for the procedure should be clear to the laboratory, which then will not be surprised by the finding of cells of respiratory origin. Sputum specimens are unlikely to be included in oral cytology, but it is important to note that the presence of highly keratinised cells, or merely free pieces of keratin in such smears is very significant, in the diagnosis of lung or bronchial carcinoma.

When smears are made from fluid aspiration of oral or para-oral swellings, laboratory knowledge of the site from which the fluid was obtained may be of critical importance. This point can be illustrated by a case encountered in this study.

The patient presented at the Radiotherapy Oral Clinic, for follow-up after treatment for carcinoma of the parotid region and numerous facial skin cancers, with a fluctuant swelling on the lateral surface of the nose extending infra-orbitally. As the swelling was palpable through the buccal sulcus, an 18 gauge needle attached to a 20 ml. syringe was passed up through the oral tissues into the swelling. Pus-like fluid was aspirated and reduction in size of the mass occurred.

Radiographically there was no bone loss in the region, and no ulceration or fistulae evident on the skin, buccal

mucosa or in the nose. Cytology smears were prepared from the aspirated material and contained poorly differentiated malignant cells, some free pieces of keratin, and highly keratinised cells. Had the smears only contained keratinised squamous cells, the cytology would have been suspicious, since epicholium is not usually found in the region from which the specimen was obtained.

RESULTS.

The results of the project are set out to show:-

1. The degree of morphological changes in oral exfoliated cells in the form of an 'atlas' of photomicrographs.

2. The variety in type and anatomical position of the lesions examined in those dental patients involved in the project and the results of a follow-up on each of these lesions. As cytology results are correlated here with biopsy and clinical findings, the follow-up constitutes an important part of the work.

3. Application of oral cytology under radio-therapeutic conditions.

4. The results of a 'pilot' study on the prognostic significance of changes in irradiated cells.

DENTAL PATIENTS.

The lesions and tissues from which cytological smears were taken are indicated in tables II - V. These include each lesion once only though a series of smears may have been taken from a particular lesion; but where a patient developed a number of different lesions over a period of time, these were each recorded separately. One or two smears were taken for a lesion depending on the site, accessibility, and nature of the lesion.

There were 15 Squamous Cell Carcinoma seen that were confirmed by biopsy. Of these, 11 lesions were clinically indicative of carcinoma. One of the cases in each of the four groups in tables II, III and IV marked by an asterisk (*) was suggested by cytology and later confirmed by biopsy to be malignant. The tables indicate the clinician's impression of the lesion at the time that the smear was taken.

Leukoplakia, as it appears in these tables, applies to a clinically evident white plaque.

By far the greatest number of lesions seen were non-specific ulcerations and hyperkeratotic nodules which, in the majority of cases, were the result of denture irritation and hence were found usually in sulci and alveolar mucosae. It is realised that there may be considerable overlap of lesions from one region of the mouth to another, not confined simply to those areas indicated by the tables. In such cases, there was only one lesion recorded, placed in the area most affected at the time the smear was taken.

Table VI shows the findings on follow-up of the lesions from which smears were obtained. As is indicated in the following discussion, a truly correlative study has not been attempted, but, assuming that a lesion that

undergoes clinical resolution is not malignant, table V indicates that in only 8.35 percent of cases was there any reasonable chance of a malignant lesion being overlooked, regardless of its smear receiving a suspicious or negative report. Criticism that has been levelled at various oral cytological studies which have not confirmed that lesions which were given negative cytology reports were indeed benign, has, therefore, been circumvented here, except in a very small percentage of cases.

All lesions in this study which were considered cytologically suspicious or positive for malignancy were subsequently either -

1. Confirmed to be malignant by biopsy.
2. Shown by biopsy to be benign - although in some cases, where resolution has not taken place, the lesion is being closely watched.
3. Seen to resolve completely.
4. Still under treatment and observation, except for three cases, where the patient failed to keep subsequent appointments.

TABLE II.

Simple ulcerating and/or erythematous lesions from which smears were taken.

Clinical description	Tongue	Buccal mucosa	Floor of mouth	Buccal sulcus	Gingival mucosa	Hard palate	Soft palate	Retromolar area	Lip	Generalized	Total
Non-specific ulceration	4	4	6	24	23	1	1	3	2	-	68
Superficial erosion	3*	5	1	2	1	1	1	-	-	-	14
Ulceration and erythema	2	2	1	1	-	2	1	2	1	-	12
Erythema or atrophy	3	-	-	1	-	2	4	-	-	2	12
	12	11	8	28	24	6	7	5	3	2	106

*One case in this group was suggested to be malignant by cytology and later confirmed by biopsy.

TABLE III.

White lesions from which smears were taken.

Clinical description	Tongue	Buccal mucosa	Floor of mouth	Buccal sulcus	Gingival mucosa	Hard palate	Soft palate	Retromolar area	Lip	Generalised	Total
Hyperkeratotic nodule	3	1	1	7	22	3	1	3	7	1	48
Discrete white film or spot	3*	10	-	1	1	-	1	-	1	2	19
Hyperkeratosis and erythema	-	-	-	-	2	2	-	-	-	1	5
Leukoplakia	1	6	2	-	3	2	1	1	1	1	18
	7	17	3	8	28	7	3	4	9	4	90

*One case in this group was suggested to be malignant by cytology and later confirmed by biopsy.

TABLE IV.

Elevated lesions from which smears were taken.

Clinical description	Tongue	Buccal mucosa	Floor of mouth	Buccal sulcus	Gingival mucosa	Hard palate	Soft palate	Retromolar area	Lip	Generalised	Total
Elevated: ulcerated) erythmic)	*	*	2	*	2	3	*	2	1	*	9
Granuloma	-	-	-	-	1	-	-	-	-	-	1
Papillomatous	-	-	-	1	1	3*	-	-	-	-	5
Warty naevus	1	1*	-	-	-	-	-	-	-	-	2
	1	1	1	1	4	6	-	2	1	-	17

*One case in each of these two groups was suggested to be malignant by cytology and later confirmed by biopsy.

Clinical description	Basal cell carcinoma	Squamous cell carcinoma	Malignant melanoma	Metastatic deposits from elsewhere	Not recorded	
Tongue	1	4	1	1	5	
Buccal mucosa	1	1	1	1	1	
Floor of mouth	1	2	1	1	2	
Buccal sulcus	1	4	1	2	2	
Gingival mucosa	1	3	1	1	4	
Hard palate	1	1	1	1	3	
Soft palate	1	1	1	2	3	
Retromolar area	1	1	1	1	1	
Lip	1	1	3	1	2	
Generalised	1	2	1	1	3	
Not recorded	1	1	1	1	2	
Total	10	19	12	11	26	

TABLE VI.

Follow-up of lesions from which smears were taken.

	Clinically healed and/or shown benign by biopsy	Patients conditionally discharged	Long term follow-up and observation	Confirmed squamous cell carcinoma	Still under observation and treatment	Miscellaneous	Total
Number	145	12	31	15	17	20	240
Per cent	60.40	5.00	12.90	6.25	7.08	8.35	100

Patients conditionally discharged were those which, after treatment and observation, were referred back to their own dentist for continual observation and those told to return if any change was noted in the oral tissues or if worried. These lesions were all considered benign and consisted of the following:-

Leukoplakia	-	2
Hyperkeratosis	-	2
Fibrous hypertrophy tissue	-	3
Erythema or recurrent ulcers	-	5

Long term follow-up and observation patients consisted of those with the following lesions:

Reourrent ulcers	- 2
Leukoplakia	- 12
Lichen planus	- 3
Hyperkeratosis	- 1
Persistent denture irritation	- 7
Erythema	- 3
Candidiasis	- 2
Basal cell carcinoma	- 1
<u>Total</u>	- 31

Lesions still under observation and treatment are those not included in the above, but exclude the diagnosis of squamous cell carcinoma.

<u>MISCELLANEOUS</u>

Failed to return	- 10
No follow-up (smears sent in by a private dentist)	- 8
Lesion almost healed when last seen, but final appointment not kept.	- 2
<u>Total</u>	- 20

The smears prepared from these lesions were examined and graded. Because inexperience was felt to be a factor after the project had been in operation one year, the first year's slides were re-examined, thus 'updating' them.

Of 336 smears involved in this section of the project, 243 (72.3 per cent) were graded as negative; 55 (16.4 per cent) were graded atypia indeterminate; 13 (3.8 per cent) were regarded as suggestive of malignancy; 16 (4.7 per cent) as highly suspicious of malignancy; and 9 (2.6 per cent) as conclusive for malignancy.

DISCUSSION OF PRECEDING RESULTS

The present study was aimed at instituting an oral-cytology cancer detection programme and thus demonstrating the value, reliability and practicability of oral cytology as a clinical aid. It became obvious quite early that purely attempting to obtain correlative results as reported by other workers on the accuracy of cytology, using biopsy as a standard, would be neither valid, desirable nor practicable for a number of reasons.

Firstly, the literature contains numerous correlative studies performed by authoritative people, which show the high degree of accuracy of cytology under control conditions. To repeat or improve upon such work, it would be obligatory to obtain at least one biopsy specimen from every lesion at the time it was smeared, which was not feasible.

Secondly, under normal conditions, the basis of diagnosis is usually clinical observations and histopathology, neither of which on their own are absolute. It seemed reasonable, therefore, to conduct this project on the basis of clinical observations, and biopsy evidence when possible. In some cases, a 'suspicious' cytology report was made, but clinical observation next visit (at which time a biopsy was planned) showed complete, or almost complete, healing

in response to routine treatment. The conclusions drawn from these situations were that for all practical purposes the lesion had not undergone malignant change, but the cytology could have reflected a degree of reversible epithelial dysplasia or severe atypia in response to inflammation in the lesion at the time the smear was taken.

Thirdly, it has been clearly shown that interpretation of pathological material can vary markedly from one experienced pathologist to another, and even with the same individual over a period of time. Cocker, Fox and Langley (1968)[27]. This inconsistency could be expected to be greater with less experience and obviously in this project, where previous cytology experience was nil, a degree of variation must occur between the early reports, and those twelve months later, making accurate correlation impossible. With this in mind, a review of the routine smears was undertaken after the project had been in operation approximately twelve months to assess the extent to which a shift in applying the cytological criteria had occurred during this period, and also attempt to ensure that no obviously incorrect gradings had been made among the early smears, on the basis of subsequent experience. It was found that a degree of variation had occured chiefly associated with degrees of

atypia, with the tendancy being to 'over grade' smears initially. On review 17 smears were 'upgraded', but 31 smears were asigned to a lower cytological grading.

Summary of those results.

1. Smears were obtained from 240 different lesions of widely different types and in various oral soft tissue situations.
2. In some cases smears were taken at more than one appointment, and the number of smears taken from each lesion was not standardised for reasons stated. In all, 336 smears were included.
3. Of the 240 lesions, 15 were finally diagnosed as squamous cell carcinoma (11 being clinically obvious and four being suggested as malignant by cytology). Follow-up established that malignancy was not overlooked in any of 220 of the 240 patients, so that the possibility of an early cancer being overlooked by an innocuous appearance clinically and receiving a negative cytology report, was extremely remote and, in fact, possible in only 20/240 or 8.3 percent of cases.
4. By virtue of the corrolation between cytology and biopsy/clinical findings the accuracy of the oral cytology programme was found to be high. Of the

336 smears examined, only 6 "false negatives" occurred, of which three were considered, on review after twelve months not to be positive, and originally three smears were falsely graded positive, but with greater experience, were later down-graded.

The indefinite smears - "doubtful" or "suspicious" were a fairly regular occurrence, reflecting, firstly, uncertainty in microscopic interpretation, true dyskaryotic cells from "disquiet", premalignant, or actual malignant epithelium; or where indirect criteria suggested that the smear may not contain cells truly representative of the lesion. However, it is worth noting that about 80 percent of the total smears were graded definitely positive or negative.

It is felt that these results indicate the reliability of the oral cytology technique.

The following figures are interesting with regard to the increasing incidence of squamous cell carcinoma detection among Dental Hospital patients:-

Number of squamous cell carcinomas confirmed by biopsy in twelve months -

June, 1963 - 1964 = 3

June, 1964 - 1965 = 4

June, 1965 - 1966 = 4

June, 1966 - 1967 = 5

June, 1967 - 1968 = 10.

The oral cytology programme was effective as a clinical routine from April, 1967. Of the squamous cell carcinomas diagnosed over the last twelve months, three were lesions that had been previously biopsied (up to three years before), which did not appear malignant clinically but were reported as positive cytologically. Diagnosis was later confirmed by biopsy.

ESTABLISHMENT OF AN ORAL CYTOLOGY SERVICE.

In this project, experience was gained by the examiner, in oral cytology, through -

1. the staining, scanning and examination of a large number of oral smears,
2. familiarisation with methods and techniques through texts and journals,
3. most importantly, frequent discussion of various 'problem' cases with a trained and experienced cytologist, in particular atypia and suspicious smears.

It was further found valuable to obtain understanding

of the lesions involved by personally examining the patient clinically, and taking the appropriate specimen. This simplified a routine approach to the technique and obtained a fairly consistent type of smear, at the same time giving first hand information on the nature of the lesion.

However, since oral cytology offers the greatest advantage to clinicians as a diagnostic aid for innocuous appearing lesion, or as a screening technique for large numbers of patients, it is essential that an oral cytology service be of optimum efficiency and reliability, the following suggestions can be made from this investigation.

OBTAINING OF SPECIMENS.

The method used here is a simple proceedure for obtaining smears of consistent quality. Saline was found suitable as a vehicle for mouthwash and gargling and irrigation techniques, but centrifugation of the collected specimen and preparation of the resultant sediment into smears was done almost immediately. Where there is likely to be a delay of days between collection and preparation of smears, an alternative solution such as Geys balanced salt solution (Helsper and Sharp, 1964)[59] may be necessary to preserve the cells. Obviously, the

less the delay the greater the accuracy of the result.
The sediment is simply prepared into smears by transferring onto a glass slide with a swab stick, this smear is then fixed as for a normal smear.

FIXATION.

A number of fixatives may be used, Carnoys being found best here. As a last resort methylated spirits will suffice. When more than one smear is taken from the same patient it is important that the slides, if placed in the same bottle of fixative be 'back to back', to avoid dislodging cells from one slide to another.
Never should slides from different patients be placed in the same jar of fixative, as the risk of contamination of cells from one to the other is great.

Smears can be removed from fixative after 20 - 30 minutes, or left in fixative indefinitely. However, smears should be removed from the solution and mailed to a laboratory 'dry'.

MAILING OF SMEARS.

Slides may be sent to the cytology laboratory, suitably wrapped and packaged against breakage, or in special slide carriers with patient's name and clinical details.

It is felt that the routine taking of cytological

smears, using these simple sampling and fixing techniques, by dental practitioners, is to be encouraged. This could be achieved by distributing to them pamphlets outlining techniques, indications and contra-indications, and making readily available oral cytology kits containing spatulas, glass slides, clinical history card and slide carrier.

LABORATORY ASPECTS.

Staining is a simple laboratory procedure.

Scanning. The use of 'untrained' personnel as scanners has been found satisfactory in major general cytology laboratories in this country. These scanners are taught to recognise simply normal cells, and smears containing any variations from normal are dotted and set aside to be examined by cyto-technologists, who further classify the smears, giving those that are doubtful, suspicious or positive to the cytologist for final diagnosis. The majority of oral smears are normal, and oral cytology is, in general, less complicated by physiological variations in normals as found in cervical smears.

PRESENTATION OF RADIOTHERAPY CASES INVOLVED IN THE PROJECT.

The second group of subjects consisted of patients who attended an oral clinic of the Radiotherapy Department of Royal Prince Alfred Hospital, Sydney. It included some patients, principally those with squamous cell carcinoma, seen in the main group of Dental Hospital patients.

This section of the project fulfilled a number of objectives, with regard to oral cytology.

(1) It provided cytological material and first hand clinical information from a large number of oral cancers from various regions.

(2) It enabled demonstration of cytological changes during and following radiotherapy.

(3) It enabled the prognostic implications of oral cytology to be briefly looked at.

(4) It provided a limited service in aiding the clinical evaluation of a lesion's progress during or after radiotherapy.

(5) Experience was gained with post radiation smears, as a clinical adjunct in differentiation of residual or recurrent neoplasm from radionecrotic ulcerations with or without secondary infection, this being a situation where biopsy is undesirable. The following cases were included.

Lip lesions

Smears were taken from eleven patients.

Biopsy indicated squamous cell carcinoma in seven cases, the corresponding smears of which were -

positive in three cases

suspicious in two cases

negative in two cases.

Biopsy showed no malignancy in four cases and cytology was negative in all.

Carcinomata of the lip are generally treated with a short course of radiotherapy over a period of usually nine days. As shown by above results, cytology of lip squamous cell carcinoma is rather unreliable, due to the dry, often crusty nature of the lesions. Although no false positive smears from lips were encountered, it could be expected that false negatives would not be uncommon. Because of this unreliability of the cytology and the usually good clinical response of the lesions to treatment, repeated smears were not made in these cases.

Two lesions involving lip and buccal mucosa were included, one squamous cell carcinoma (class IV cytology report), the other severe papillomatous leukoplakia (class II cytology report).

Buccal mucosal lesions

Leukoplakias - One case in which cytology was negative.

Squamous cell carcinoma:- Three cases

Case 1 - Cytology immediately after completion of radiation, showed some doubtful features but subsequent cytology was negative.

Case 2 - Clinical response to radiotherapy was good and lesions appeared to regress. Cytology during and after treatment was positive. Recurrent disease subsequently developed with fatal outcome in six months.

Case 3 - Cytology before treatment was obviously malignant, class V, and radiotherapy failed to control the neoplasm.

Neoplasms involving the tonsillar and pharyngeal region

The following lesions were included:- Three squamous cell carcinoma; 1 reticulum cell sarcoma; 1 lymphosarcoma. In all cases where pretreatment smears were taken (including both non-squamous lesions) cytology was positive.

Post treatment smears in two cases of squamous cell carcinoma were negative despite the clinical appearance of residual tumour, but both lesions subsequently underwent complete healing.

Palatal lesions

Case 1 - Biopsy indicated squamous cell carcinoma. Pretreatment cytology was positive. The

lesion responded well to treatment and healed. Post-treatment cytology was negative.

Case 2 - Biopsy report equivocal for squamous cell carcinoma or inflammatory response to irritation (pseudo-epitheliomatous hyperplasia). Cytology was suggestive of malignant change. Lesion slowly healed after removal of irritant (denture). Subsequent cytology was negative at various stages of the healing process.

Case 3 - Pyogenic granuloma, the cytology of which was negative.

Lesions involving the buccal sulcus

Two cases of squamous cell carcinoma were encountered.

Case 1 - Smears taken during treatment were graded as III. Smears taken after treatment at 1,3,9 and 12 months post-treatment were negative. This patient showed a good response to treatment and the lesion healed completely.

Case 2 - Smears taken during treatment indicated a IV grading. Post-treatment smears at 6, 9 months were positive for malignancy. The clinical response to treatment was good with apparently complete regression, although an oro-antral fistula persisted. Recurrent or

residual tumour was not obvious clinically until 12 months after treatment when it advanced rapidly into the orbit.

Alveolar mucosal lesions

This area included two lesions, one being histologically an acanthotic nodule clinically suggestive of recurrent neoplasm, the cytology of which appeared suspicious. The other was a squamous cell carcinoma which showed complete regression after treatment. Cytology in this case, before, during and after treatment correlated with biopsy and clinical findings.

Tongue lesions

Five cases of squamous cell carcinoma.

Pre-treatment smears taken in four of these were positive. Four cases showed good clinical response to treatment with regression of tumour. In three of these, post-treatment smears were suggestive of recurrent malignancy, which was clinically evident in two cases shortly afterwards, and the third case developed severe radionecrosis.

Complete clinical healing with no sign of recurrence was observed after 12 months in the fourth case, post-treatment smears from which had been negative.

Post-treatment smears from a fifth case, with probable

residual tumour, supported clinical findings.

Lesions of tongue extending to and including floor of mouth

Six cases of squamous cell carcinoma.

Pre-treatment smears were obtained in four cases; three being definitely positive and the fourth suspicious (this lesion was covered by necrotic debris). In the other two cases, post-treatment smears only were taken. These were positive and correlated with clinical findings of residual or recurrent neoplasm. In all six cases, recurrent disease occurred.

Retromolar region neoplasms.

Pre-treatment smears in the three cases suggested malignancy. Treatment failed to control the disease in all cases.

Miscellaneous

Antrum

Smears were taken from the unbroken palatal mucosa overlying a large swelling extending orally from an antral carcinoma. Only normal oral cells were obtained.

Side of Nose - Infra-orbital region

Needle aspiration of fluctuant swelling in this region was carried out through buccal sulcus. The resultant pus and fluid was prepared into smears examined cytologically and seen to contain poorly differentiated squamous carcinoma cells. This was subsequently confirmed histologically.

Discussion of these results

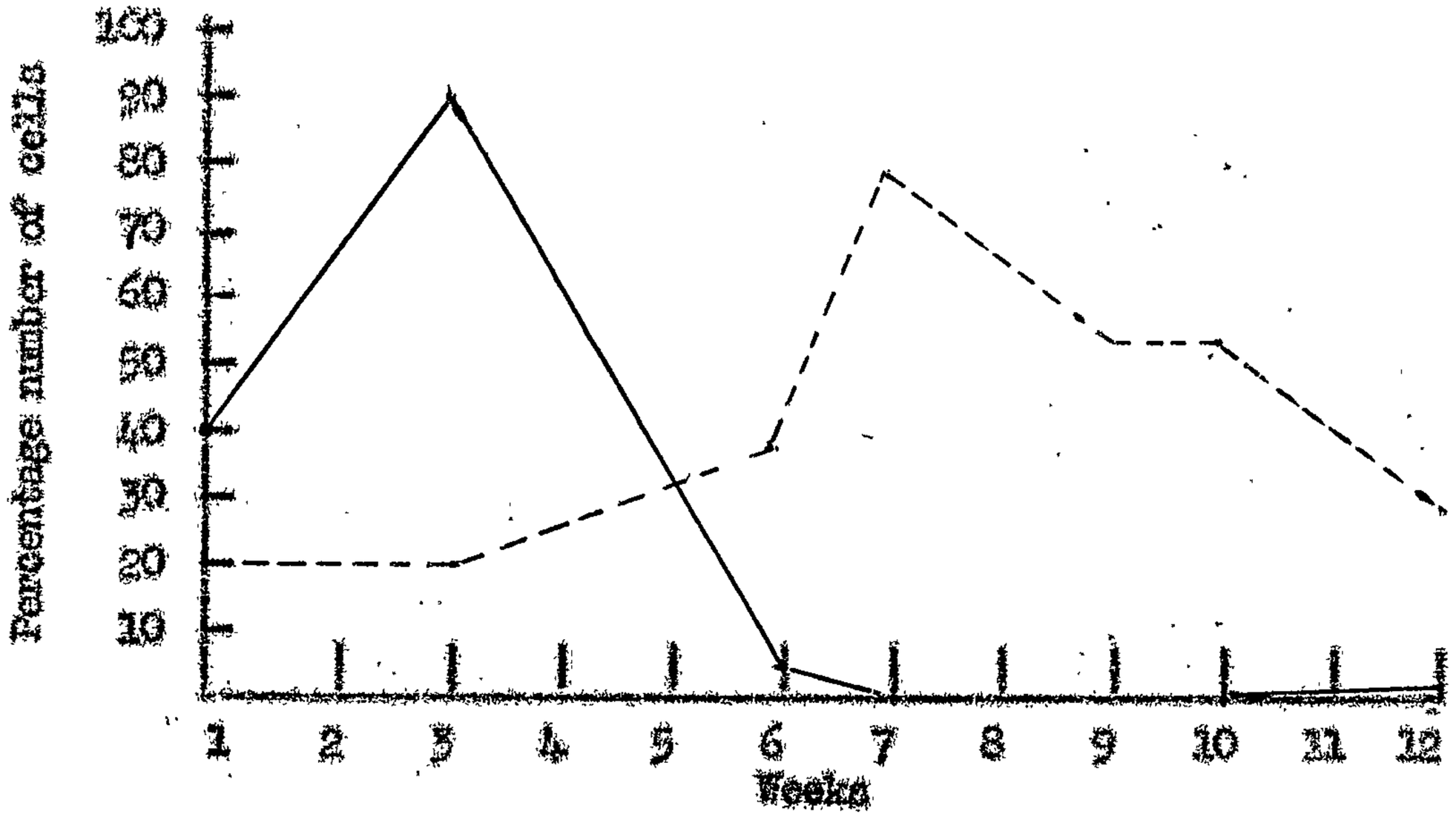
Oral cytology was introduced to the oral radiotherapy clinic with generally favourable results. In all, smears were taken from 45 cases from different regions of the mouth at different stages in treatment. In four cases the cytology was positive some months prior to clinically evident recurrence. On the other hand, in two cases post-treatment smears were suspicious from lesions which were shown histologically to be benign. The first of these, an alveolar acanthotic nodule, occurred early in the project, and it could be expected that it would not have been considered troublesome cytologically towards the end of the project when more experience had been gained. The other was a severe radionecrotic ulcer, the atypia of which were quite extreme. Three cases yielded false negative pre-treatment smears; of these two were encrusted lip lesions, and the smear from the other was taken from the unbroken palatal mucosa overlying a bulging antral squamous cell carcinoma. In all other cases, cytology reflected histological and clinical findings.

PILOT STUDY OF EFFECTS OF RADIOTHERAPY ON ORAL CYTOLOGY.

Cell counts were made on cytological smears taken from patients during radiotherapy. It must be assumed that each smear is representative of the lesion with respect to the types of cells exfoliated, at the time that the smear is taken. The standard spatula technique employed throughout this project was used to obtain these smears.

Procedure

Counting of a random selection of fields, under high magnification (X 450), was made on each slide. The number of cells showing malignant characteristics were recorded and the numbers of benign cells showing effects of radiation per 100 cells were also noted. These figures were then plotted against time on a graph for each patient.



Weeks after commencement of treatment	Percentage of malignant cells	Percentage of radiation effects
1	40	-
3	90	-
6	5	35
7	0	77.5
9	0	52
10	0	52
12	1	26

Case 1. Advanced squamous cell carcinoma of tongue.

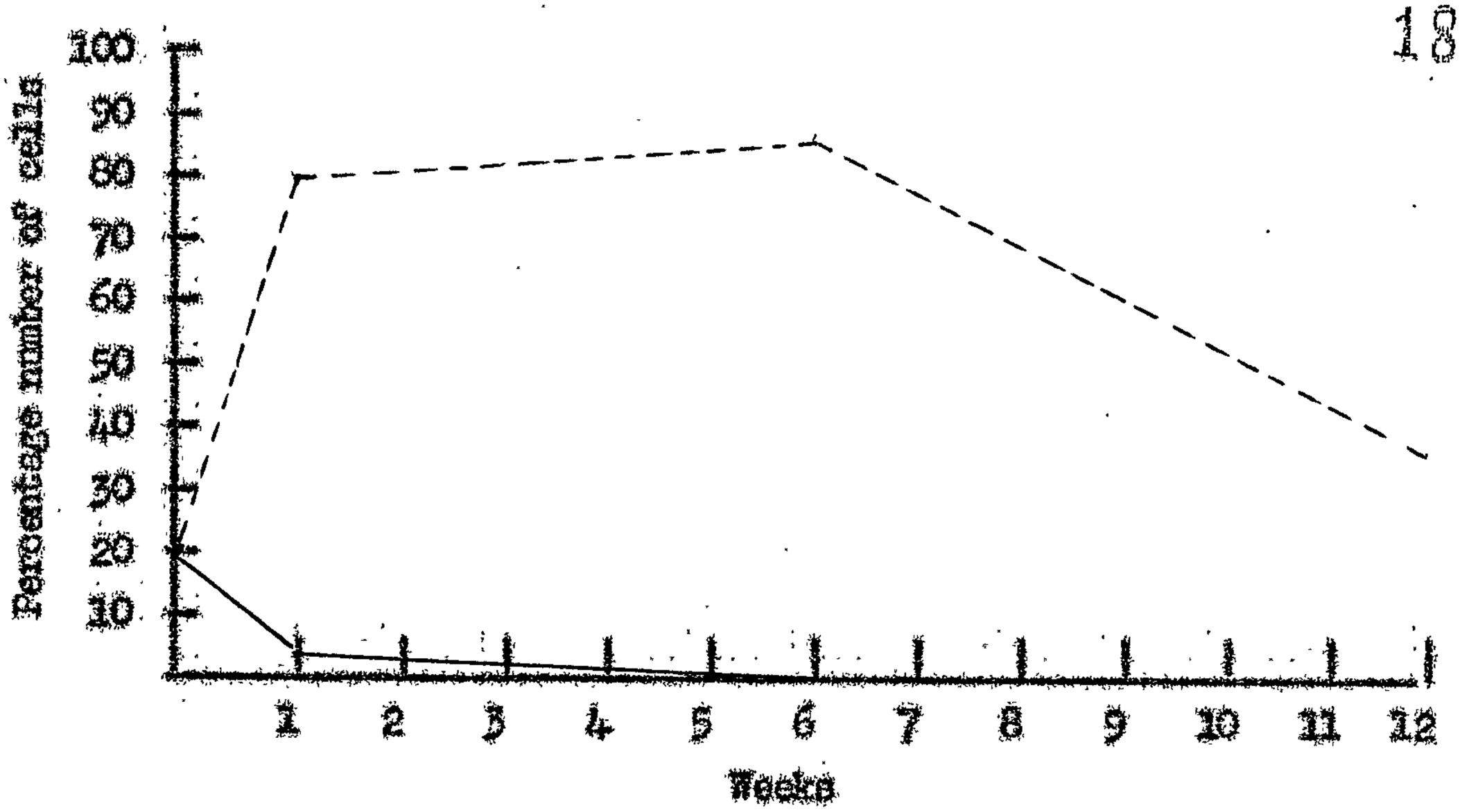
Radiotherapy lasted 10 weeks with 2 one-week interruptions at end of 4th and 7th weeks.

Clinically: Initial response to treatment was good with regression of tumour.

Recurrent disease evident after 6 months.

---- = percentage of normal cells showing radiation effect.

——— = number of malignant cells per 100 epithelial cells.



Weeks after commencement of treatment	Percentage of malignant cells	Percentage of radiation effects
0	20	-
1	2.5	80
6	0	85
12	0	37.5

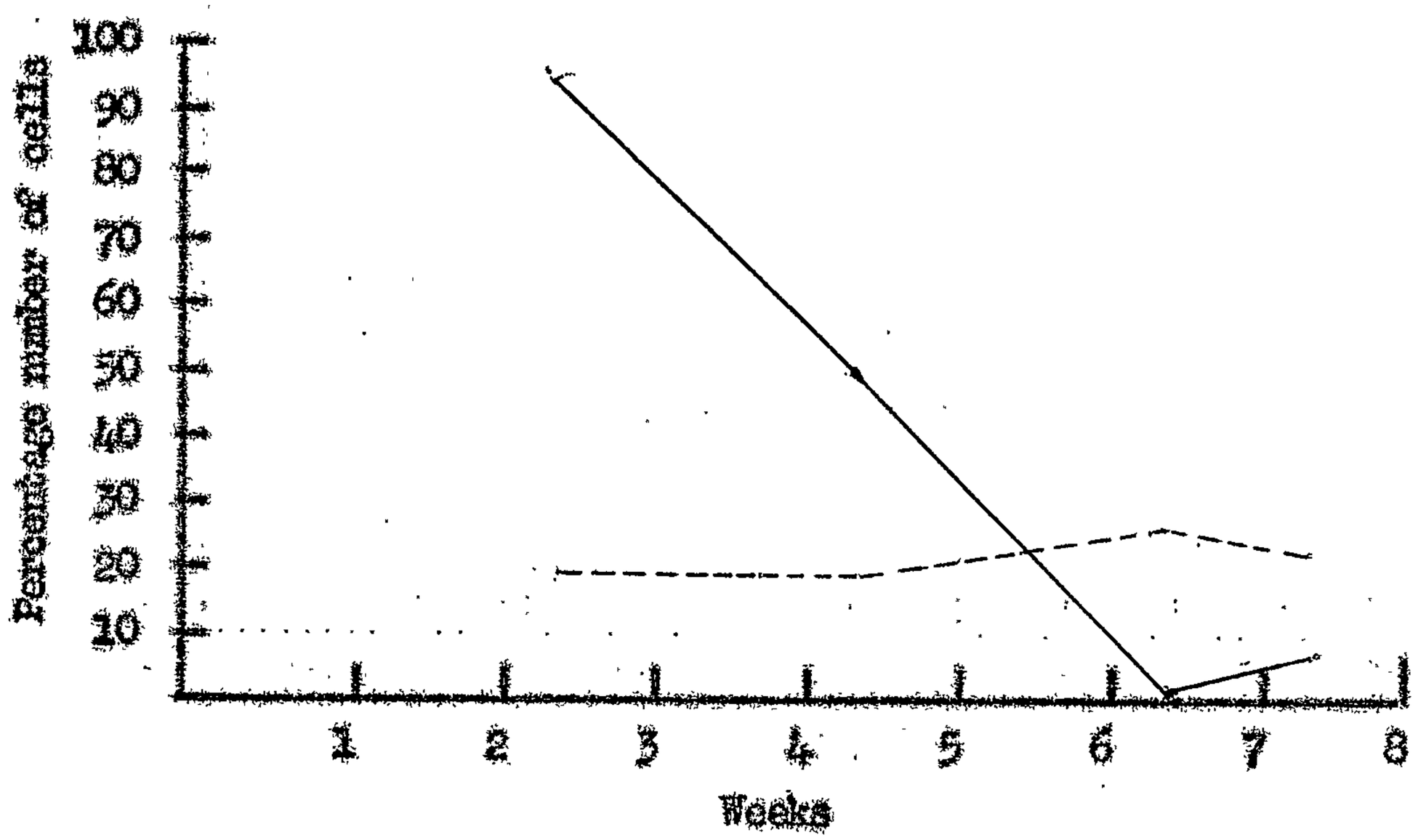
Case 2. Squamous cell carcinoma maxillary buccal sulcus.

Duration of radiotherapy - 5 weeks.

Clinically: Initial response to treatment was good. Free of neoplasm at 6 months, and 12 months.

---- = percentage of normal cells showing radiation effect.

———— = number of malignant cells per 100 epithelial cells.



Weeks after commencement of treatment	Percentage of malignant cells	Percentage of radiation effects
2.2/7	95	-
4.2/7	50	-
6.2/7	0.5	27.5
7.2/7	7.5	25

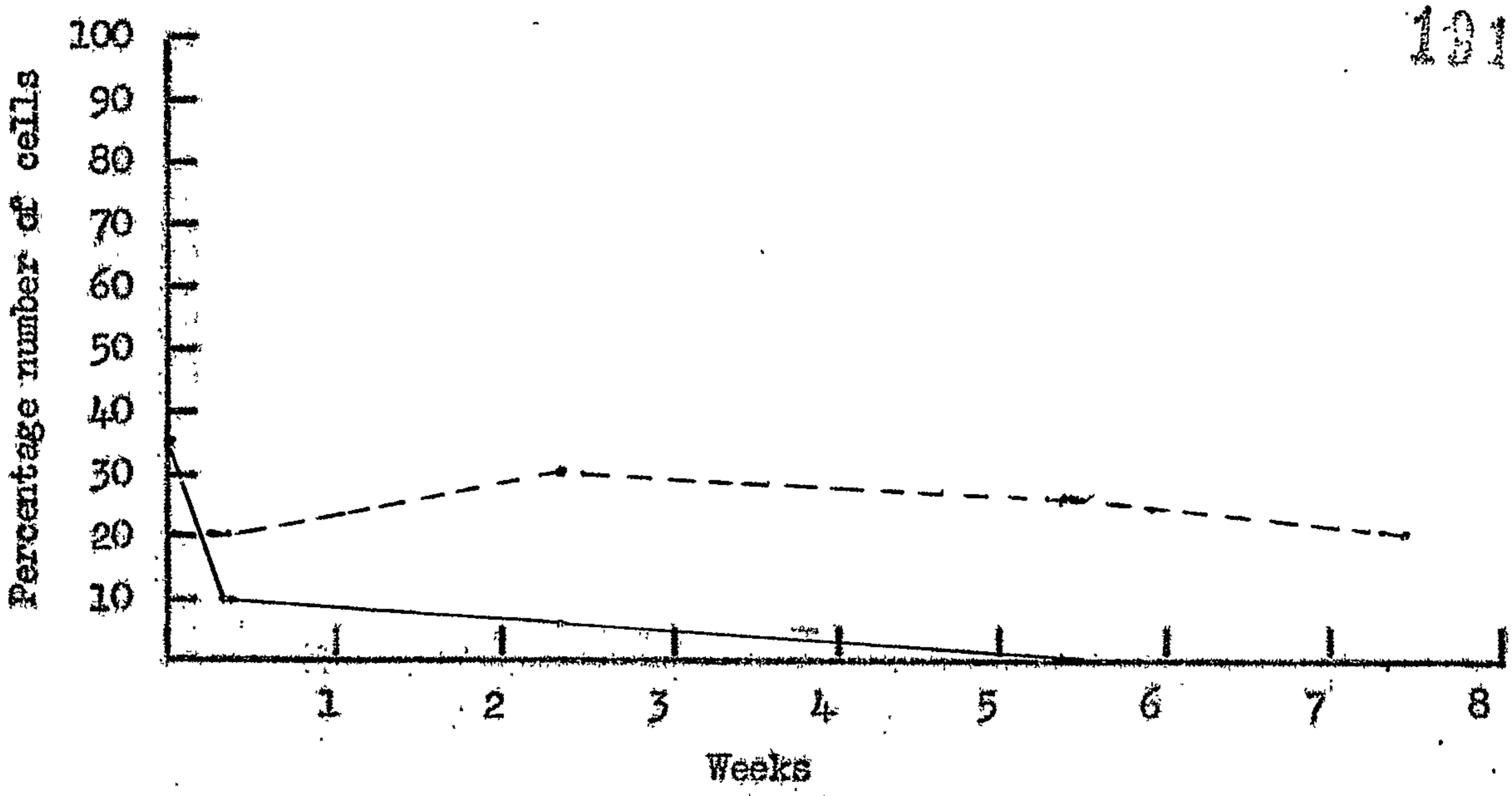
Case 3. Advanced squamous cell carcinoma in tongue and floor of mouth.

Duration of radiotherapy - 7 weeks.

Clinically: Initial response to treatment was fair with regression of tumour. Recurrent disease evident within 6 months.

---- = percentage of normal cells showing radiation effect.

———— = number of malignant cells per 100 epithelial cells.

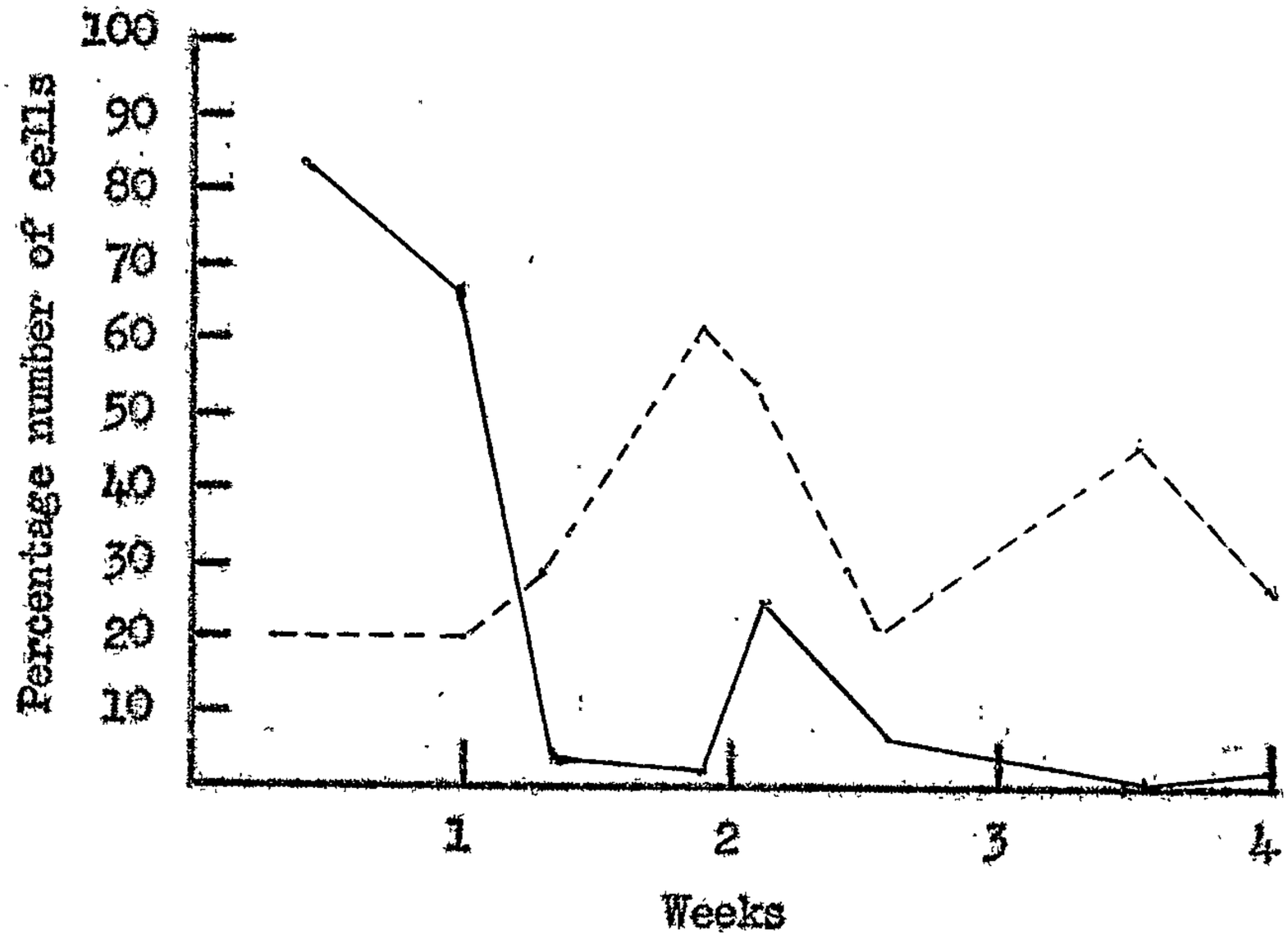


Weeks after commencement of treatment	Percentage of malignant cells	Percentage of radiation effect
0	35	-
2/7	10	-
2.2/7	7.5	30
5.2/7	0	25
7.2/7	0	-

Case 4. Advanced squamous cell carcinoma in tongue and floor of mouth.
Duration of radiotherapy = 7.1/2 weeks.
Clinically: Initial response was poor. Neoplasm uncontrolled.

_ _ _ _ = percentage of normal cells showing radiation effect.

_____ = number of malignant cells per 100 epithelial cells.

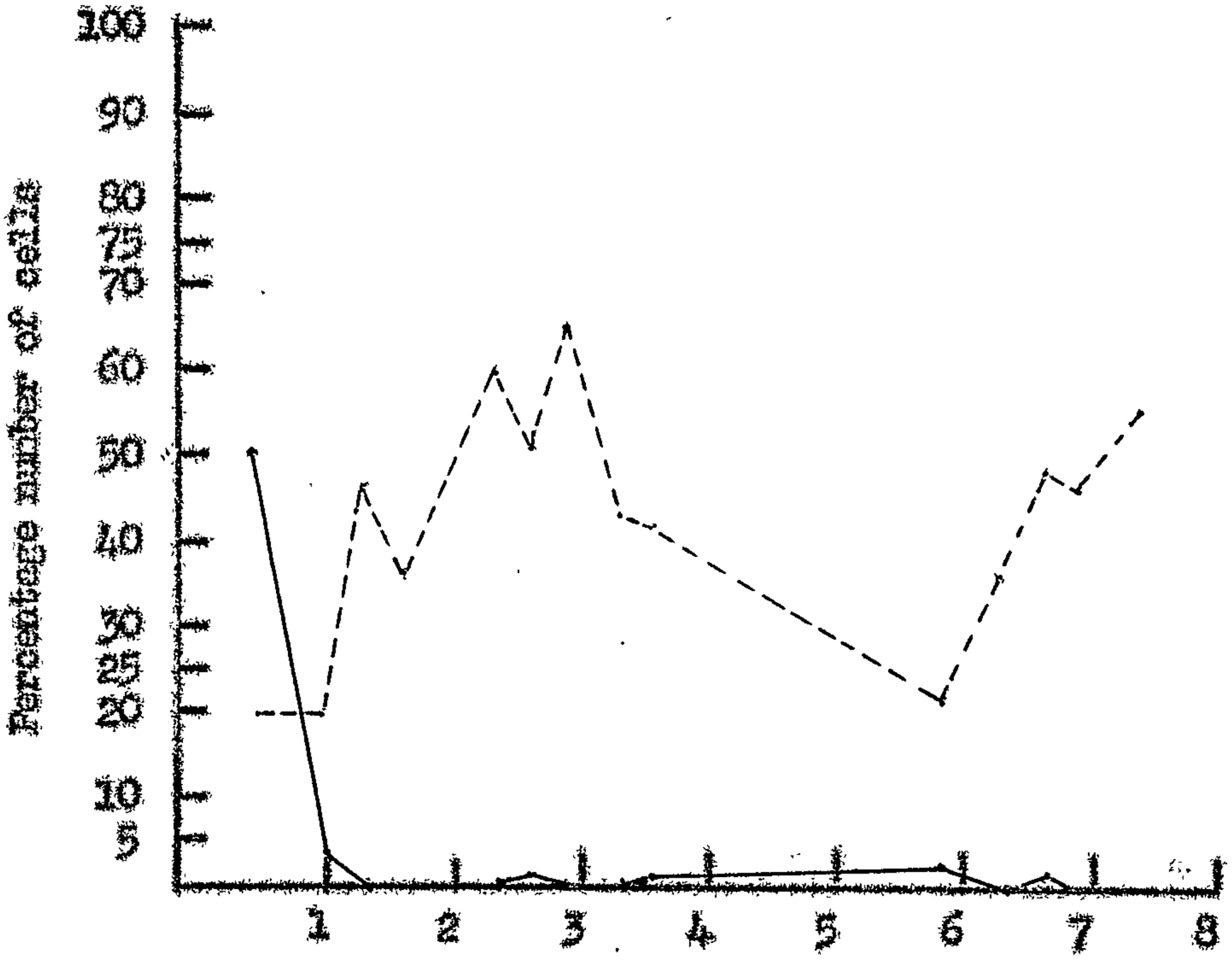


Weeks after commencement of treatment	Percentage of malignant cells	Percentage of radiation effects
2/7	82.5	-
1	65	-
1.2/7	3	28
1.6/7	1	59
2.1/7	25	53
2.3/7	9	-
3.3/7	0	44
4	1.5	26

Case 5. Advanced squamous cell carcinoma in floor of mouth and tongue.
Duration of radiotherapy - 6 weeks.
Clinically: Initial response was fair - evidence of residual tumour.

– – – – = percentage of normal cells showing radiation effect.

——— = number of malignant cells per 100 epithelial cells.



Week after commencement of treatment	Percentage of malignant cells	Percentage of radiation effects
4/7	50	-
1	40	-
1.2/7	0	46
1.4/7	0	56
2.2/7	0	59
2.4/7	10	50
2.6/7	0	63
3.2/7	0	41
3.4/7	0.5	40
5.3/7	2	22.5
6.2/7	0	35
6.4/7	10	47.5
6.6/7	0	46
7.2/7	0	54

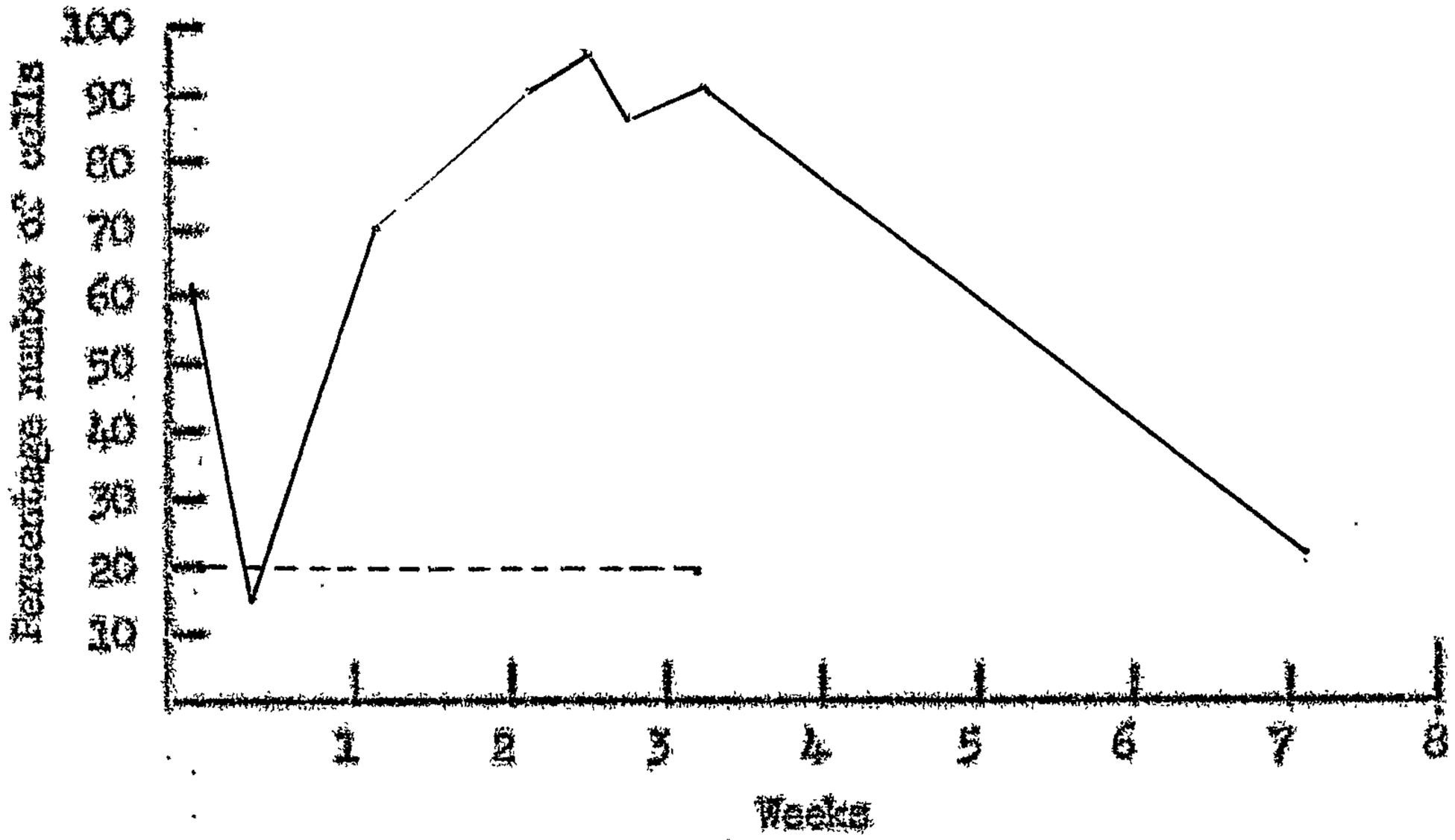
Case 6. Advanced squamous cell carcinoma in tongue and floor of mouth.

Duration of radiotherapy = 7 weeks with a two-week interruption at end of 3rd week.

Clinically: Initial response was good. Recurrent disease within 6 months.

---- = percentage of normal cells showing radiation effect.

——— = number of malignant cells per 100 epithelial cells.



Weeks after commencement of treatment	Percentage of malignant cells	Percentage of radiation effects.
1/7	60	-
3/7	15	-
1.1/7	70	-
1.3/7	Smear unreadable - necrosis	
2.1/7	90	-
2.3/7	95	-
2.6/7	85	-
3.2/7	90	-
7.1/7	95	Not able to be counted

Case 7. Squamous cell carcinoma buccal mucosa.

Duration of treatment - 4 weeks.

Clinically: Initial response was good with regression of tumour. Recurrent disease developed subsequently.

_____ = percentage of normal cells showing radiation effect.

_____ = number of malignant cells per 100 epithelial cells.

DISCUSSION OF PILOT STUDY RESULTS

Radiation effects were looked for in benign squamous cells which, according to Graham (1964)[52], reflect the changes in both malignant and benign cells of the tissue irradiated. The cellular effects noted are:-

(1) Marked increase in cellular and nuclear size.

(2) Multinucleation.

(3) Vacuolisation.

(4) Fragmentation of nucleus, or other abnormal nuclear change.

Only benign cells showing these changes were counted. Although multinucleation and giant cells are normally rarely seen in oral smears, vacuolisation and nuclear degeneration is not quite so common, so that percentage radiation effect of less than 20 percent was not considered significant as this could represent the range of normal cellular variation by other factors, such as viral infections, inflammation, or systemic disorders. Thus it will be noted from the graphs, that in no case was radiation effect registered below 20 cells per 100. This was an arbitrary level chosen, after examination of some non-irradiated specimens.

The graphs indicate for each case, at different points in time, the number of malignant cells seen in each smear per 100 cells; and the percentage number of

cells showing radiation effects. Usually two smears were made at each appointment and the figures represent the mean of counts of the two smears. In order to obtain objectivity with the counting, the smears were examined and numbers of cells recorded without knowledge of which case the mean represented. That this precaution was effective, and that the counts were reasonably accurate was indicated by the fact that rarely did a variation of more than 10 percent occur between the counts of two smears from the patient taken at the same time.

The following factors should be noted.

(1) That in all of these cases the type of treatment used was external irradiation. It is interesting that Graham (1964)[52] has recorded finding different cytological effects with different forms of radiotherapy. Recent work by Andrews (1968)[5], however, suggests that radiation sensitivity is the same in all cells, regardless of the source of energy applied, though the response exhibited by normal cells is different from that of malignant cells because of the different rate of cell removal and replacement.

(2) The nature, site or degree of advancement of the lesions included was not at all standardised. These could be influencing factors on the type of treatment, response to it, and thence on the cytological cell counts.

Andrews (1960)[5] refers to the oxygen effect influencing a tumour's apparent radiosensitivity, such that with decreasing oxygen tension in the tissues, the capacity of cells to survive radiation is increased.

(3) The duration and dosage of treatment differs with individual cases, and in some advanced lesions may be aimed more at palliation rather than cure.

Although it is probably ideal for smears that are to be counted to be taken every second day during treatment, this was not possible in four of the seven cases presented. From the cases presented the following points can be made.

(1) In only two cases, did radiation changes in cells exceed 75 percent, the level set by Graham (1964)[52] as indicative of good prognosis. In case 2, the high percentage of affected cells was prolonged over a period of at least five weeks and was associated with complete absence of malignant cells. This patient had remained clinically free of recurrent disease.

In case 1, the radiation-induced changes at only one point exceeded 75 percent before falling off again. The fact that recurrent disease developed in this patient may be more a result of the advanced state of the lesion rather than the effectiveness of the irradiation, with the result that apparently radio-resistant cells, perhaps

existing in an environment of low oxygen tension, remain after the more "radiosensitive" cells have been destroyed. (2) In all cases, treatment produced a marked reduction, or an initial increase followed by a reduction (as noted by Umiker (1959-1960)[172, 174]), in number of malignant cells. (3) There appears to be no consistent relationship between the clinically evident response to treatment initially and the radiation induced changes in the cells. It is interesting that in cases where the lesion underwent apparently complete regression, but the percentage numbers of radiation effects in the normal cells was low, recurrent disease subsequently developed. (4) The graph in three cases, showed radiation-induced changes to be minimal, that is, not above 30 percent. The changes in each of the other four at some stage exceeded 50 percent. Whether or not this is suggestive of different response of individual tumours to irradiation, is open to further investigation.

The examination of this small number of patients receiving radiation therapy shows clear changes in the cells immediately following treatment. The changes in both clinical features and cellular morphology if sufficient numbers of examples could be studied, are such that a relationship between them and the subsequent course

of the disease might be established in the case of oral squamous carcinoma. This would require swabs to be taken at frequent and appropriate intervals during and immediately after radiotherapy. The practical difficulties of such a regime are recognised and whether such information could be of value in modification and manipulation of various forms of treatment to improve the patient's prognosis is a matter for speculation. Nevertheless, it is an avenue worthy of further study.

C O N C L U S I O N

This study on the diagnostic value of oral exfoliative cytology, has shown the technique to be practicable as a routine procedure, reliable, and of great value to the clinician. It can be a reasonable indication of the nature of certain benign conditions, such as bullous eruptions, but its principal value lies in the early detection of malignant change in lesions appearing clinically benign. As such, oral cytology provides the clinician with a diagnostic tool with which to assess mucosal changes when biopsy is not desirable, and further may guide selection of an area of tissue for biopsy. There are, however, certain specific situations where cytological smears are of doubtful reliability and should, therefore, not be expected to yield accurate reports. These are (1) encrusted squamous carcinomata of the lip;

(2) oral neoplasms covered by a thick necrotic layer.

(3) lesions advancing through deep tissue without involving the surface of the oral mucosa.

This work, however, does not support the contention that exfoliated cells fail to accurately reflect changes in oral

leukoplakia. Only rarely does malignant change in leukoplakia occur under a thick keratinous layer, but generally takes place in an area of ulceration, erythema or granulation within or at the edge of the white patch. Smears from such lesions are reliable.

Oral exfoliative cytology is indicated for the follow-up of oral cancer patients after radiotherapy to help detect recurrent disease.

An 'atlas' of oral exfoliated cell types has been compiled and an attempt made to correlate the various degrees of atypia encountered with various types of lesions.

The simplicity of the technique and equipment involved in oral cytology as applied here recommend further the use of this aid as an essential part of the oral diagnostic regimen in private dental or medical practice, as well as institutional clinics.

As a research tool, exfoliative cytology has considerable potential. Mucosal changes are accurately reflected without trauma, and it is evident from the study of irradiation effects that tissue responses to various factors can be assessed using those methods. A number of points for further investigation using exfoliative techniques per se, or in a modified form, have been highlighted by this project. Oral epithelial proliferation and maturation processes are not fully understood and could be investigated

in this way. Of more clinical significance is the controversy over the problem of whether lichen planus has or has not in fact a role in carcinogenic processes. The frequency and significance of field cancerisation effects still appears to be subject to considerable speculation.

A T L A S

OF

EXFOLIATED CELL TYPES

Papanicolaou stain used in all coloured preparations.

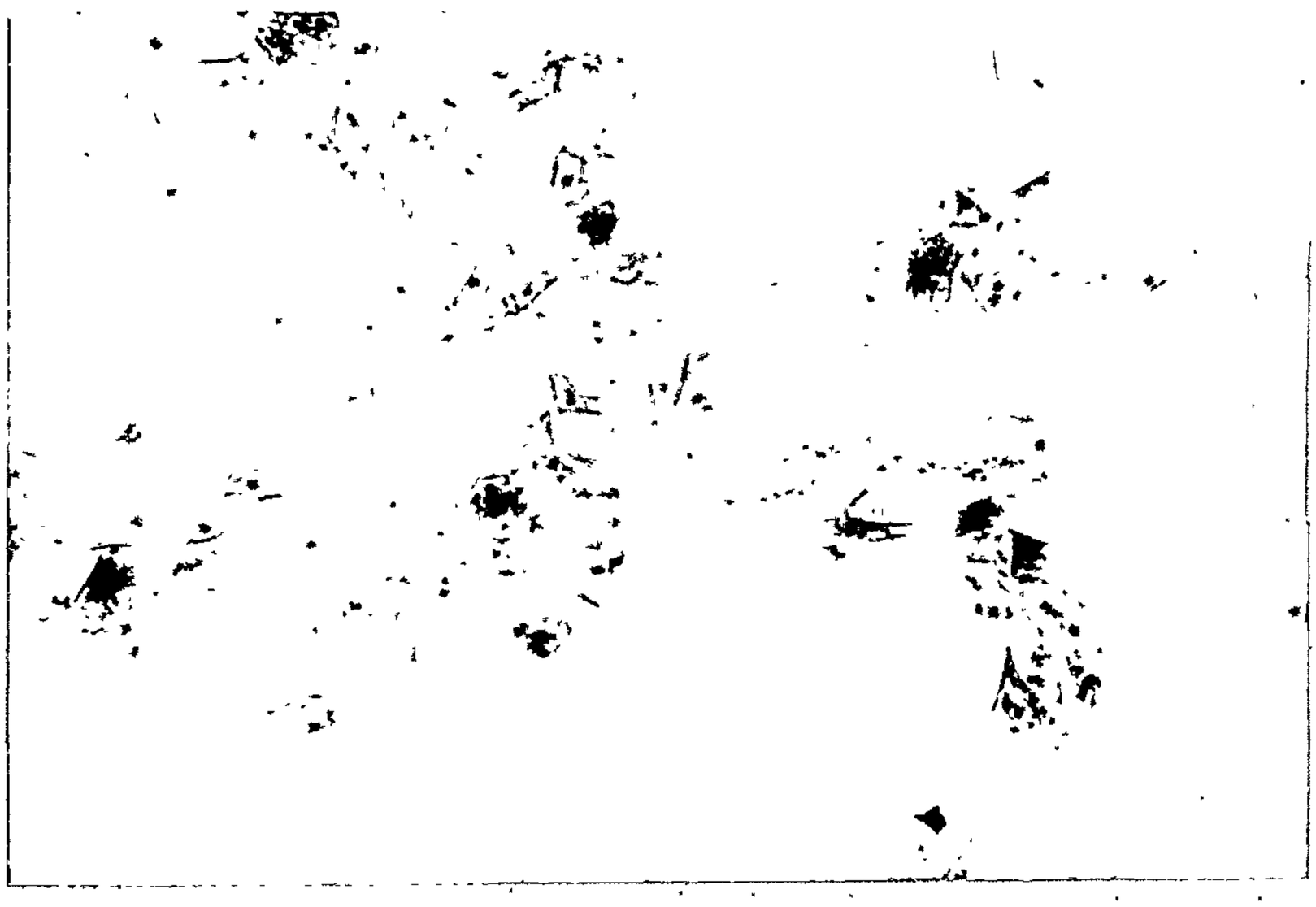


Fig. 6. Typical field of normal gingival cells. Pentachlorophenol stain. X 210.

Fig. 7. Normal oral mucosa folded to resemble a tadpole cell. X 2,100.

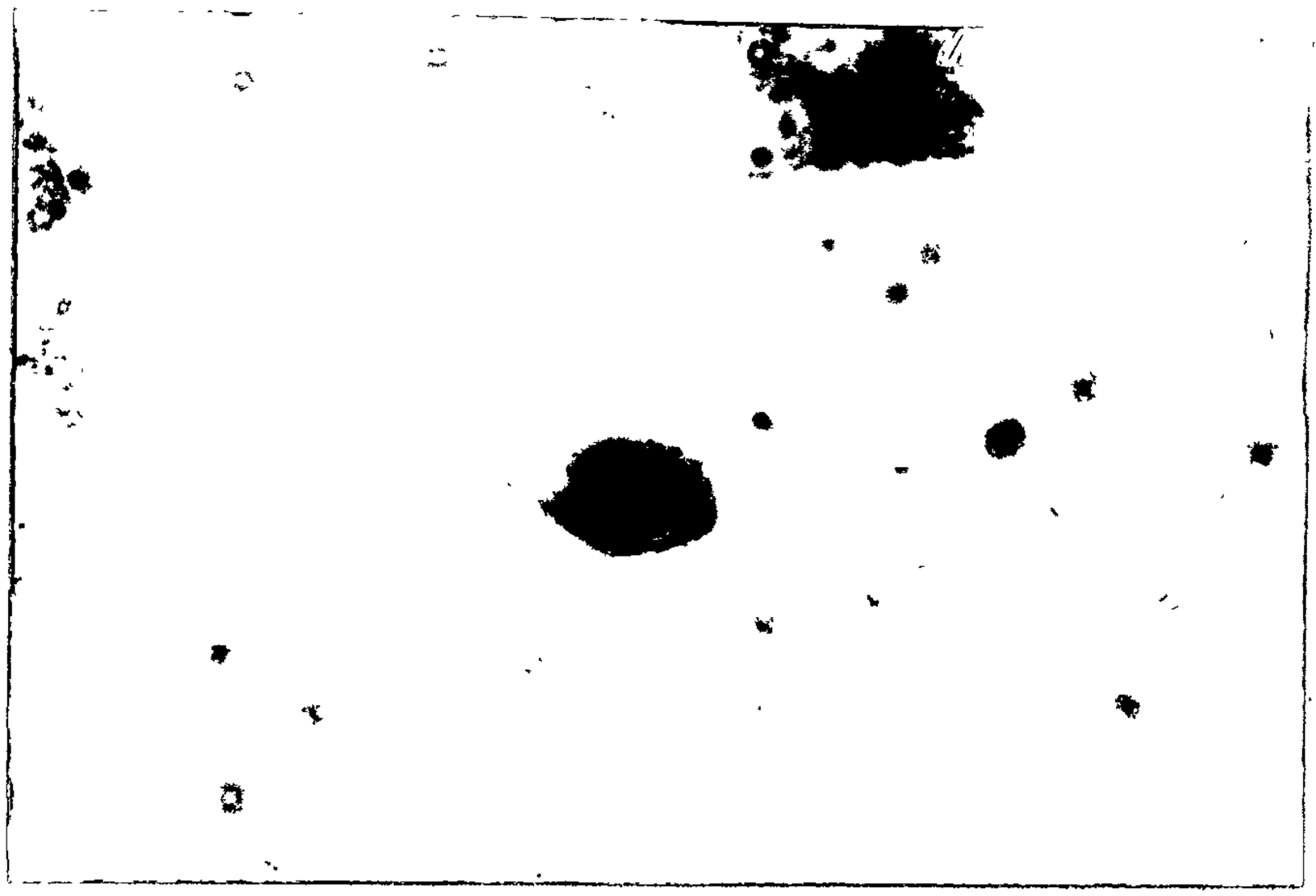


Fig. 8. Inner layer parabasal cell. X 2,400.

Fig. 9. Dividing parabasal cells. These cells are not common in oral smears. X 2,400.

Fig. 10. Phagocytic histiocyte. X 1,000.

Fig. 11. Field of small histiocytes and polymorphs around degenerate keratinised cells. The vague cellular outline and vacuolated consistency of the histiocytes is obvious. X 1,000.

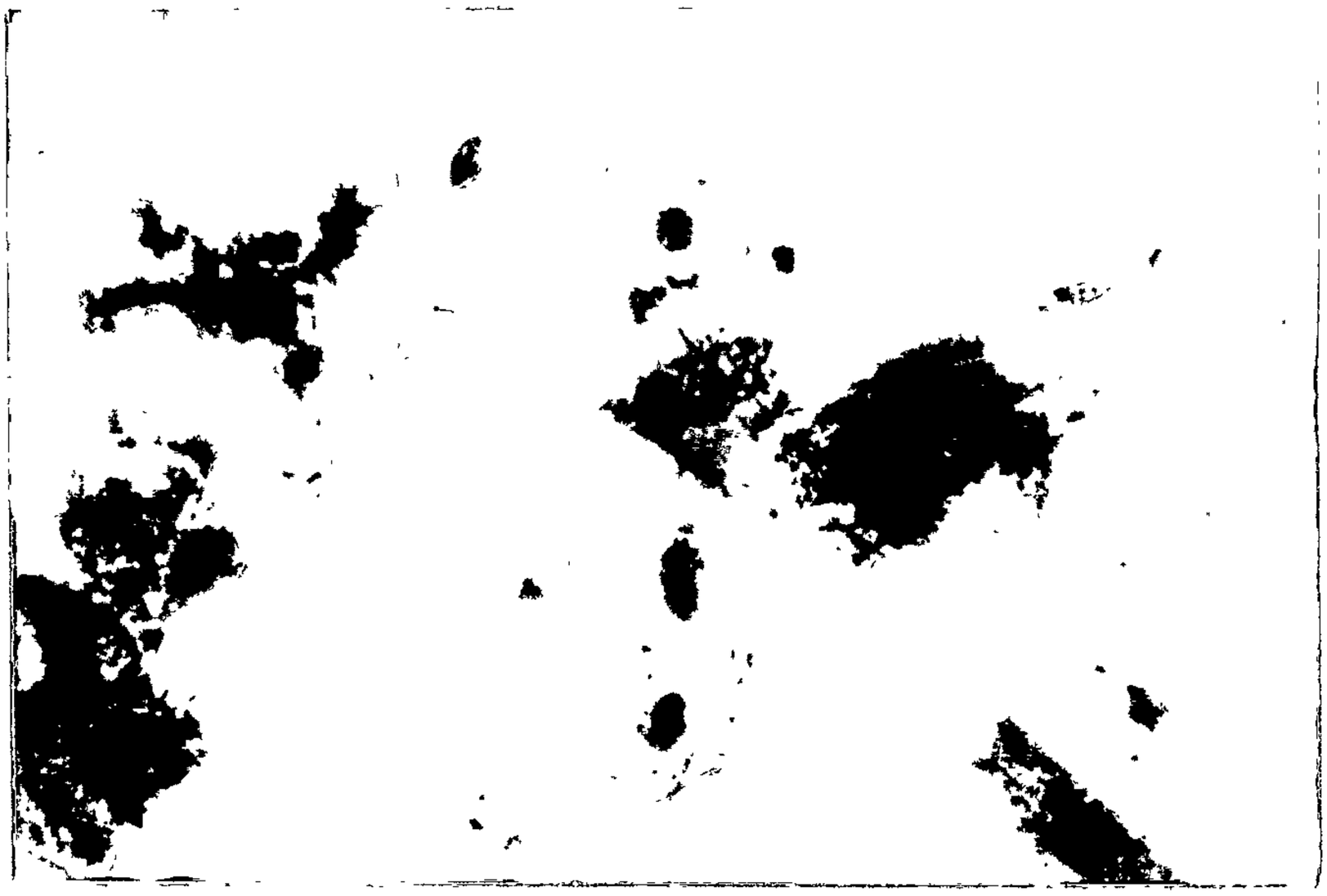


Fig. 12. Leptothrichia organisms are not infrequently found, particularly in smears from the tongue. X 1,000.

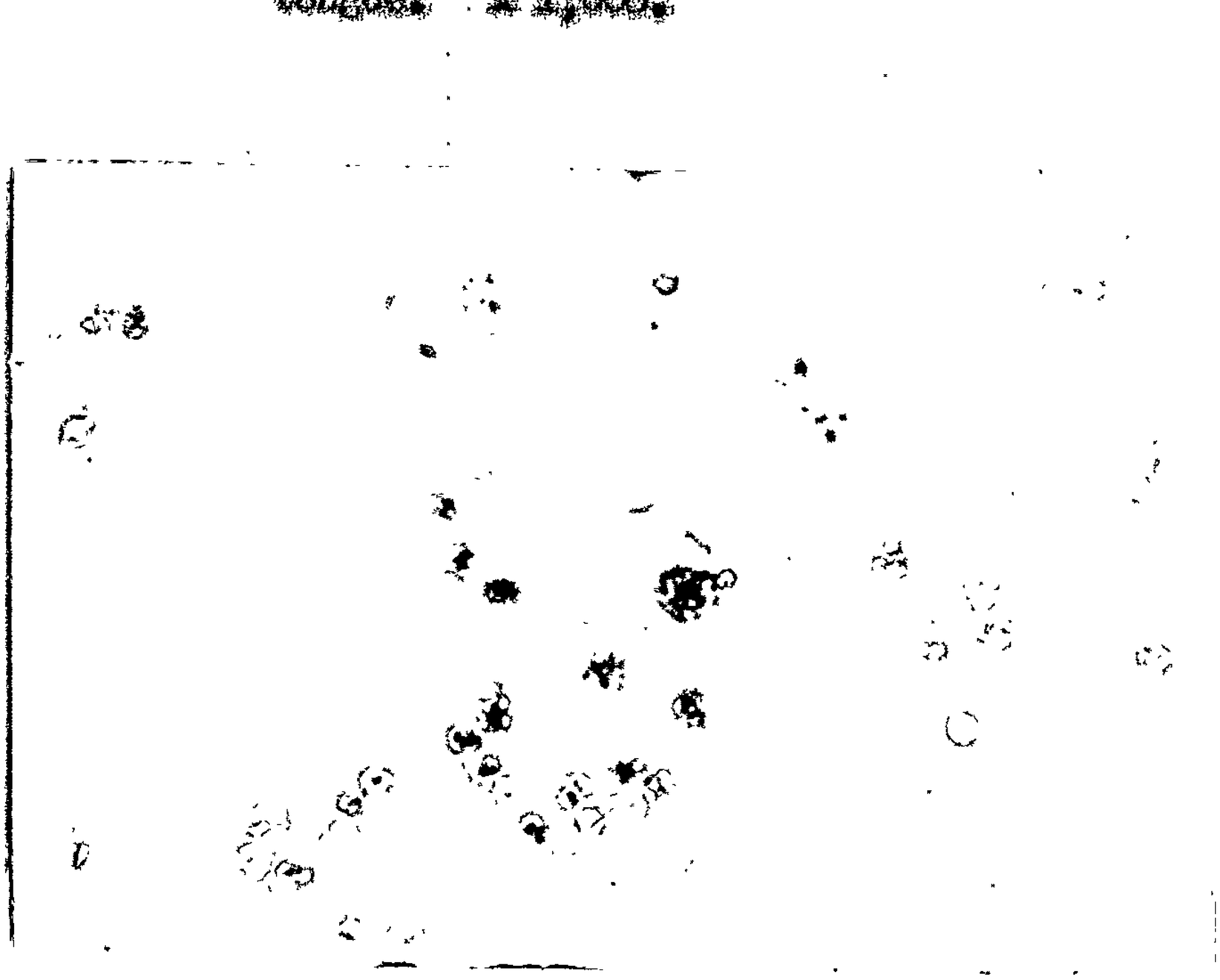


Fig. 13. Smears from an oral aphthous ulcer. The cells are fairly normal, although there may be some tendency for increased cell size and vacuolization. X 1,000.

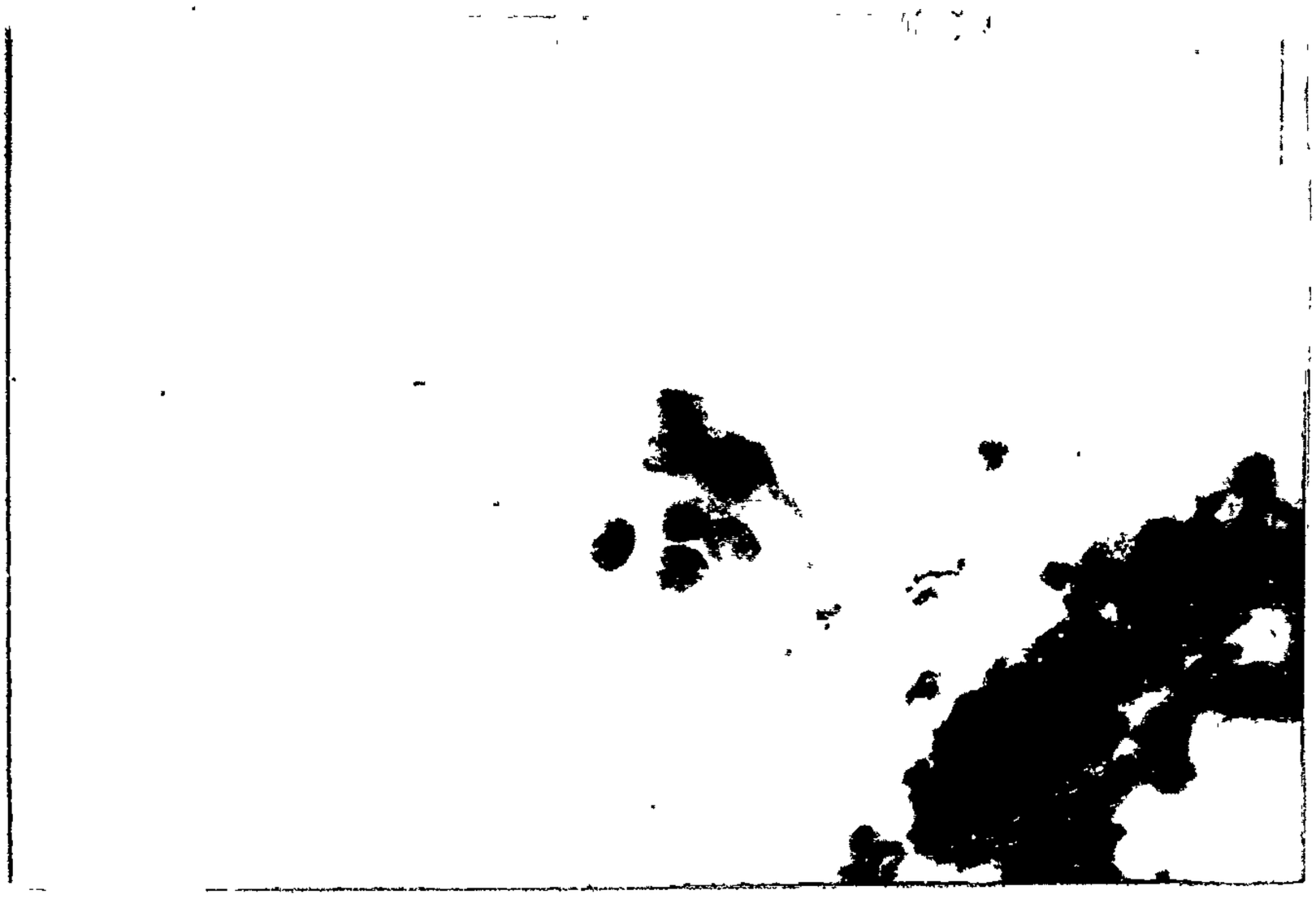


Fig. 14.

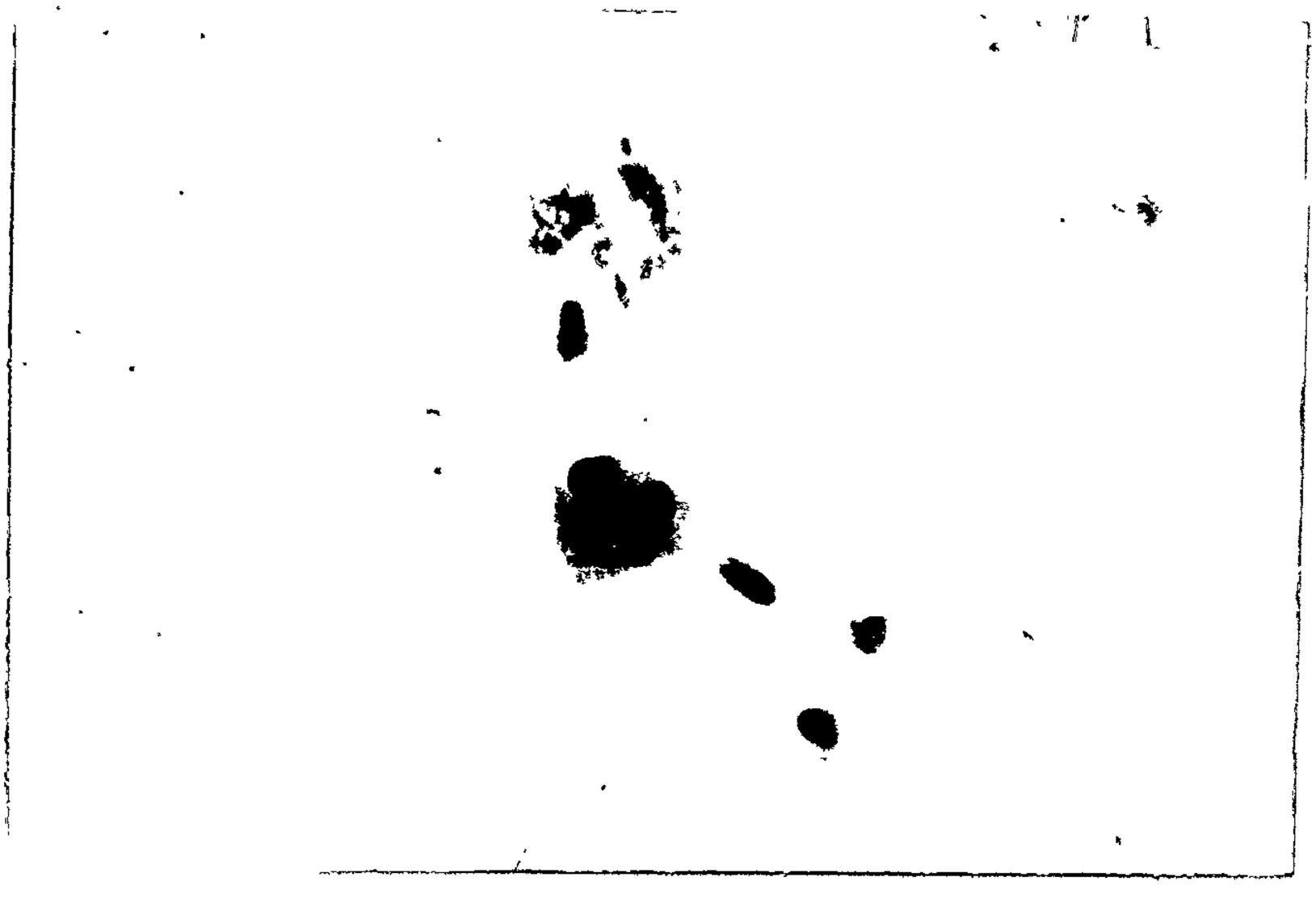


Fig. 15.

Figs. 14 and 15. Multinucleated cells with vague cell borders and irregular shape, in a smear from herpes simplex. X 1,000.

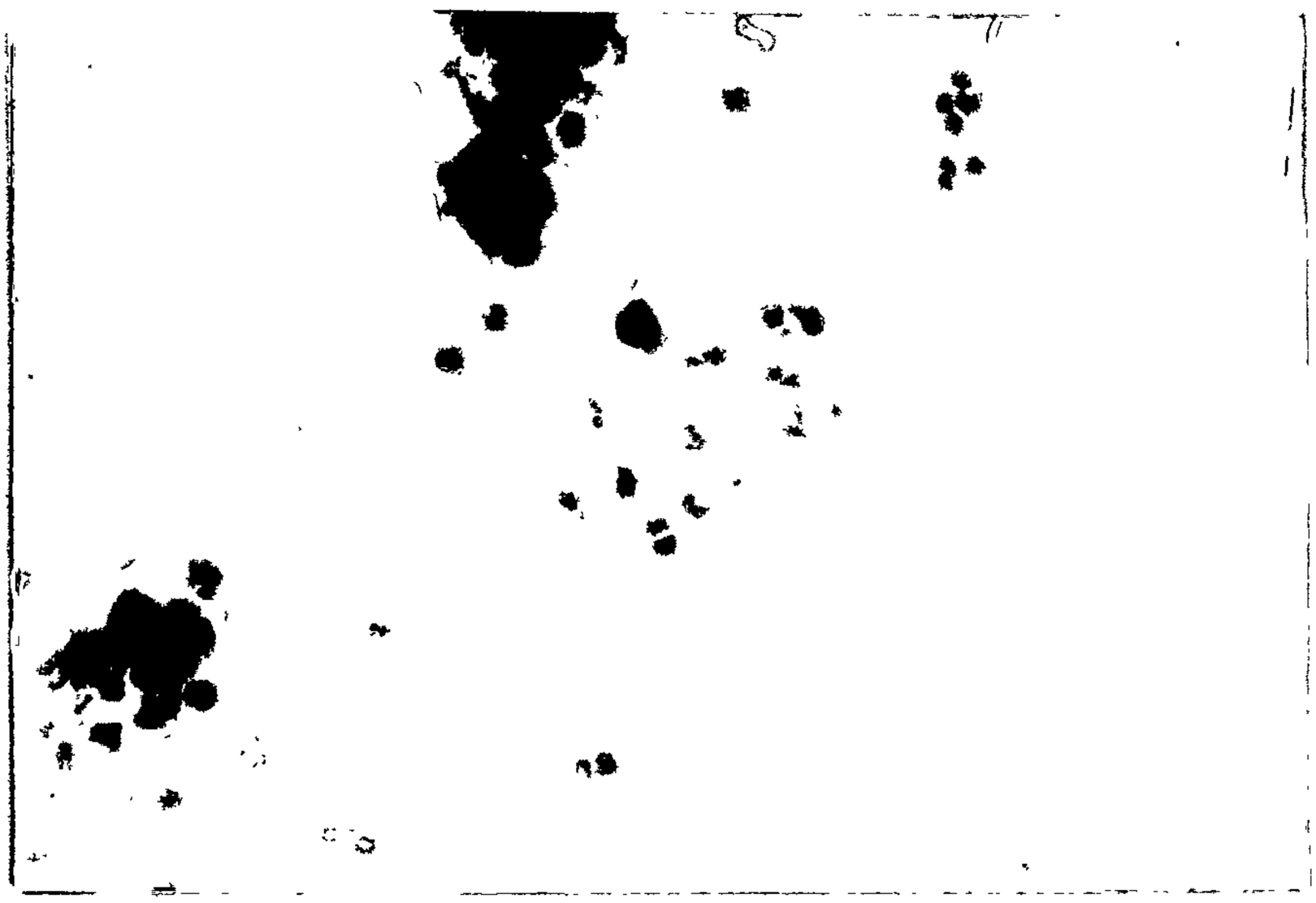


Fig. 16. Partially acantholytic cells from an oral bulla. The patient has recurring lesions suggestive of a benign pemphigus. X 1,600.

Fig. 17. Duct formation of ectopic gland cells in a lesion from the floor of the tooth. X2,400.

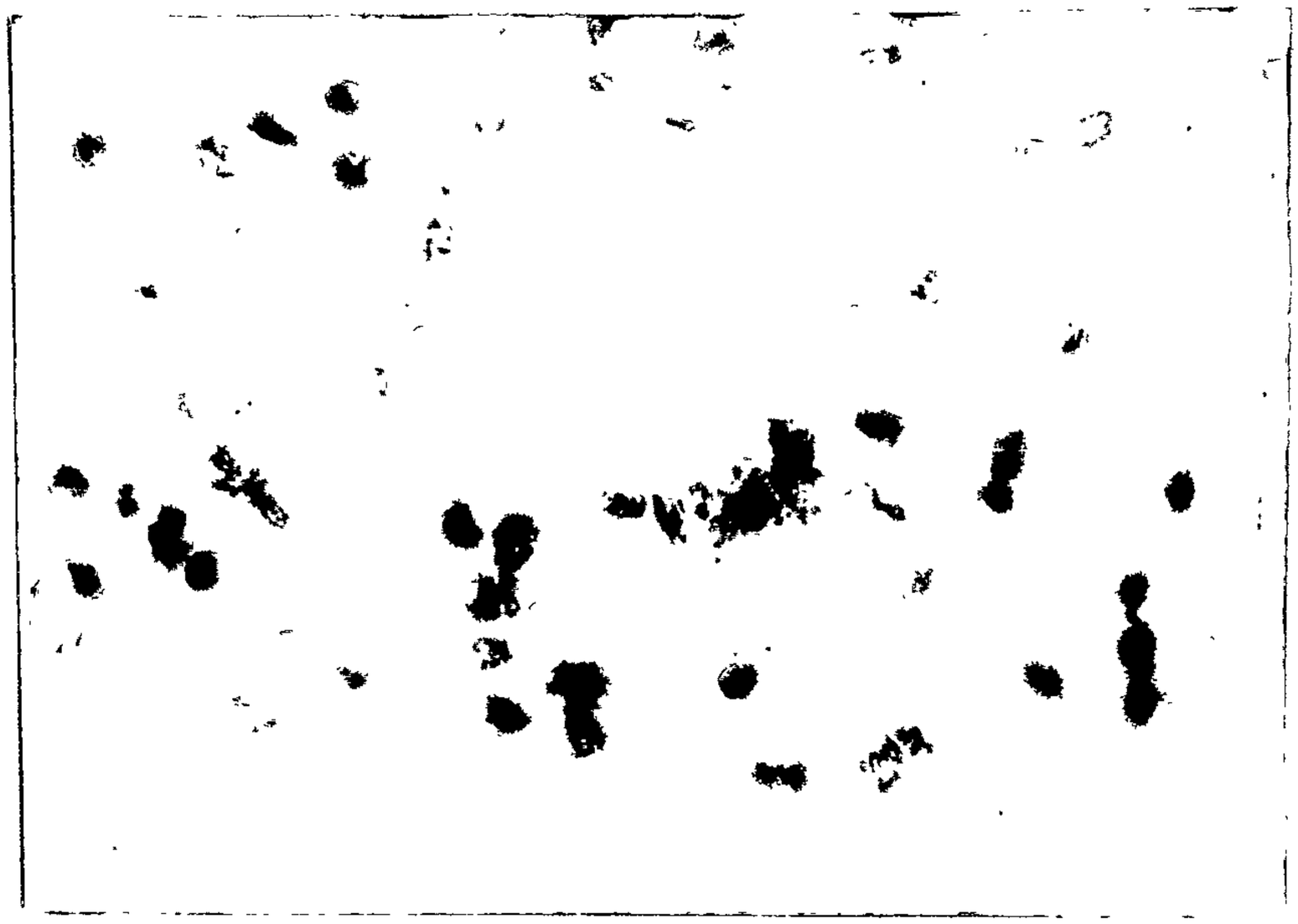


Fig. 18.

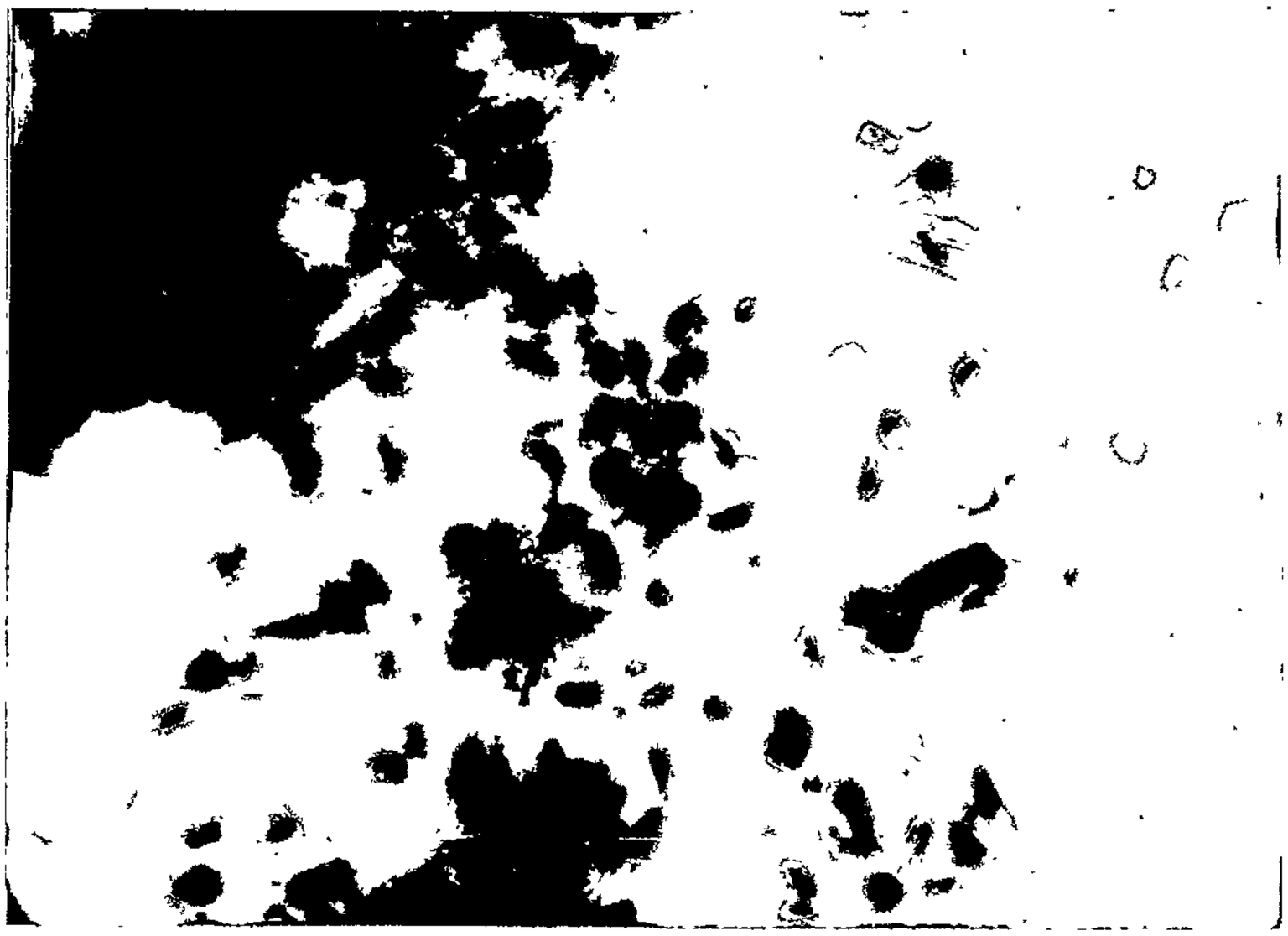


Fig. 19.

Figs. 18 and 19. Minor salivary gland cells in smears from a traumatic ulcer in the floor of the mouth. Histological section of a biopsy taken at the same appointment showed salivary glands very close to the surface. Note the long rectangular shape of the gland cells, with foamy vacuoled cytoplasm but definite cell outlines. The nuclei are well preserved and of uniform consistency. X 1,000.

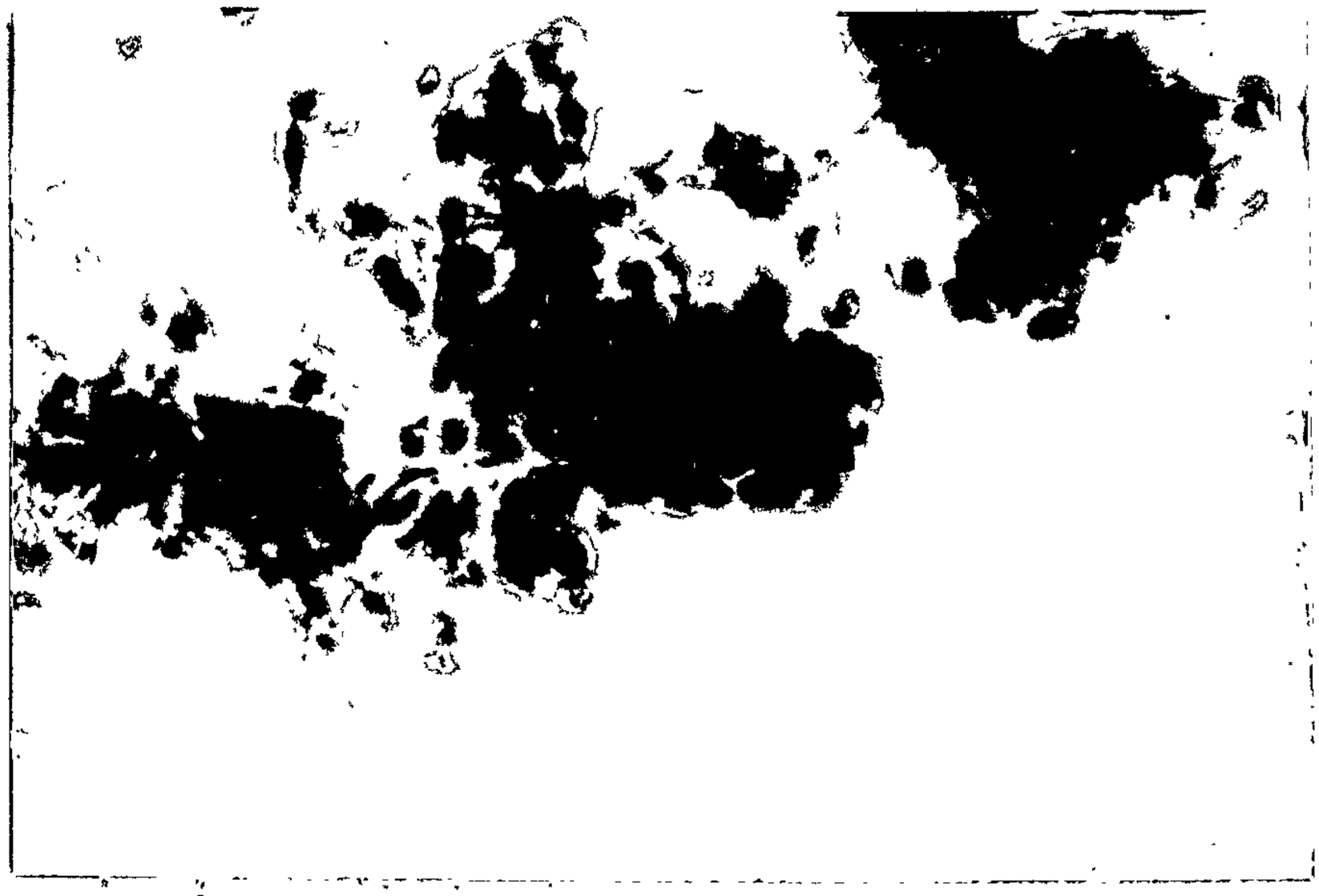


Fig. 20. Cluster of contaminate respiratory tract cells in an oral smear. These cells probably originate from the nasopharynx and are found only occasionally. X 1,000.

DESCRIPTIVE ORAL LESIONS

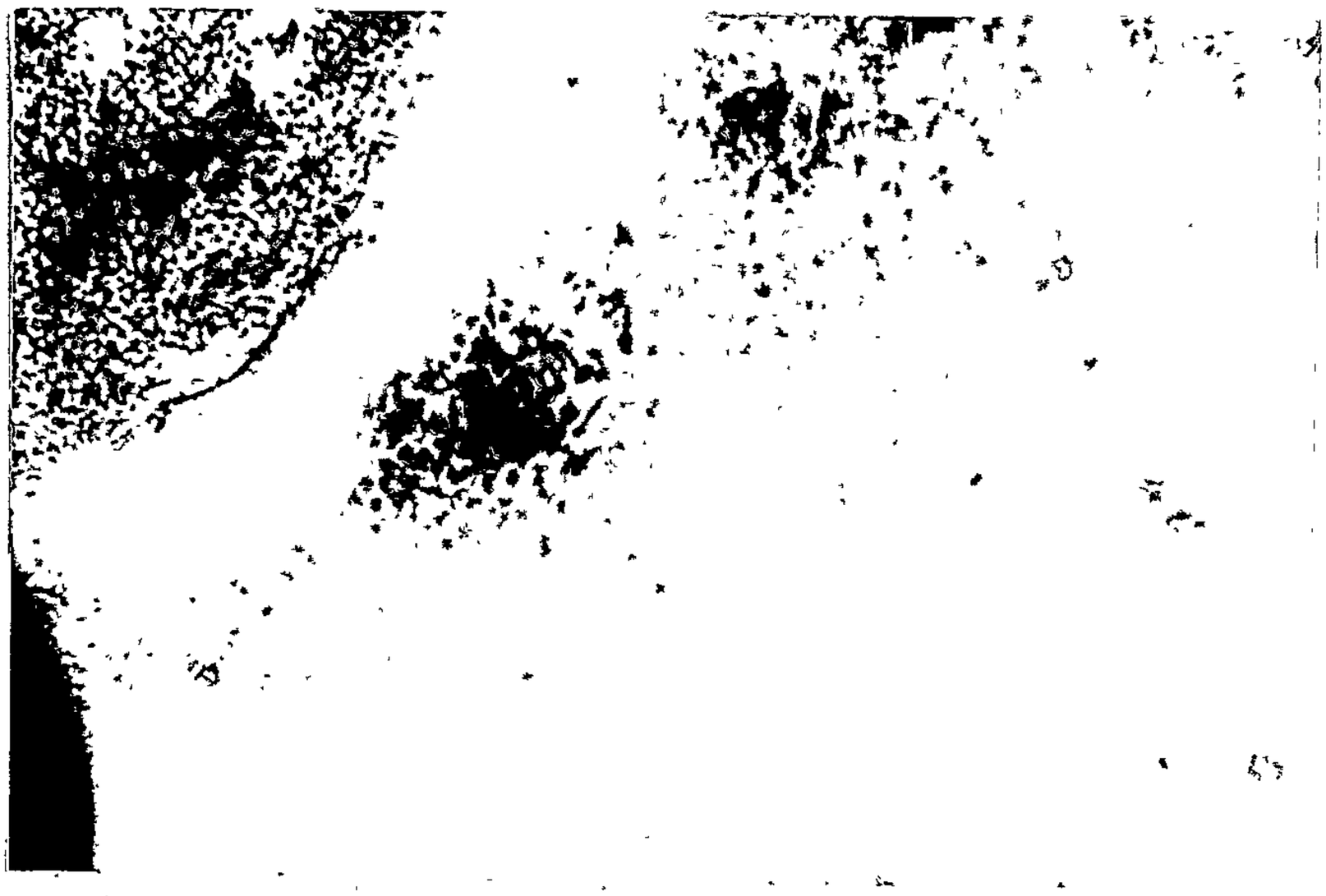


Fig. 21. A cocoon field from an acute oral ulceration. X 240.

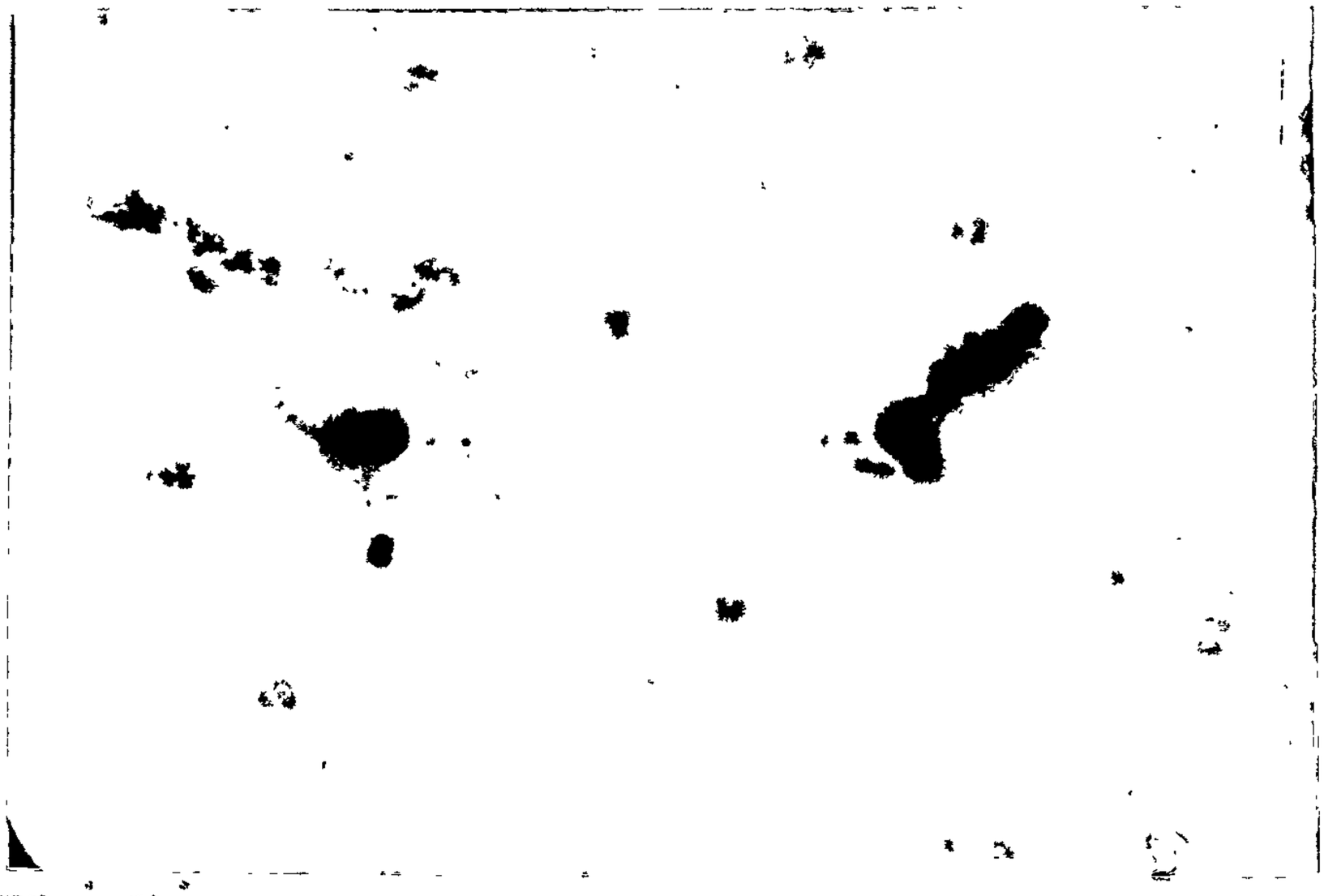


Fig. 22.

Fig. 23.

Figs. 22 and 23. Small cells with scanty cytoplasm but large nuclei, such as these, may be seen in inflammatory oral lesions, especially in non-cornified mucosa, such as in the cheek and soft palate. X 1,008.

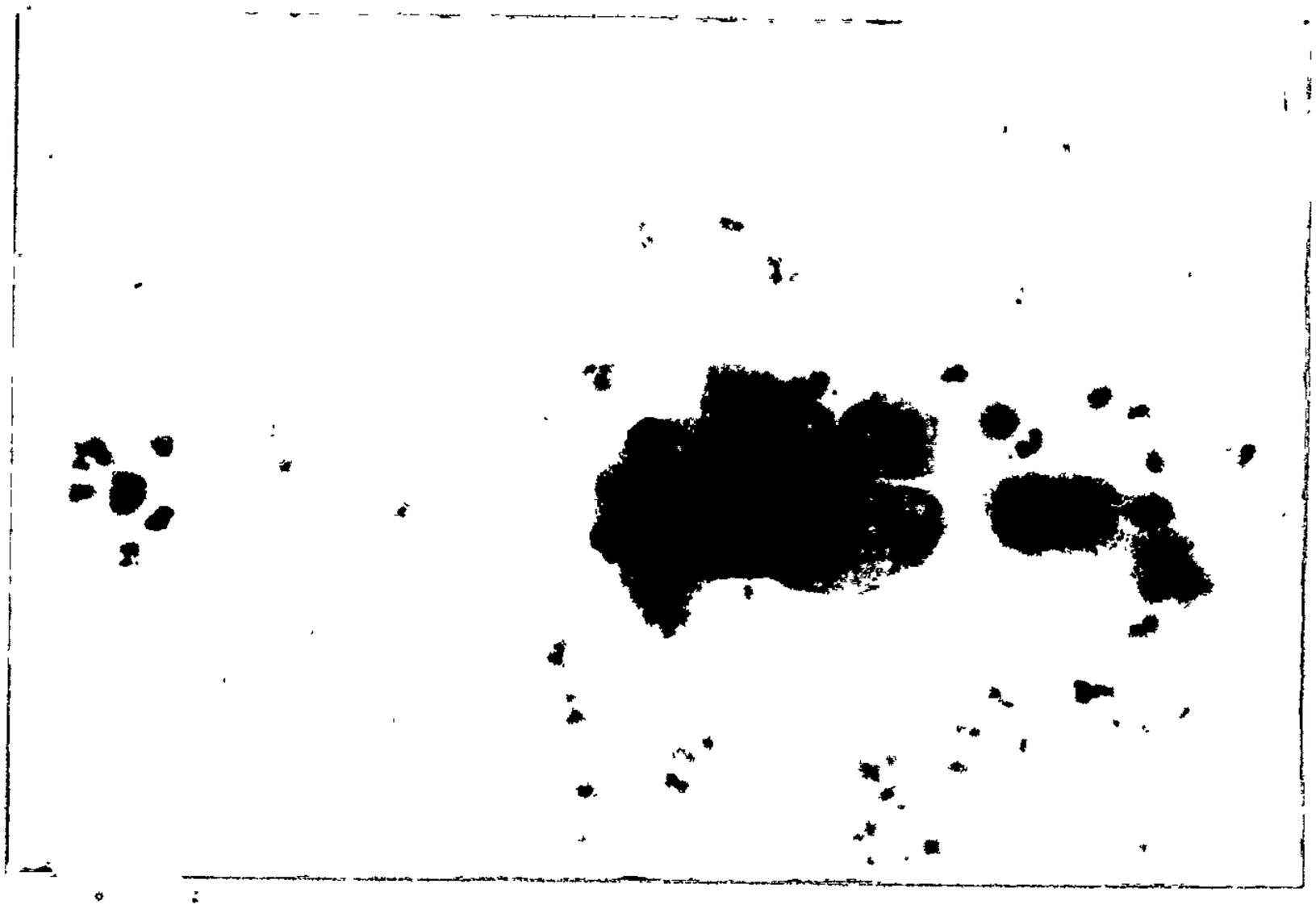


Fig. 24.

Fig. 25.

Figs. 24 and 25. Intermediate and superficial cells with large, fairly dark nuclei in smears from benign acute buccal erosions. The nuclei, however, are uniform in size and shape and do not display clumps of chromatin, although nucleoli are often prominent. These lesions heal quickly after removal of irritants. X 1,000.

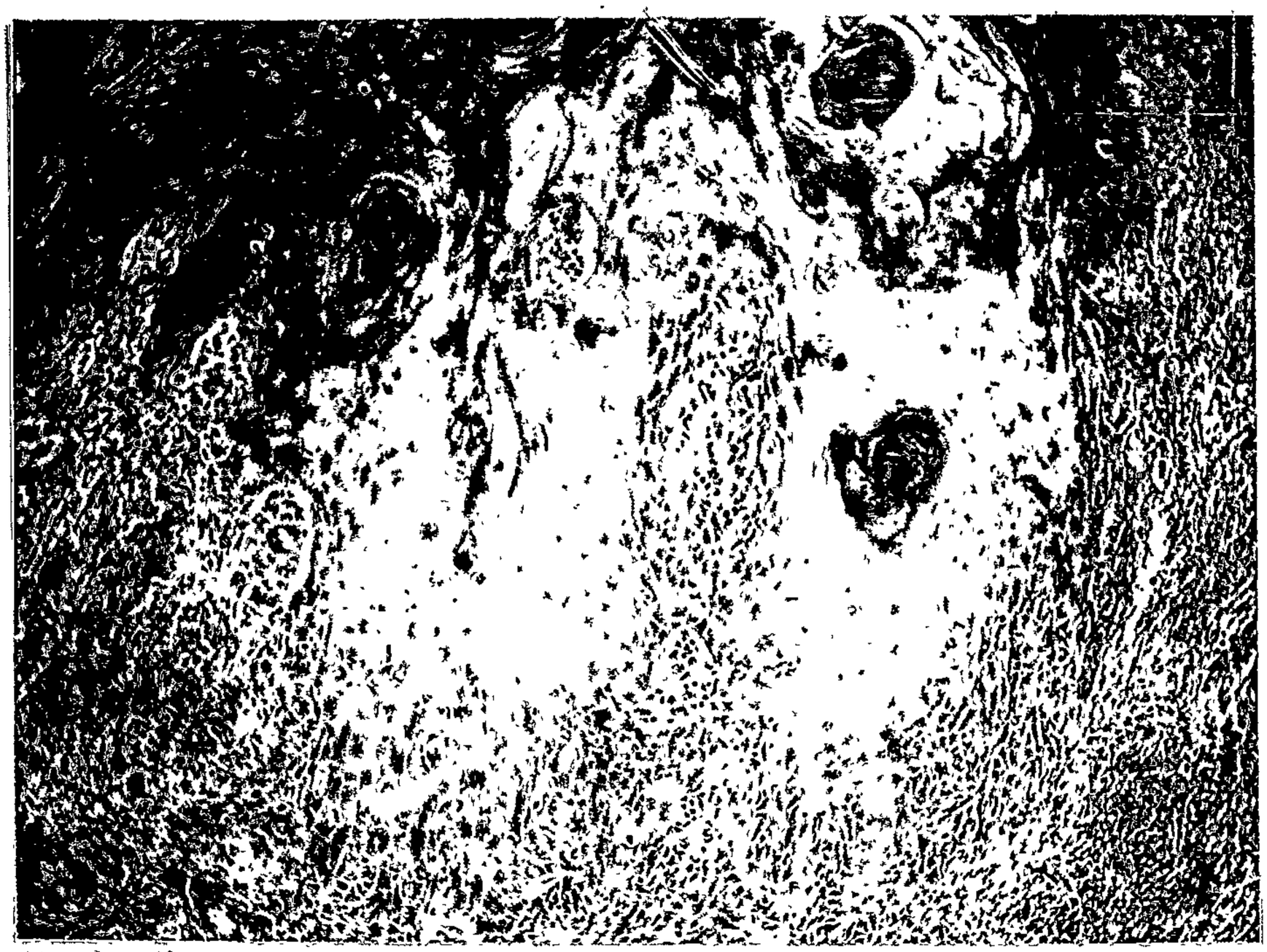


Fig. 26. Severe epithelial hyperplasia and inflammatory reaction to chronic irritation of the hard palate. The irritant in this case was a vacuum chamber in the palate of a maxillary denture; when the patient stopped wearing the prosthesis, the lesion slowly healed. H. & E. stain. X 300.

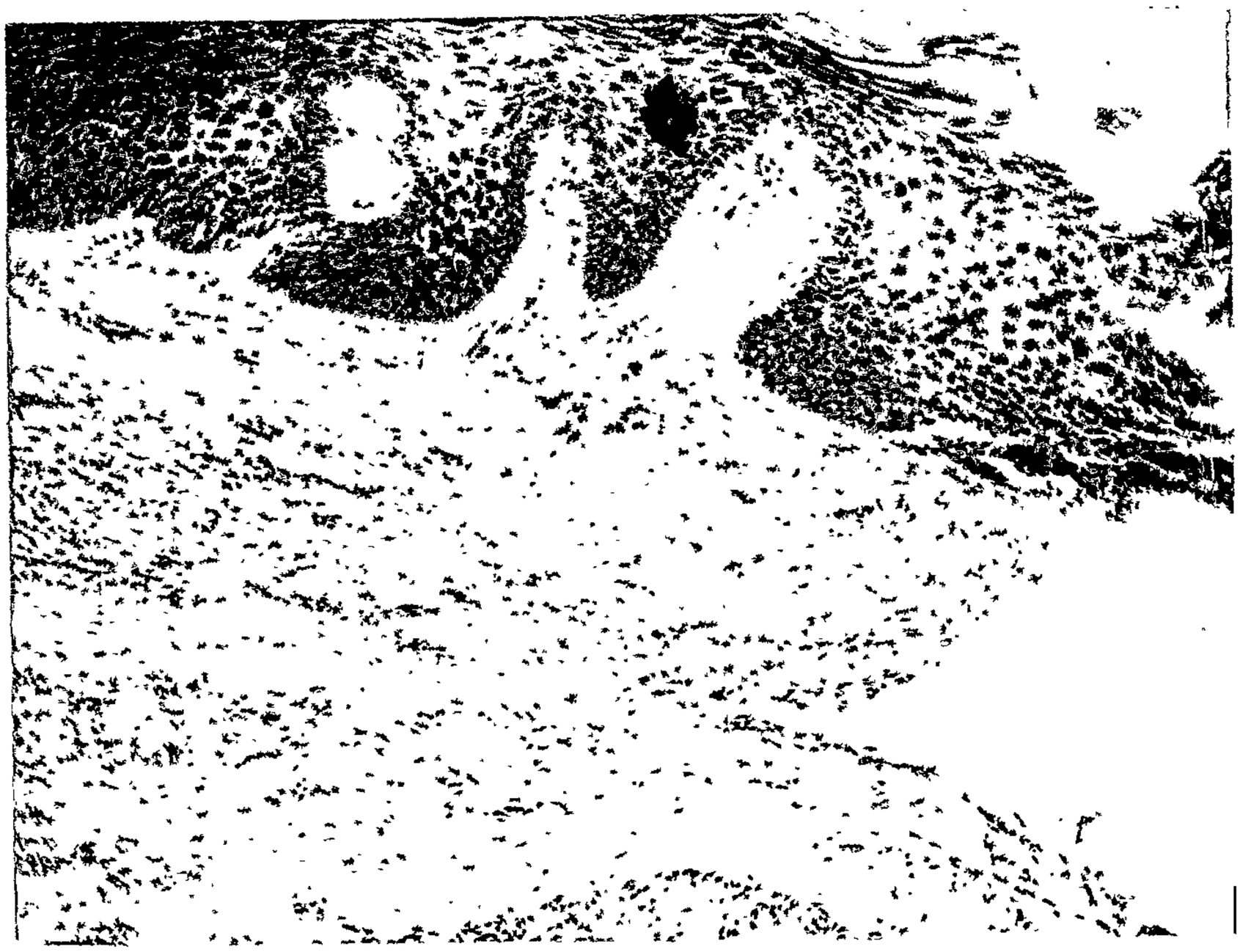


Fig. 27. Abnormal mitotic activity in epithelium around a traumatic ulcer in the lower labial sulcus. H. & E. stain. X 300.

Fig. 28. Malignant basal cells in a cytological smear from an oral carcinoma. These "third type" cells which characterize carcinoma in situ in vaginal smears, are not usually found in oral smears. X 1,000.

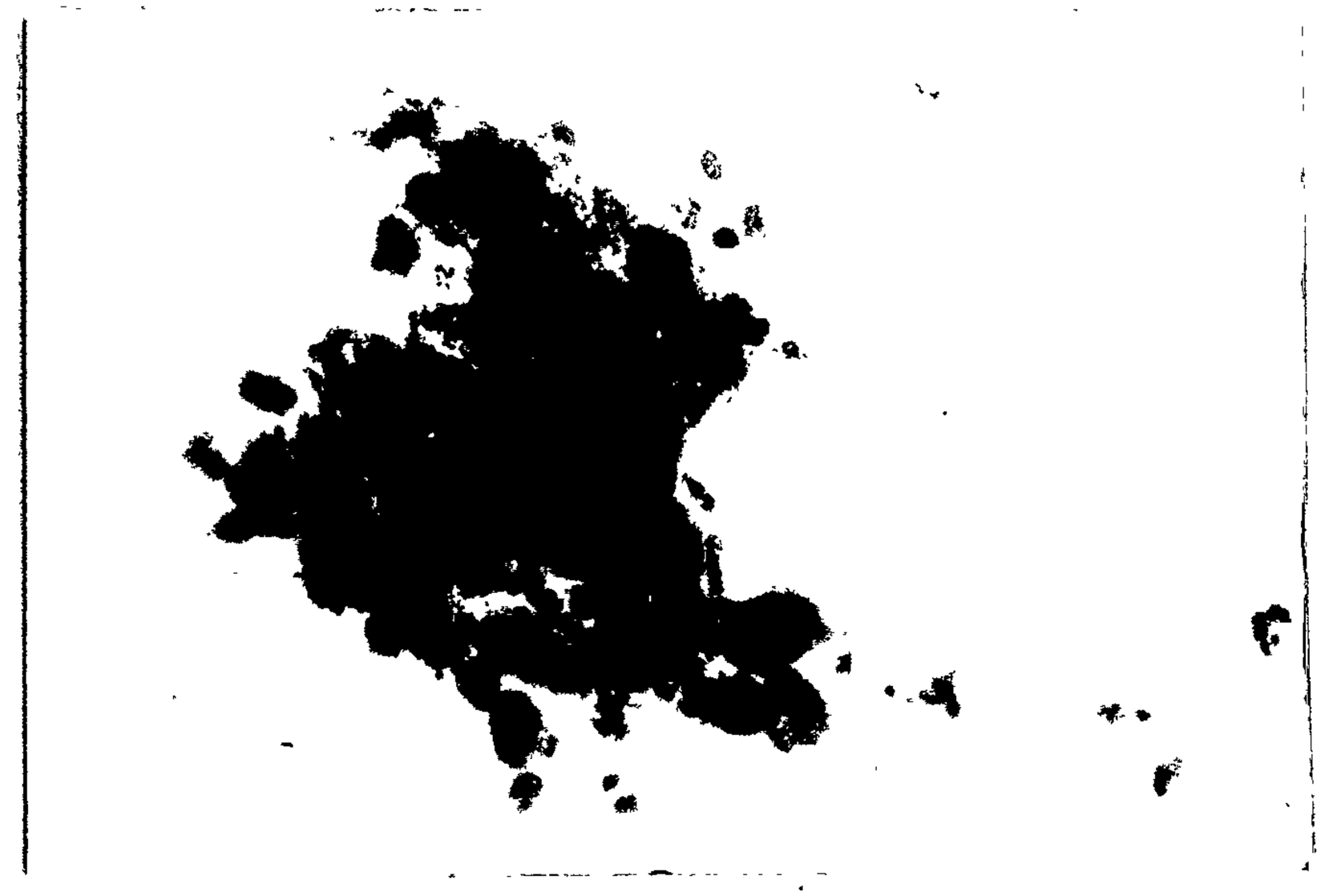


Fig. 29. Malignant cells in a smear from a very superficial erosive carcinoma of the tongue. The cells here are more typical of an oral lesion, in the degree of keratinization, but it can be noted that the nuclei in the cells in both Figs. 28 and 29 show chromatin clumping and lack of uniformity in size and shape. X 1,000.

Fig. 30. Early erosive squamous carcinoma. H. & E. stain. X 300.

Fig. 31. Well differentiated squamous cell carcinoma of oral mucosa. H. & E. stain. X 300.

Fig. 32. Section showing acanthosis and parakeratosis in epithelium of the soft palate, as a result of pipe smoking. H. & E. stain. X 300.

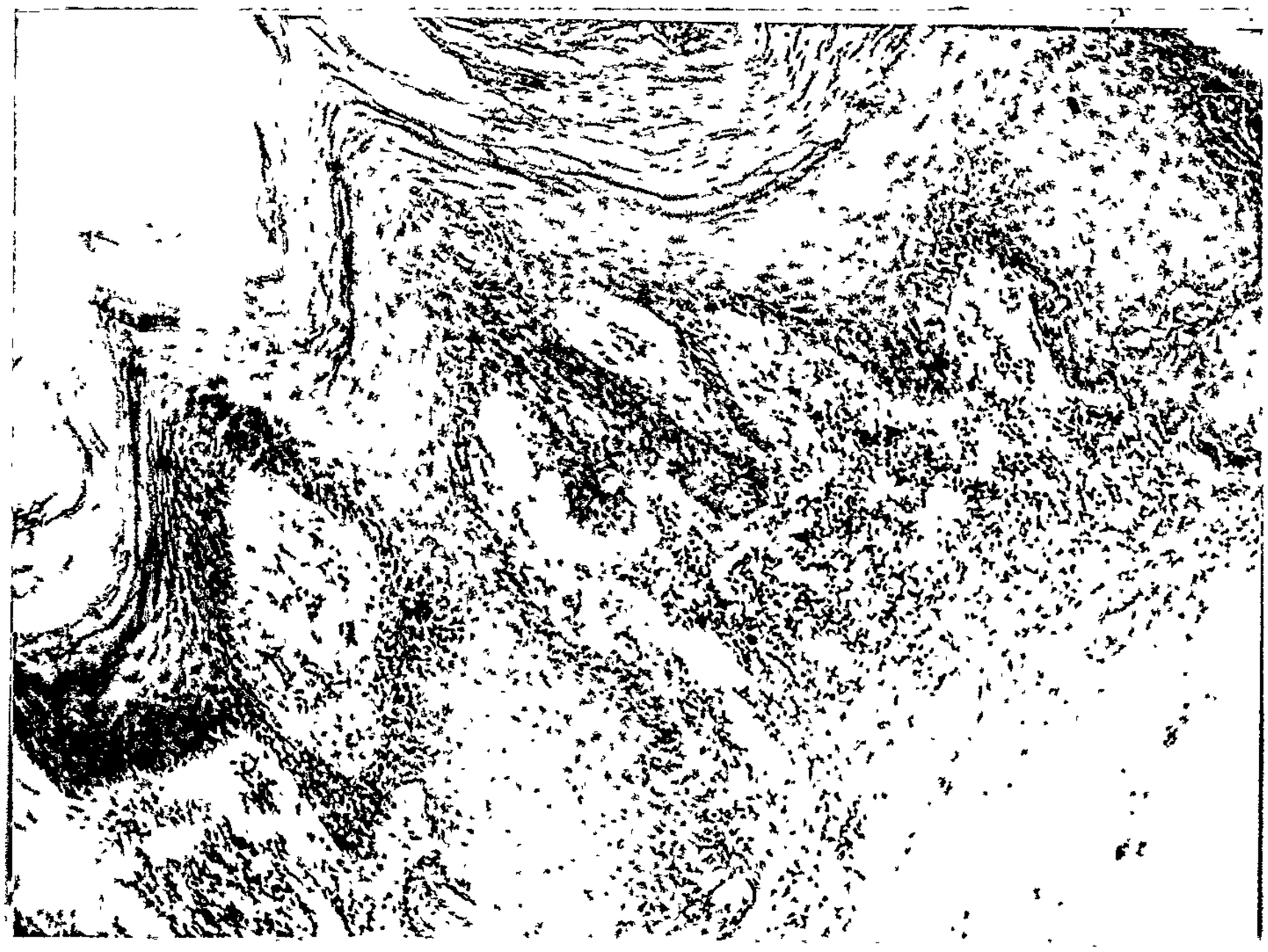


Fig. 33. "True" leukoplakia in oral epithelium showing hyperkeratosis, some dyskeratosis and inflammatory reaction in stroma. H. & E. stain. X 300.

Fig. 34. Sheet of parakeratotic cells from a non-malignant oral white patch. X 1,000.

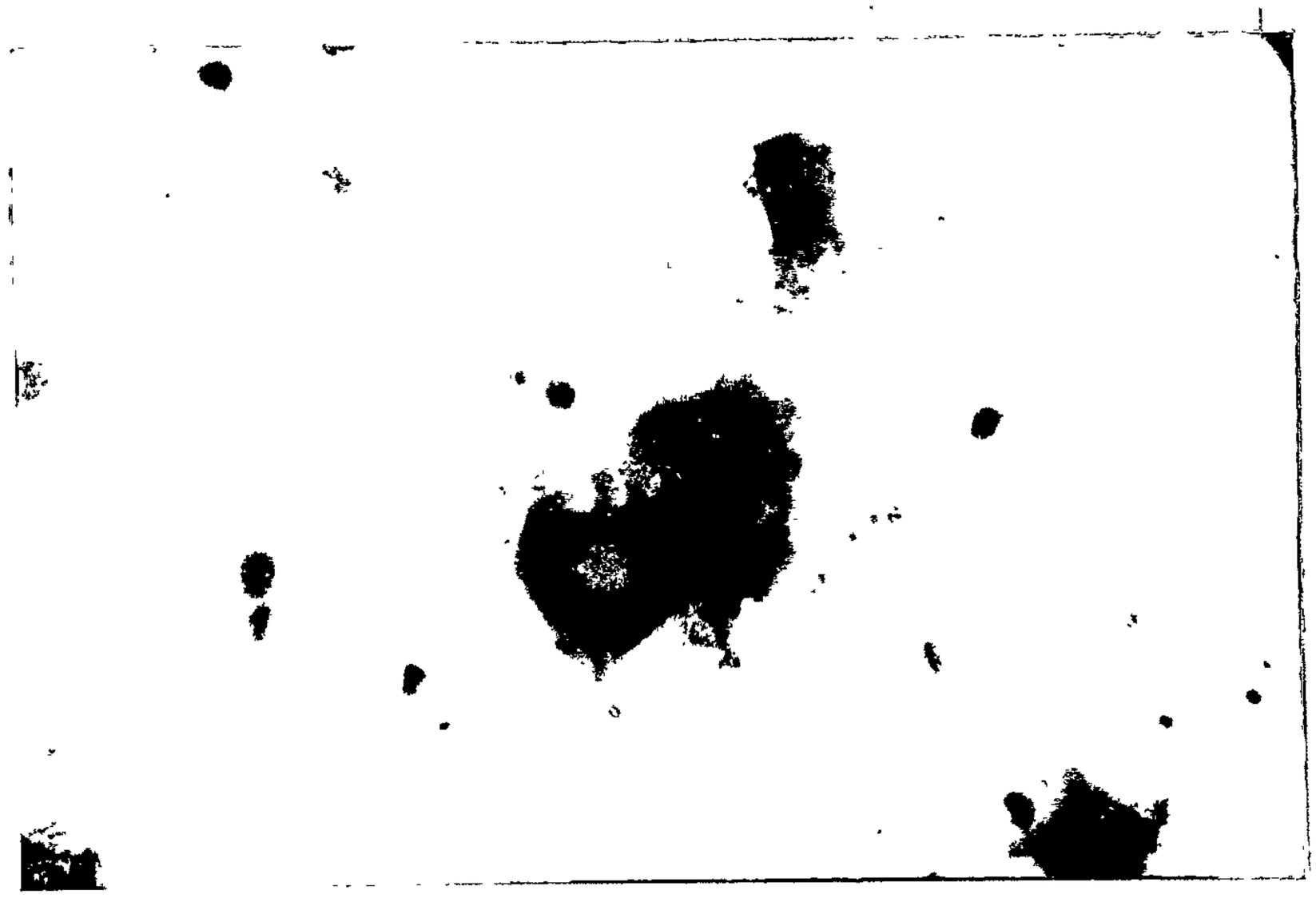


Fig. 35. Cell showing a perinuclear vacuole. These may be seen in smears from oral white lesions but are not typical of leukoplakia. X 1,000.

Fig. 36.

Fig. 37.

Figs. 36 and 37. Degenerating cells in neuro from chronic leukoplakia. X 1,000.

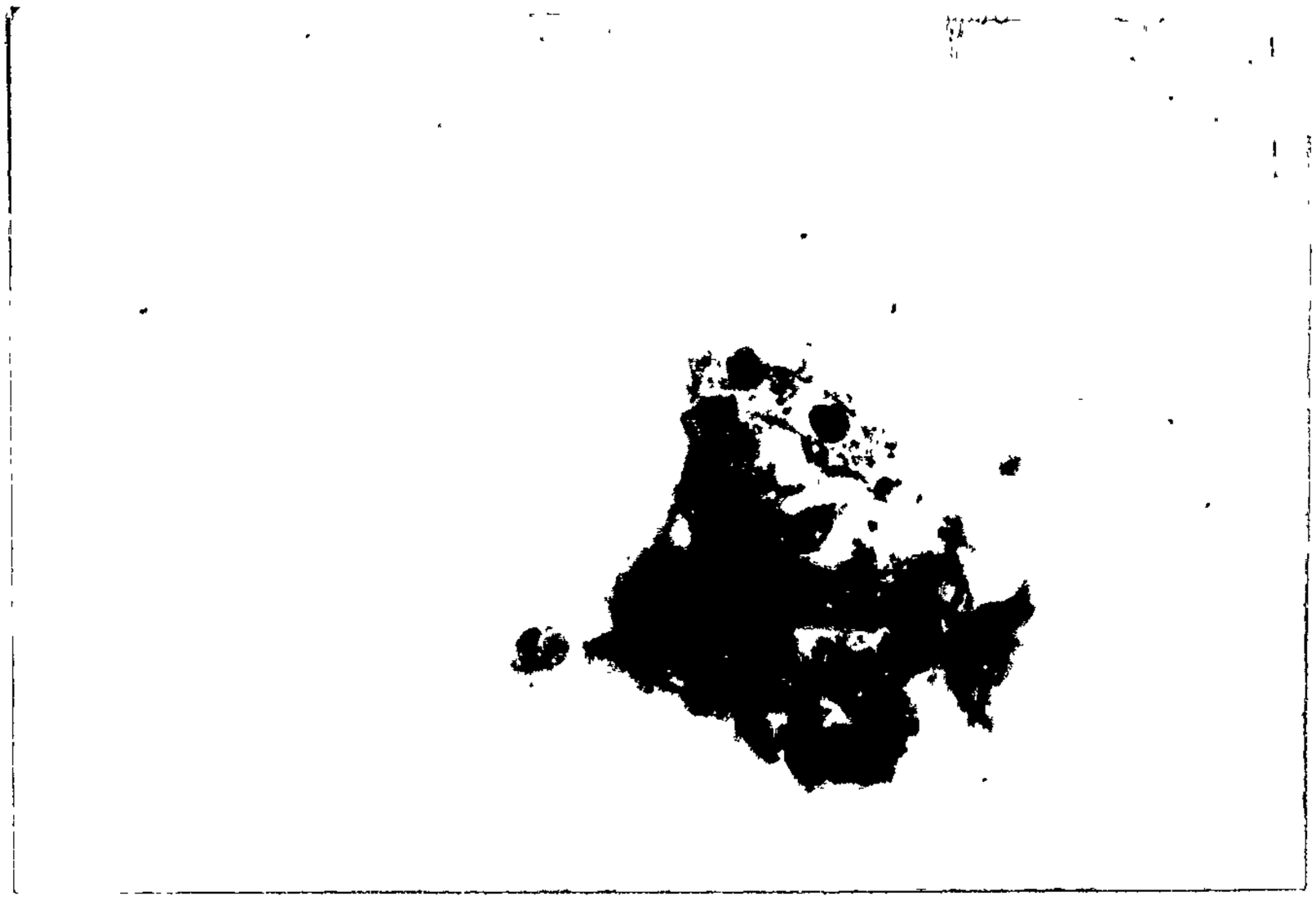


Fig. 38. A group of superficial cells showing large, well preserved nuclei, from an irregular elevated white lesion, resulting from a chronic candidiasis. X 1,000.

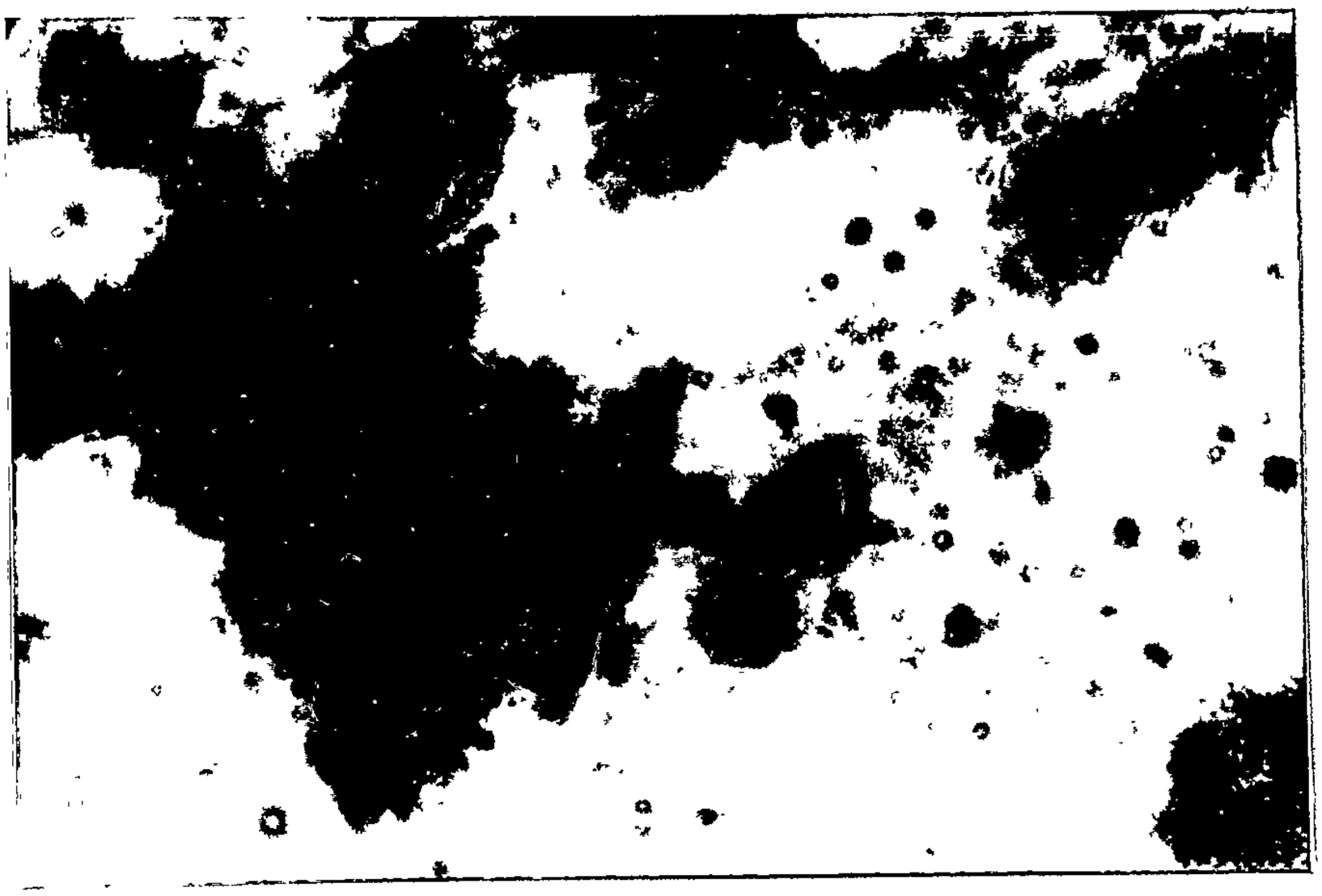


Fig. 39. Cells showing cytocytic features (large hyperchromatic, somewhat irregular nuclei surrounded by adequate cytoplasm) from chronic verrucous leukoplakia of the buccal mucosa. X 1,000.

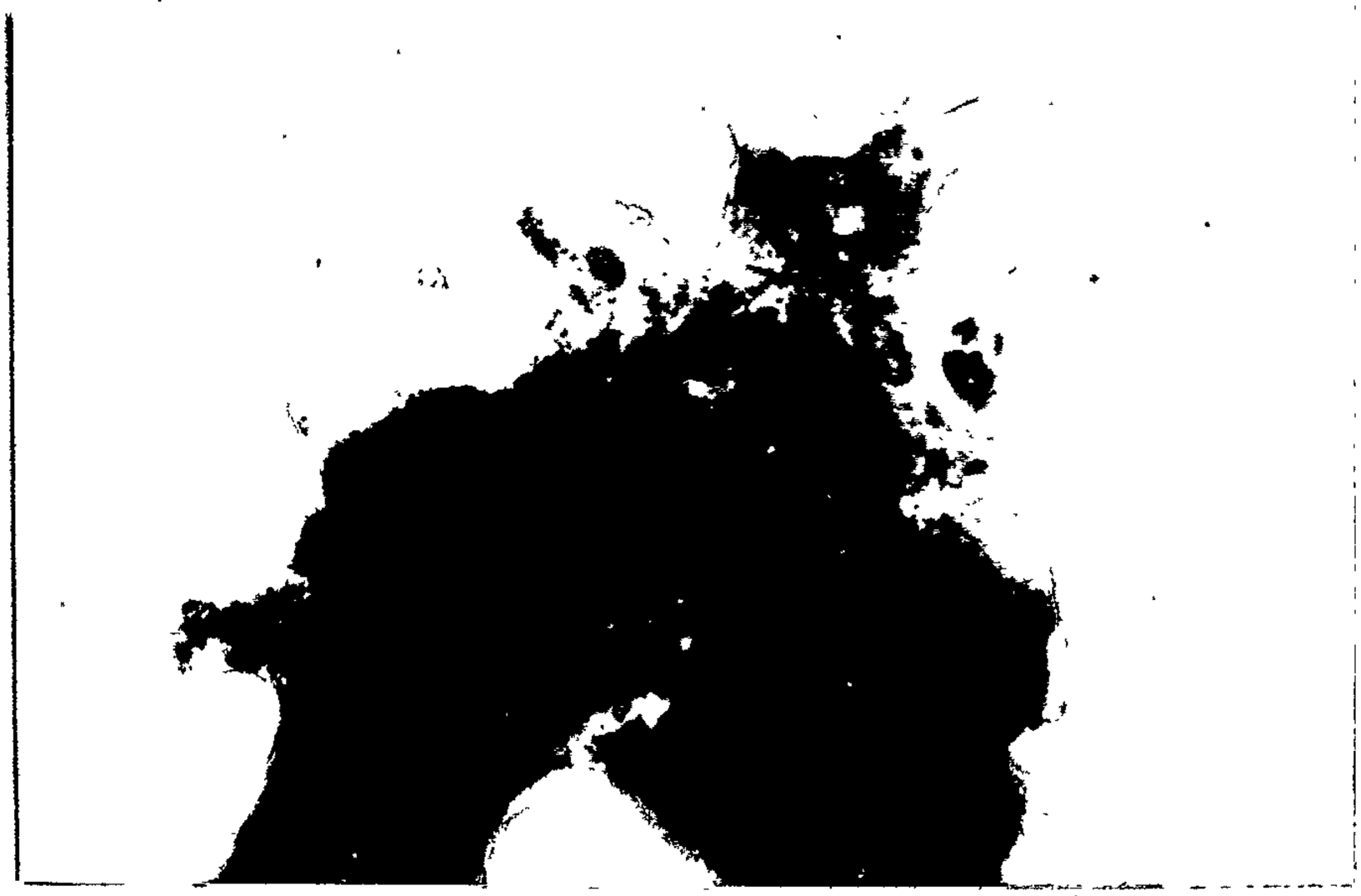


Fig. 40. Large, hyperchromatic, irregular nuclei in a sheet of superficial cells from an ulcerated, squamous carcinoma of the lip. Benign white lesions frequently shed clumps of cells with well preserved nuclei, but none as large, irregular and black as these (compare with Fig. 32). X 1,008.

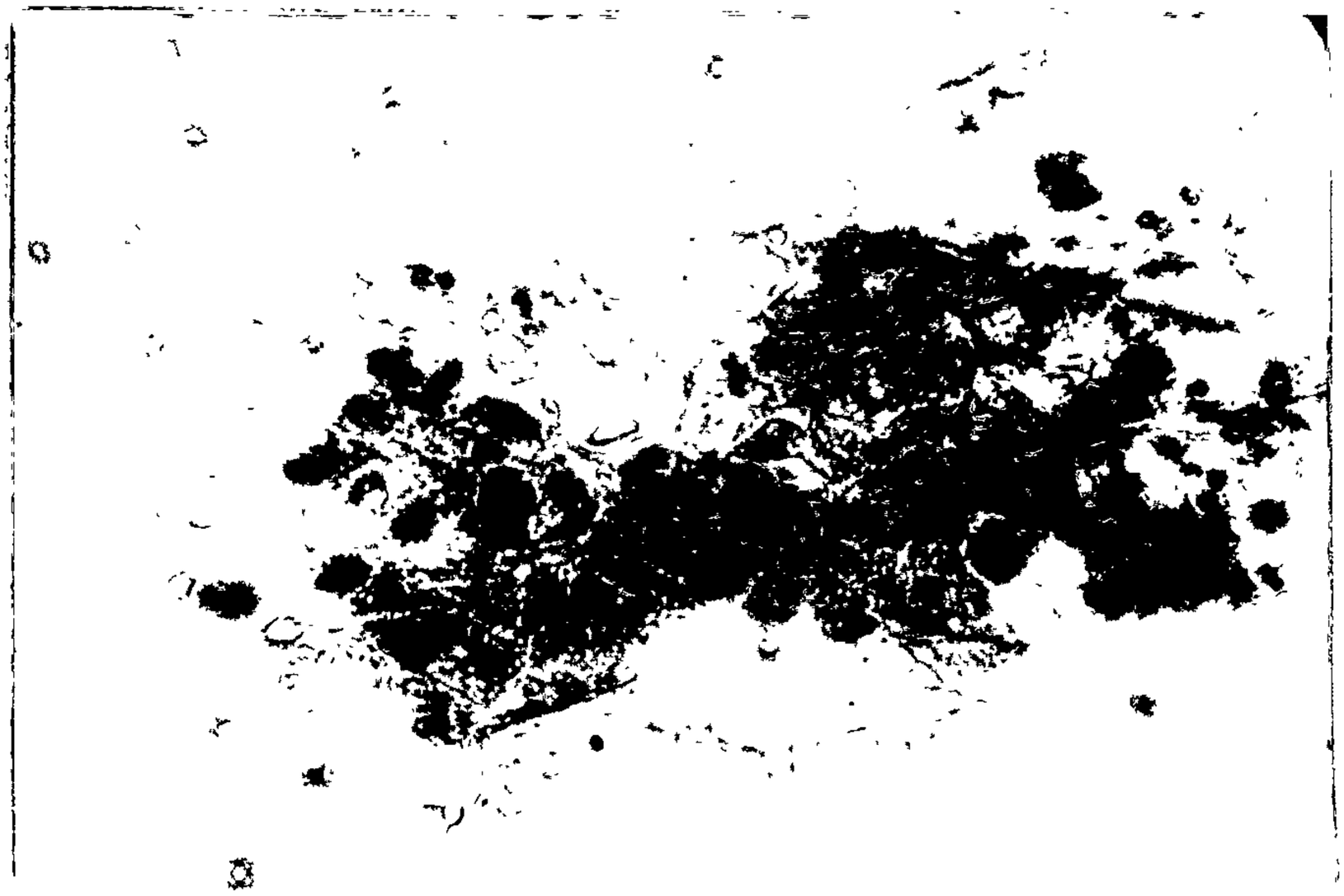


Fig. 41. Smear of superficial cells from an oral carcinoma showing typical malignant nuclei. In some cases, lightly taken superficial smears may be of greater value than deeper scrapings. X 1,008.

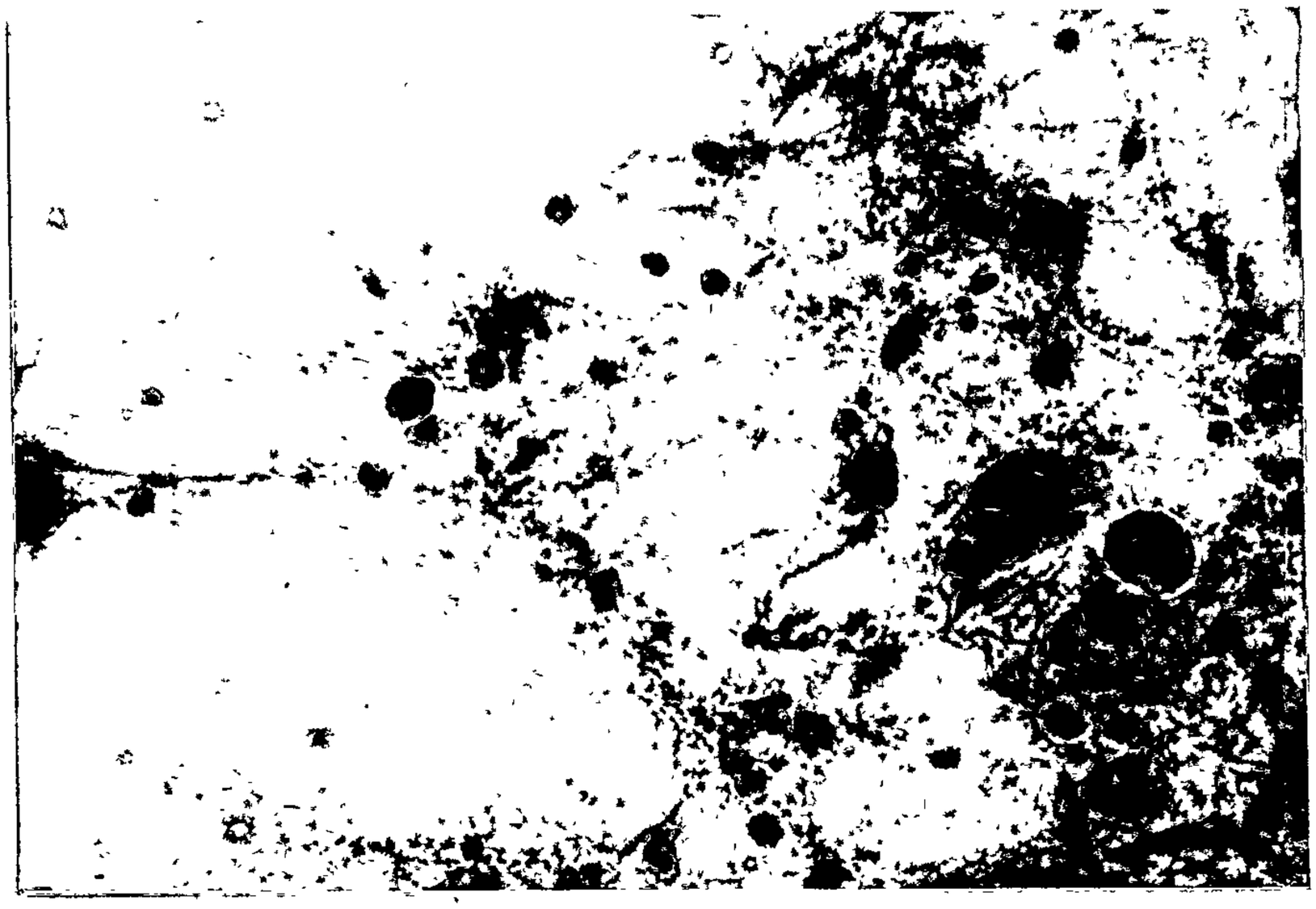


Fig. 42. Smear from a deep scraping of the same lesion as Fig. 41 showing inflammatory cells and exudate, which tend to obscure the epithelial elements. X 1,008.

Fig. 43. Keratin pearl from an oral squamous cell carcinoma. X 1,008.

Fig. 44.

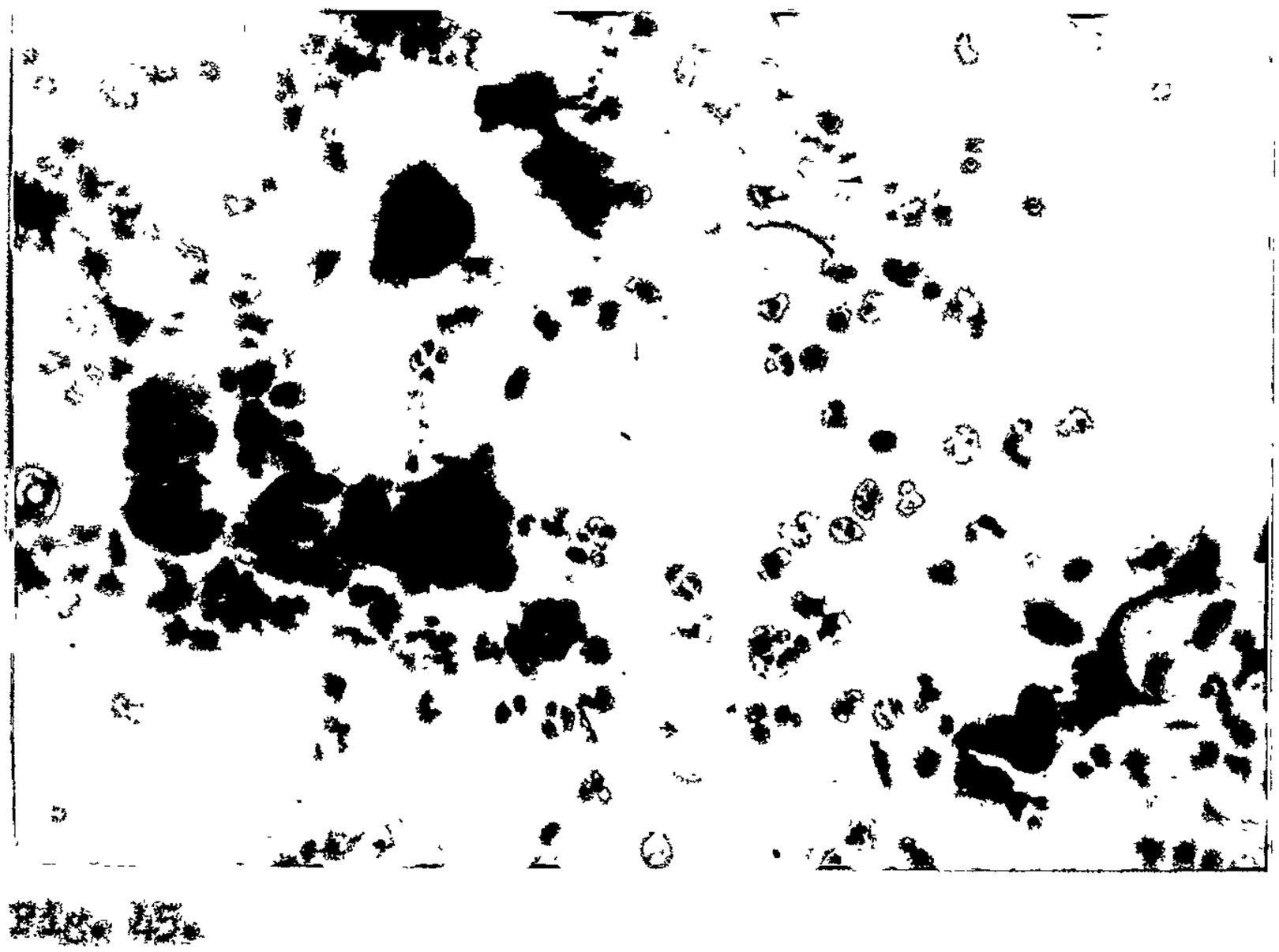


Fig. 45.

Figs. 44 and 45. Malignent oral carcinoma cells, X 1,008.

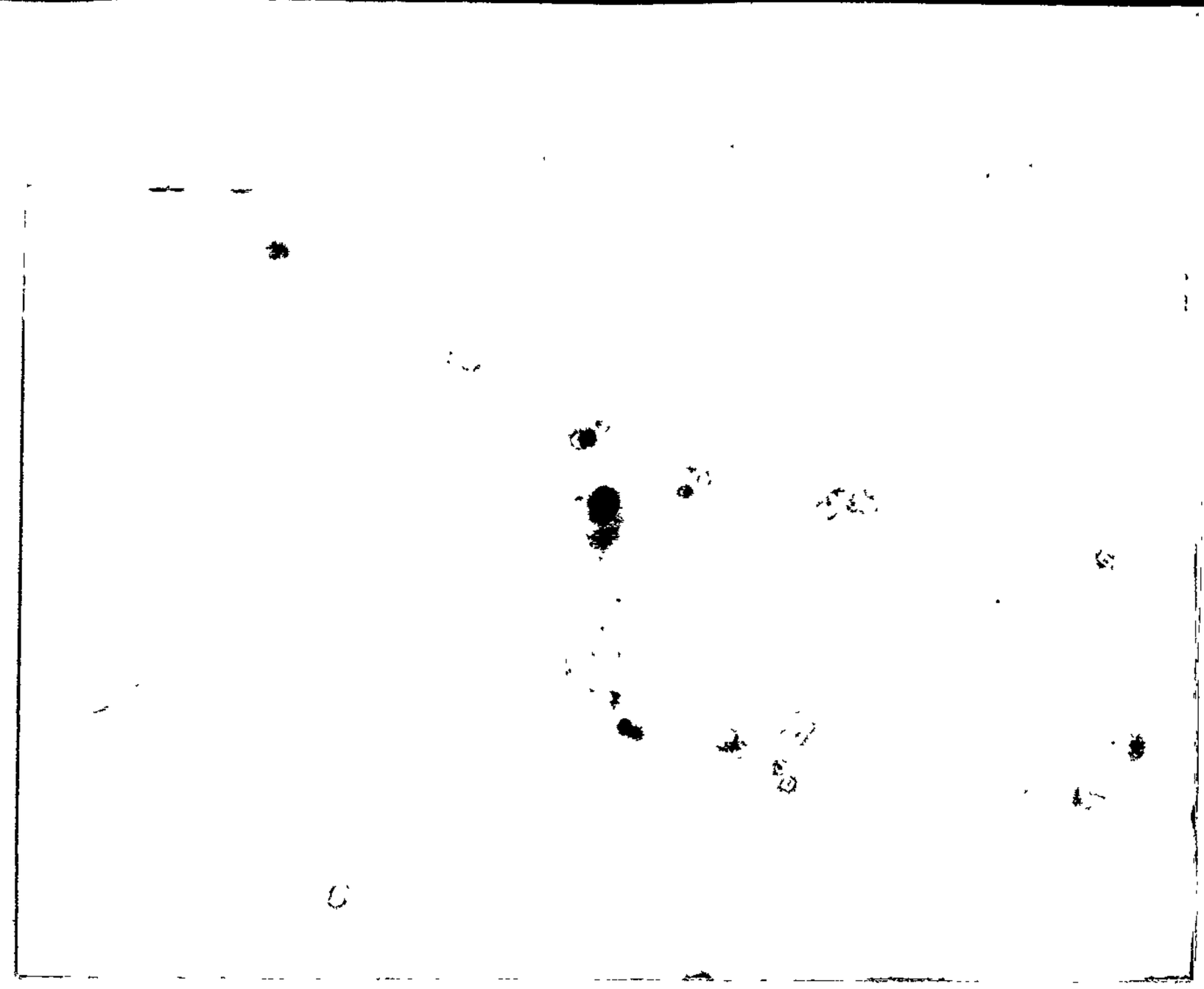


Fig. 46. 'Free' nucleus in a malignant oral smear. X 1,008.

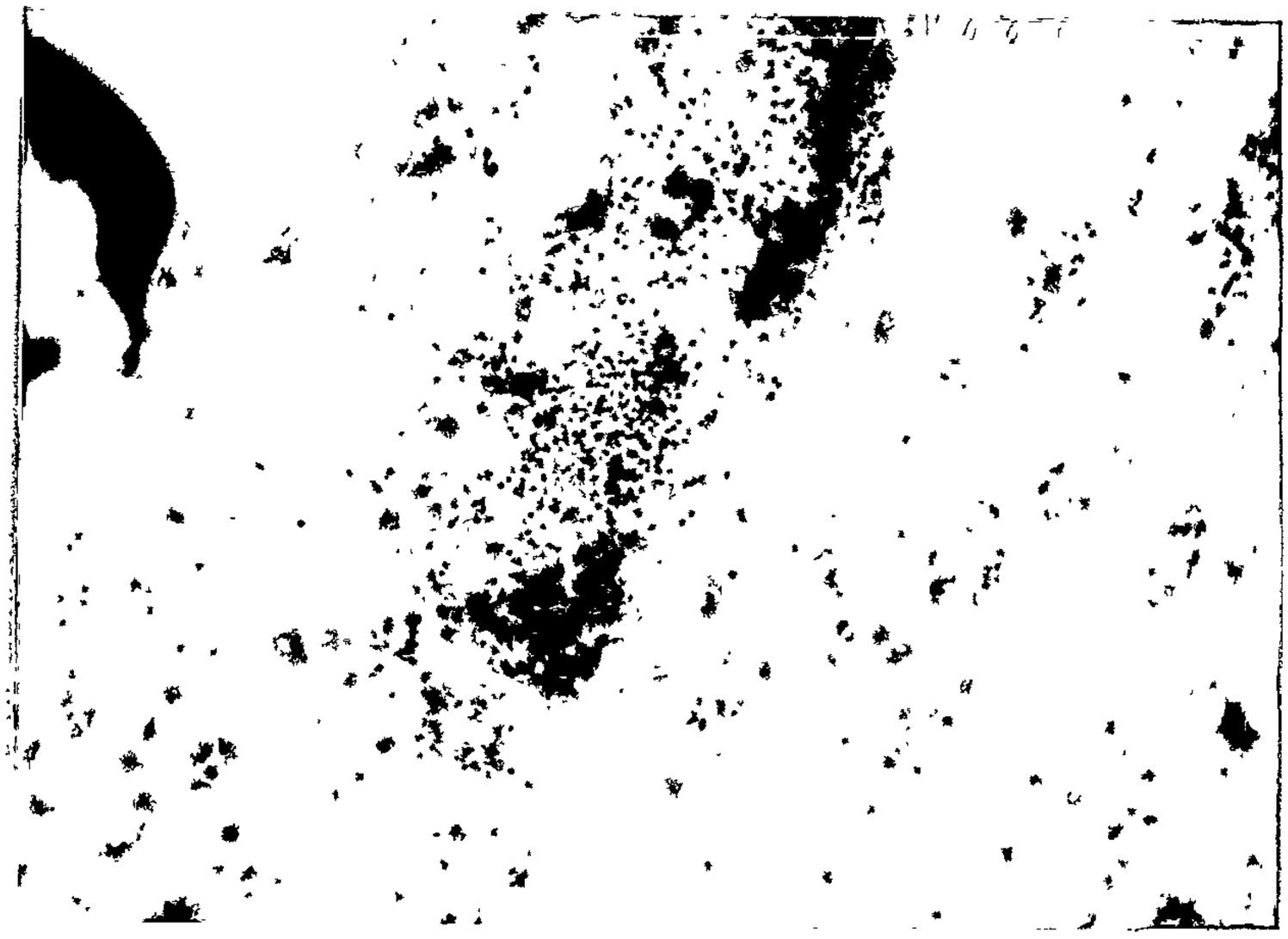


Fig. 47. Low power field of prolific numbers of malignant cells. During the initial stages of radiotherapy, oral carcinomata tend to show greatly increased exfoliation. X 240.

Fig. 48. Cells from irradiated normal oral mucosa appear normal cytologically but tend to be increased in size, show vacuolization and somewhat pyknotic nuclei. X 1,000.

Fig. 49. A folded multinucleated giant cell with vacuoles in a smear from irradiated oral tissue. X 1,000.

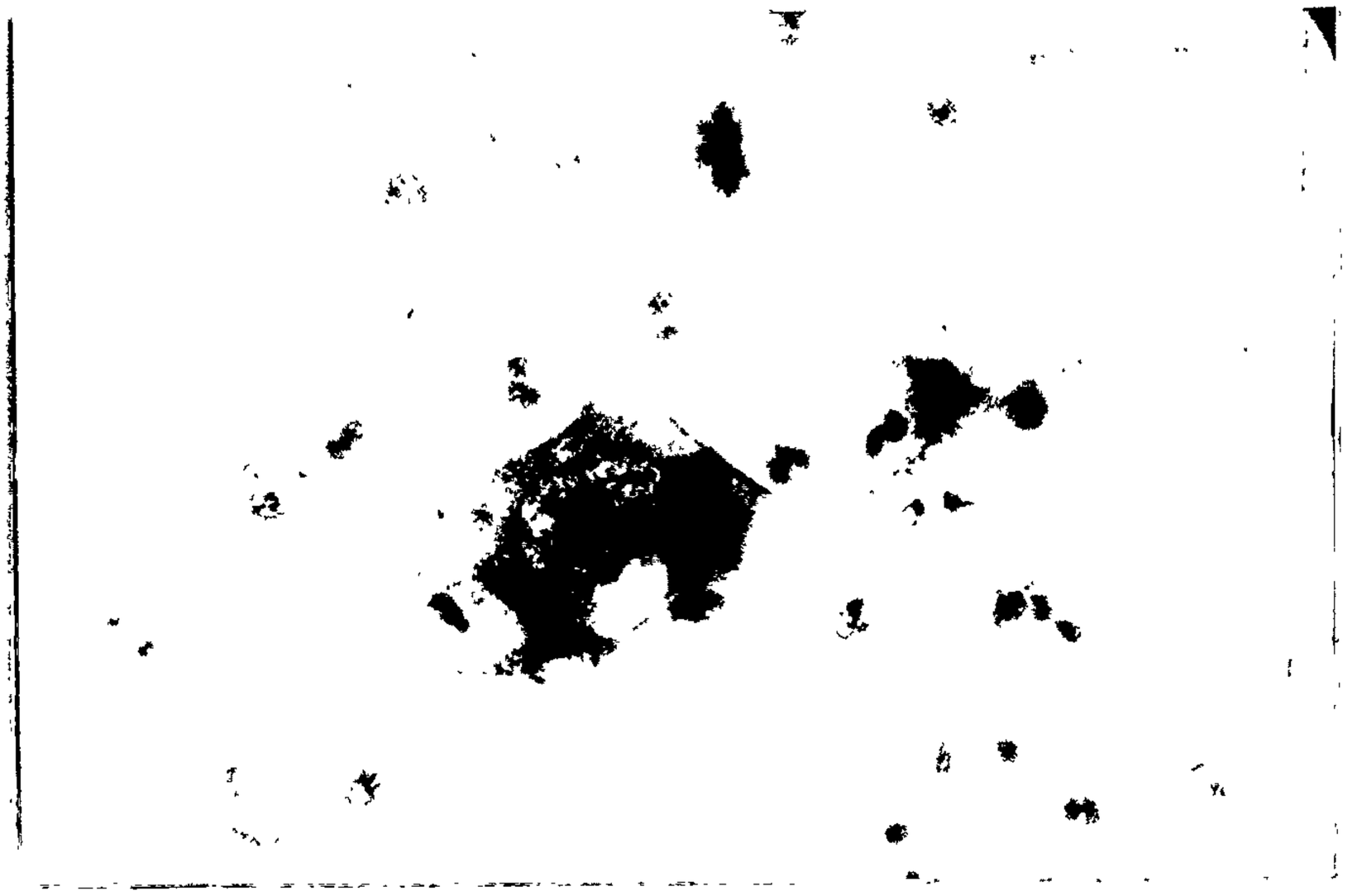


Fig. 50. Cell showing marked vacuolization in response to radiotherapy. X 1,000.

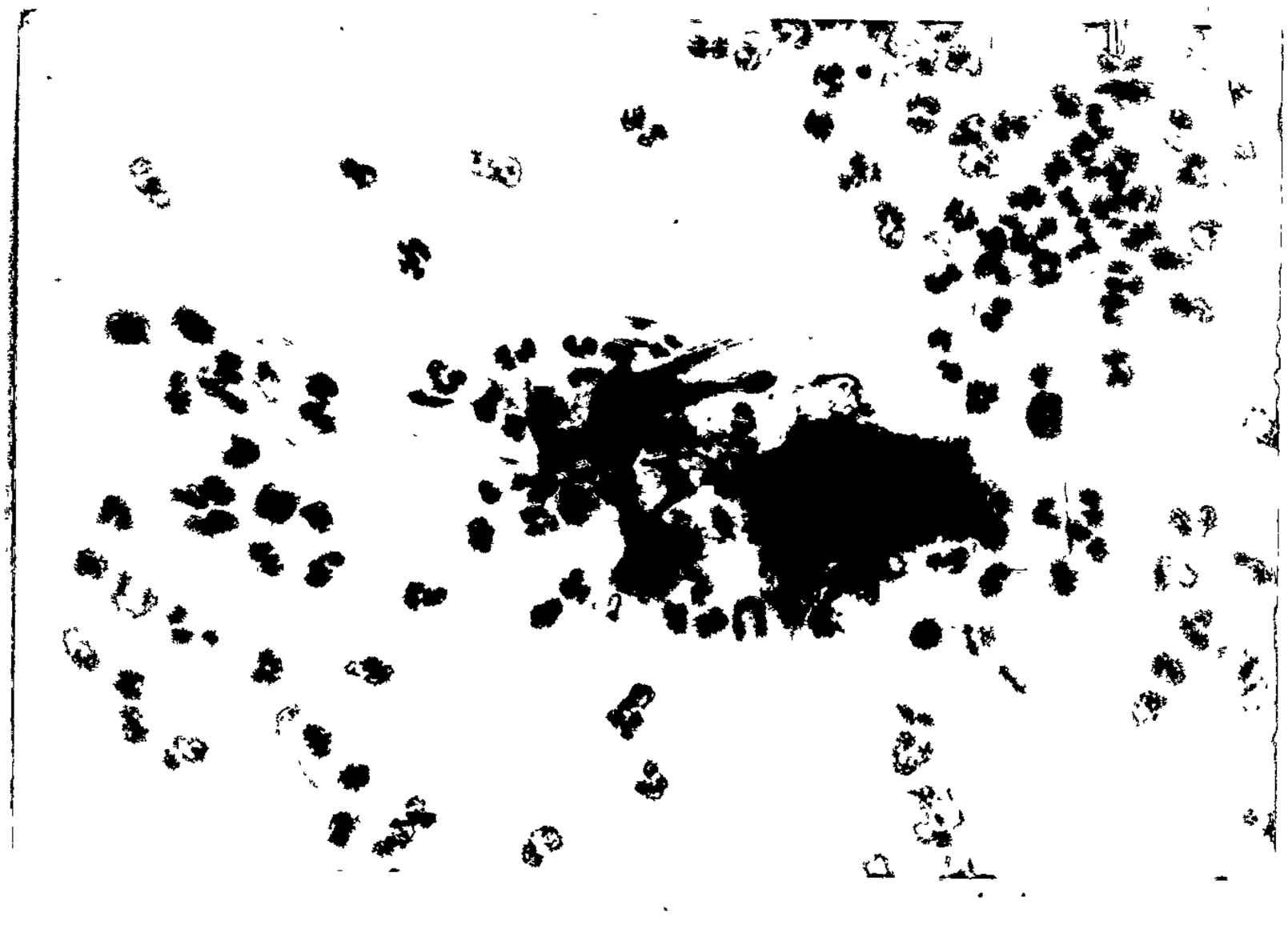


Fig. 51. Bizarre cellular shapes resulting from radiotherapy to oral tissues. X 1,000.

Fig. 52. Low power field of aggregates of keratinized cells with fairly large nuclei from a non-malignant radionecrotic ulcer. Cytological smears reliably reflect recurrent malignancy in irradiated oral tissue by showing cells with definite malignant tendencies, even prior to clinical evidence. The atypia seen here is insufficient to indicate recurrent disease. X 94.

CAUSES OF FALSE NEGATIVE REPORTS.

Fig. 53. Section of an ulcerated squamous cell carcinoma of the lower lip. Obviously, smears of superficial cells of such lesions are of very little value. X 300.

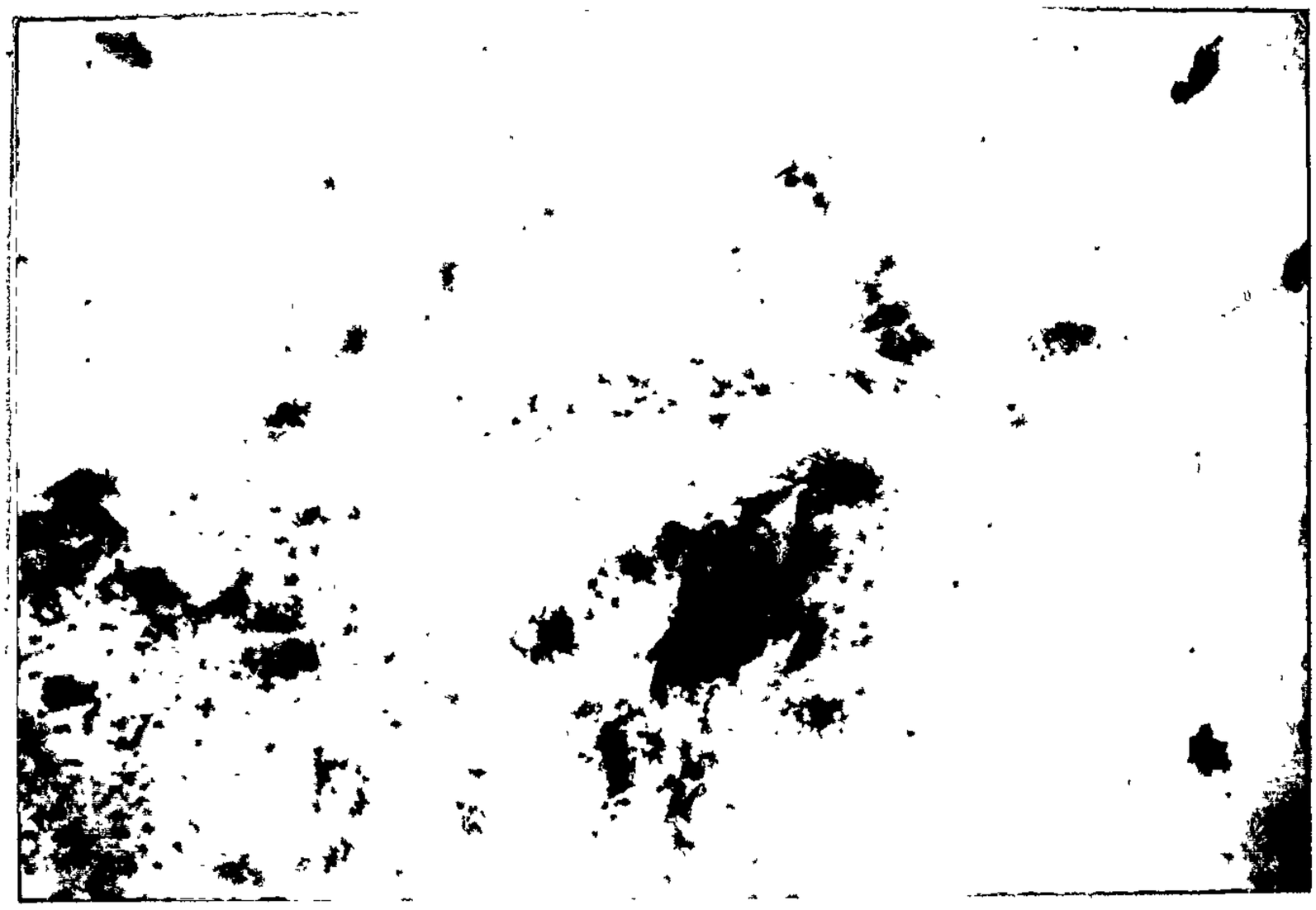


Fig. 54. Low power field of cellular debris in a smear from an oral carcinoma covered by necrosis. The degenerate cells here give no indication of the nature of the underlying tumour. X 240.

BIBLIOGRAPHY

1. Alling, C.C. and Secord, R.T. - A technique for oral exfoliative cytology. Oral Surg., 17:5, 668-676 (May) 1968.

2. Annies, A. - Report of National Health and Medical Research Council, Dental Health Committee, February, 1967.

3. Anderson, W.A.D. - Synopsis of pathology. St. Louis, C.V. Mosby Co., 5th ed., 1960.

4. Anderson, W.A.D. and Gunn, S.A. - Cytological detection of cancer: Considerations for its future. A comparative examination of the Papanicolaou and acridine-orange techniques. Acta Cytologica, 6, 468-470 (Sept.-Oct.) 1962.

5. Andrews, J.R. - The radiobiology of human cancer radiotherapy. Philadelphia, London and Toronto, W.B. Saunders Co., 1968.

6. Archer, W.H. - Oral surgery. Philadelphia and London, W.B. Saunders Co., 4th ed., 1966.

7. Bahr, G.F. - Changes in liver cell elements during stimulated protein synthesis: A cytochemical study. Acta Radiologica, Suppl. 147, 1-111, 1957.

8. Bahr, G.F. and Mohberger, G. - Histochemical methods for the demonstration of sulphydryl groups in normal tissues and malignant tumours. Acta Path. Microbiol. Scand., 42, 109-132, 1958.

9. Barnett, R.J. and Seligman, A.M. - Histochemical demonstration of protein-bound sulphydryl groups. Science, 116, 323-327 (Sept.) 1952.

10. Beale, L.S. - Examination of sputum from a case of cancer of the pharynx and adjacent parts. Arch. Med. 2, 44-46, 1860-1861.

11. Bertalanffy, F.D. and Nagy, K.P. - Fluorescence microscopy and photomicrography with acridine-orange. Med. Radiog. & Photog., 38:3, 32-91, 1962.

12. Bertalanffy, F.D. - Cell renewal as a basis of diagnostic exfoliative cytology. Amer. J. Obstet. & Gynaec., 85:3, 383-396 (Feb.) 1963.

13. Bertalanffy, L. von and Bickis, I. - Identification of cytoplasmic basophilia (ribonucleic acid) by fluorescent microscopy. J. Histochem. Cytochem., 4, 481-493 (Sept.) 1956.

14. Bertalanffy, L. von, Masin, M. and Masin, F. - A new and rapid method for diagnosis of vaginal and cervical cancer by fluorescent microscopy. Cancer, 11:5, 873-883 (July-Aug.) 1958.

15. Diehl, H., Burgeon, C.F., Baldridge, E.D., McCarthy, P.L. and Urbach, F. - Cytologic smears in diagnosis of herpes simplex, herpes zoster and varicella. J. Amer. Med. Assoc., 146:15, 1410-1412 (Aug.) 1951.

16. Dlouhy, E.G. - Oral cancer detection. J. Amer. Dent. Assoc., 70:6, 1472-1475 (June) 1965.

17. Boddington, H.H., Cordell, R.H. and Spriggs, A.I. - Development of carcinoma of the cervix, uteri (observations resulting from cytological examination of 10,000 cervical smears). Brit. J. Cancer, 14:2, 151-164 (June) 1960.

18. Boyle, P.E. - Differential diagnosis of soft tissue lesions of the mouth, with a discussion on biopsy procedures. Oral Surg., 7:5, 507-523 (May) 1954.

19. Bullough, W.S. - Growth control of mammalian skin. Nature, 193:4815, 520-523 (Feb. 10) 1962.

20. Cahn, L.R. - The early detection of cancer of the mouth. Brit. Dent. J., 111:8, 285-290 (April 24) 1961.

21. Cahn, L.R. - Comment on S.S. Stahl article "Correlation of cytodiagnosis and biopsy in evolution of an experimentally induced carcinoma". Editorial. Oral Surg., 16:8, 992 (Aug.) 1963.

22. Cahn, L.R. - Oral exfoliative cytology. Editorial. Oral Surg., 16:11, 1281 (Nov.) 1963.

23. Camilleri, G.E. and Smith, C.J. - Exfoliative cytology in experimental oral carcinogenesis. Acta Cytologica, 8:1, 85-90 (Jan.-Feb.) 1964.

24. Castigliano, S.G. - Oral cancer. (In Burkett, L.W., ed. Oral Medicine. Philadelphia, London and Montreal, J.B. Lippincott Co., 5th ed., 1955).

25. Coulter, S.L. - Fluorescence microscopy utilizing acridine-orange in oral cytodiagnosis. Oral Surg., 23:3, 343-350 (Mar.) 1967.

26. Corson, R.A. - The cytological diagnosis of oral cancer. Brit. Dent. J., 108:8, 294-298 (April 19) 1960.

27. Cocker, J., Fox, H., and Langley, F.A. - Consistency in the histological diagnosis of epithelial abnormalities of the cervix uteri. J. Clin. Path., 21:1, 67 (Jan.) 1968.

28. Conroy, C.W. - Fluorescent microscopy for cytodiagnosis of oral cancer. (Abstract). J. Dent. Res., 40:4, 649 (July-Aug.) 1961.

29. Cooke, B.E.D. - Epithelial smears in the diagnosis of herpes simplex and herpes zoster affecting the oral mucosa. Brit. Dent. J., 104:3, 97-99 (Feb. 4) 1958.

30. Cooke, B.E.D. - Biopsy procedures. Oral Surg., 11:7, 750-761 (July) 1958.

31. Cooke, B.E.D. - The diagnosis of bullous lesions affecting the oral mucosa. Part I. Brit. Dent. J., 109:3, 83-96 (Aug. 2) 1960.

32. Cooke, B.E.D. - The diagnosis of bullous lesions affecting the oral mucosa. Part II. Brit. Dent. J., 109:4, 131-138 (Aug. 16) 1960.

33. Cooke, B.E.D. - Exfoliative cytology in evaluating oral lesions. Proceedings of the 2nd International Conference on Oral Biology in Bonne, July 2 - July 5, 1962. J. Dent. Res., 42, Suppl. to No. 1, 343-347 (Jan.-Feb.) 1963.

34. Cooke, B.E.D. - Keratinising lesions affecting the oral mucosa. Proc. Royal Soc. Med., 60:8, 819-822 (Aug.) 1967.

35. Dowsett, H. - Normal buccal mucosa. M.D.S. Thesis, Sydney University, 1964.

36. Einhorn, J. and Jakobsson, P. - Multiple primary malignant tumours. Cancer, 17:11, 1437-1444 (Nov.) 1964.

37. Fischman, S.L. and Greene, G.W. - Cytologic changes during experimental carcinogenesis. Acta Cytologica, 10:4, 289-295 (July-Aug.) 1966.

38. Fleming, W.E. and Orams, H.J. - Ulceration of the oral mucosa. Aust. Dent. J., 13:1, 25-31 (Feb.) 1968.

39. Frazelle, E.L. and Lucas, J.C. - Cancer of the tongue; Report of the management of 1,554 patients. Cancer, 15:6, 1085-1099 (Sept.-Oct.) 1962.

40. Gaither, W.D. - Comparison of exfoliative cytodiagnosis and histodiagnosis of oral lesions: Review of the literature and report of 75 cases. J. Oral Surg., 25:5, 446-453 (Sept.) 1967.

41. Gardner, A.F., Hamburger, S. and Love, S. - Oral carcinoma: Analysis of 189 cases. J. Amer. Dent. Assoc., 66:4, 456-465 (April) 1963.

42. Gardner, A.F. - Investigation of the use of exfoliative cytology in the diagnosis of malignant lesions of the oral cavity. Acta Cytologica, 8:6, 436-445 (Nov.-Dec.) 1964.

43. Gardner, A.F. - An investigation of 890 patients with cancer of the oral cavity: Its incidence, etiology, prognosis and relationship to oral exfoliative cytology. Acta Cytologica, 9:4, 273-281 (July-Aug.) 1965.

44. Gause, G.F. - Microbial models of tumour metabolism. Advances in Applied Microbiology, 9:69-90, 1967.

45. Gladstone, S.A. - Sponge biopsy in carcinoma of skin and oral mucosa. Arch. Dermat. & Syph., 62, 380-390 (Sept.) 1950.

46. Glukmann, A. and Spear, F.G. - Qualitative and quantitative histological examination of biopsy material from patients treated by radiation for carcinoma of the cervix uteri. Brit. J. Radiol., 18, 313-322 (Oct.) 1945.

47. Gobbel, W.C., Adkins, R.B. and Sawyer, J.L. - Carcinoma of the tongue. Amer. Surg., 33:8, 635-641 (Aug.) 1967.

48. Goldhaber, P. - The role of saliva and other local factors in oral carcinogenesis. J. Amer. Dent. Assoc., 54:4, 517-524 (April) 1957.

49. Goldhaber, P. - Further studies on experimental oral carcinogenesis. (Abstract). J. Dent. Res., 37:1, 18 (Feb.) 1958.

50. Graham, R.M. - Radiation and hormone changes in cancer of the uterus followed by cytology. Proceedings of the Symposium on Exfoliative Cytology, Oct. 23-24, New York, 1951. American Cancer Society Inc.

51. Graham, R.M. and Graham, J.B. - Cytological prognosis in cancer of the uterus and cervix treated radiologically. Cancer, 8:59-70, (Jan.-Feb.) 1955.

52. Graham, R.M. - The cytological diagnosis of cancer. Philadelphia and London, W.B. Saunders Co., 2nd ed., June, 1964.

53. Greulich, R.C. - Aspects of cell individuality in the renewal of stratified squamous epithelia. (In Montagne, W. and Lobitz, W.C., Jnr., ed. The epidermis. New York and London, Academic Press, 1964).

54. Haam, E. von. - Some observations in the field of exfoliative cytology. Amer. J. Clin. Path., 24:1, 652-662 (June) 1954.

55. Haam, E. von. - The historical background of oral cytology. Acta Cytologica, 9:4, 270-272 (July-Aug.) 1965.

56. Haam, E. von. - Cytological examination of the oral cavity. (In Miller, S.E., ed. A textbook of clinical pathology. Baltimore, The Williams and Wilkins Co., 7th ed., 1966).

57. Hartman, F.W. - The problem of carcinoma in situ. The Proceedings of the Symposium on Exfoliative Cytology, Oct. 23-24, New York, 1951, American Cancer Society Inc.

58. Harris, M. - Pemphigoid (benign mucous membrane pemphigus). Brit. J. Oral Surg., 5:1, 42-50 (July) 1967.

59. Helsper, J.T. and Sharp, G.S. - Exfoliative cytology for the detection of abnormal oral mucous membranes and oral carcinoma. Oral Surg., 17:2, 175-177 (Feb.) 1964.

60. Hendershott, L.C. - Early detection of oral cancer (Editorial). J. Amer. Dent. Assoc., 76:4, 700 (April) 1968.

61. Hopp, E.S. - Cytological diagnosis and prognosis in carcinoma of the mouth, pharynx and nasopharynx. The Laryngoscope, 68:4, 1281-1287 (July) 1958.

62. Hughes, H.E. and Dodds, T.C. - Handbook of diagnostic cytology. Edinburgh and London, E. & S. Livingstone Ltd., 1968.

63. Hunter, D.T., Brown, M.D. and Redmond, R.F. - Extracervical cancer detected by fluorescent cytology: A preliminary report. J. Oklahoma Med. Assoc., 52; 772-777 (Dec.) 1959.

64. Ingram, R.C., Krantz, S., Mendelhoff, J. and Leslie, H. - Exfoliative cytology and the early diagnosis of oral cancer. Cancer, 16:2, 160-165 (Feb.) 1965.

65. Johnson, L.D., Hertig, A.T., Hinman, C.H. and Easterby, C.L. - Preinvasive cervical lesions in obstetric patients: Methods of diagnosis, course and clinical management. Obstet. & Gynaec., 16:133-145 (Aug.) 1960.

66. Knychalska-Karwan, Z. - Leukoplakia of the oral mucous membrane. Folia Med. Cracov, 8:3, 472-476, 1966.

67. King, O.H. - The cytology of common and uncommon oral malignancies. Acta Cytologica, 6:4, 348-354 (July-Aug.) 1962.

68. King, O.H. - Oral cytology for the general practitioner. J. Amer. Dent. Assoc., 66:4, 451-455 (April) 1963.

69. King, O.H., Coleman, S.A. and Pierce, A.E. - Definition of criteria for classification of oral cytologic preparations. Acta Cytologica, 10:5, 316-322 (Sept.-Oct.) 1966.

70. Kligman, A.M. - Biology of the stratum corneum. (In Montagna, W. and Lobitz, W.C., Jnr., ed. The epidermis. New York and London, Academic Press, 1964).

71. Koss, L.G. and Durfee, G.R. - Diagnostic cytology and its histologic bases. Philadelphia, J.B. Lippincott Co., 1961.

72. Leblond, C.P., Greulich, R.C. and Periera, J.P.M. - Relationship of cell formation and cell migration in the renewal of stratified squamous epithelia. (In Montagna, W., ed. Symposium of wound healing. New York, Pergamon Press, 1964).

73. Leibert, H. - Traite pratique des maladies cancereuses et des affections curable confondues avec le cancer. Paris, France, J.B. Bailliere, 1851.

74. Lever, W.F. - Pemphigus. Medicine (Baltimore), 32:1-123 (Feb.) 1953.

75. Levij, I.S., Polliack, A. and Thorgeirsson, T. - Correlation of cytologic smear and histologic findings during 9-10 dimethyl 1-2 benzanthracene-induced carcinogenesis in the hamster cheek pouch. Arch. Oral Biol., 12, 859-864, 1967.

76. Levy, B.M. - Experimental oral carcinogenesis. J. Dent. Res., 42, Suppl. to No. 1, 321 (Jan.-Feb.) 1963.

77. Liu, W. - Fluorescence microscopy in exfoliative cytology. Parts I and II. Arch. Path. (Chicago), 71:282-294 (Mar.) 1961.

78. McGrew, E.A. - The role of exfoliative cytology in the diagnosis and treatment of malignent disease. Post Grad. Med. J., 37:456-467 (Aug.) 1961.

79. Medak, H., McGrew, E.A., Burlakow, P. and Teicke, R.W. - Correlation of cell populations in smears and biopsies from the oral cavity. Acta Cytologica, 11:4, 279-288 (July-Aug.) 1967.

80. Miller, W.D. - Microorganisms of the human mouth. Philadelphia, S.S. White Dental Manufacturing Co., 1890.

81. Miller, S.C., Soberman, A. and Stahl, S.A. - A study of the cornification of the oral mucosa in normal males. J. Dent. Med., 7, 35-39 (Jan.) 1952.

82. Moertel, C.G. and Foss, E.L. - Multicentric carcinomas of the oral cavity. Surg. Gynaec. Obstet., 106, 652-654 (June) 1958.

83. Montgomery, P.W. - A study of the exfoliative cytology of normal human oral mucosa. J. Dent. Res., 30:1, 12-18 (Feb.) 1951.

84. Montgomery, P.W. and Heam, E. von. - A study of the exfoliative cytology of oral leukoplakia. J. Dent. Res., 30:2, 260-264 (April) 1951.

85. Montgomery, P.W. and Hoen, E. von. - A study of the exfoliative cytology in patients with carcinoma of oral mucosa. J. Dent. Res., 30:3, 302-313 (June) 1951.

86. Monto, R.W., Fino, G. and Rizol, R.A. - Exfoliative cells of oral mucous membrane in patients receiving chemotherapy for malignant disease. J. Oral Surg., Anes. & Hosp. Dent. Service, 21:2, 95-100 (Mar.) 1963.

87. Morris, A.L. - Factors influencing experimental carcinogenesis in the hamster cheek pouch. J. Dent. Res., 40:1, 3-15 (Jan.-Feb.) 1961.

88. Morrison, L.F., Hopp, E.S. and Wu, R. - Diagnosis of malignancy of the nasopharynx: Cytological studies by smear technique. Annals of Otol. Rhin. & Laryng., 58, 18-32 (Mar.) 1949.

89. Morrissey, J.B. - Positive cytology and negative biopsy. The Interim Report of Veterans Administration Co-operative Study of Oral Exfoliative Cytology, Washington, D.C., Government Printing Office, 1961.

90. Naib, Z.M., Clepper, A.S. and Elliot, S.R. - Exfoliative cytology as an aid in the diagnosis of ophthalmic lesions. Acta Cytol., 11:4, 295-303 (July-Aug.) 1967.

91. Naib, Z.M., Stewart, J.A., Dowdle, W.R., Casey, H.L., Marino, V.N. and Nahmias, A.J. - Cytological features of viral respiratory tract infections. Acta Cytologica, 12:2, 162-171 (Mar.-April) 1968.

92. Neibel, H.M. and Chomet, B. - In vivo staining tests for delineation of oral intraepithelial neoplastic change: Preliminary report. J. Amer. Dent. Assoc., 68:31, 801-806, (June) 1964.

93. Nieburgs, H.E., Herman, B.E. and Nelson, H. - Buccal cell changes in patients with malignant tumours. Lab. Invest., 2, 80-88 (Jan.) 1962.

94. Nowakowsky, S., Norgren, E.A., Vodak, M., Burlakov, P. and Hanks, S. - Manifestation of viral infections in exfoliated cells. Acta Cytologica, 12:3, 227-236 (May-June) 1963.

95. Papanicolaou, G.N. and Traut, F. - The diagnostic value of vaginal smears in carcinoma of the uterus. Amer. J. Obstet. & Gynec., 42, 193-206 (Aug.) 1941.

96. Papanicolaou, G.N. and Traut, F. - Papanicolaou staining procedure flow chart. New York, Commonwealth Fund, 1943, Ortho Pharmaceutical Co., Sydney.

97. Papanicolaou, G.N. - Atlas of exfoliative cytology. Cambridge, Harvard University Press, 1954.

98. Papanicolaou, G.N. and Bridges, E.L. - Simple method for protecting fresh smears from drying and deterioration during mailing. J. Amer. Med. Assoc., 164:12, 1330-1331 (July) 1957.

99. Pavla, Nevac. - Cytologic diagnosis from tumour tissue using the "quick method" during operation. Acta Cytologica, 11:3, 229-(May-June) 1967.

100. Peters, H. - Cytologic smears from the mouth: Cellular changes in disease and after radiation. Amer. J. Clin. Path., 29:219-225 (Mar.) 1958.

101. Peters, H. and Rijsinghani, K. - The cytologic interpretation of the mouth smear. J. Indian Med. Assoc., 20, 231-236, 1956.

102. Pindborg, J.J. - Ethnic and environmental aspects of oral cancer. Dent. Prog., 3:2, 70-74 (Jan.) 1963.

103. Pindborg, J.J., Renstrup, G., Silverman, S. and Poulsen, H.E.- Studies in oral leukoplakia. V. Clinical and histologic signs of malignancy. Acta Odont. Scand., 21, 407-414 (Nov.) 1963.

104. Pindborg, J.J., Srivastava, A.N. and Gupta, D. - Studies in oral leukoplakias. VIII. Epithelial changes in tobacco-induced leukoplakias in India. Acta Odont. Scand., 22:499-512 (Oct.) 1964.

105. Pindborg, J.J. - Oral precancerous conditions in South East Asia. Internat. Dent. J., 15, 190-199 (June) 1965.

106. Pindborg, J.J., Chawla, T.N., Misra, R.K., Nagpaul, R.K. and Gupta, V.K. - Frequency of oral carcinoma, leukoplakia, leukokeratosis, leukoderma, submucous fibrosis and lichen planus in 10,000 Indians in Lucknow, Uttar Pradesh, India: Preliminary report. J. Dent. Res., 44:615 (May-June) 1965.

107. Pindborg, J.J. - Oral epithelial changes in 30 Indians with oral cancer and submucous fibrosis. Cancer, 20, 1141-1146 (July) 1967.

108. Pindborg, J.J., Renstrup, G., Jølst, O. and Roed-Petersen, B.- Studies in oral leukoplakia: A preliminary report on the period prevalence of malignant transformation in leukoplakia based on a follow-up study of 248 patients. J. Amer. Dent. Assoc., 76:4, 767-771 (April) 1968.

109. Pindborg, J.J. - Atlas of diseases of the oral mucosa. Copenhagen, Munksgard, 1968.

110. Pomerans, H.J., and Stahl, S.S. - A correlative study of cytodiagnosis and biopsy. Oral Surg., 6, 1026-1031 (Aug.) 1953.

111. Pouchet, F.A. - Theorie positive de l'ovulation spontanee et de la Fecondation des mammiferes et de l'Espece humaine, bersa and l'observation de toute la serie animale. Paris, J.B. Bailliere, 1847.

112. Renstrup, G., Smulow, J.B. and Glickman, I. - Effects of chronic mechanical irritation on chemically induced carcinogenesis in the hamster cheek pouch. J. Amer. Dent. Assoc., 64:23, 770-777 (June) 1962.

113. Richart, R.M. - Clinical staining test for in vivo delineation of dysplasia and carcinoma in situ. Amer. J. Obstet. & Gynaec., 86, 703-712.(July 15) 1963.

114. Rothman, S. - Keratinisation in historical perspective. (In Montagna, W. and Lobitz, W.C., Jnr., ed. The epidermis. New York and London, Academic Press, 1964).

115. Rovin, S. - Assessment of the negative oral cytologic diagnosis. J. Amer. Dent. Assoc., 74:4, 759-762 (Mar.) 1967.

116. Salley, J.J. - Experimental carcinogenesis in the cheek pouch of the Syrian hamster. J. Dent. Res., 33:2, 253-262 (April) 1954.

117. Salley, J.J. - Epithelial carcinogenesis in the absence of accessory structures. Oral Surg., 14:12, 1479-1484 (Dec.) 1961.

118. Sanders, W.R. - Cancer of the bladder. Edinburgh Med. J., 10:273, 1864.

119. Sandler, H.C. and Stahl, S.S. - Exfoliative cytology as a diagnostic aid in detection of oral neoplasms. J. Oral Surg., 16:5, 414-418 (Sept.) 1958.

120. Sandler, H.C., Freund, H.R. and Stahl, S.S. - Exfoliative cytology applied to the detection and treatment of head and neck cancer. Surgery, 46:3, 479-485 (Sept.) 1959.

121. Sandler, H.C., Stahl, S.S., Cahn, L.R. and Freund, H.R. - Exfolitive cytology for detection of early mouth cancer. Oral Surg., 13:8, 994-1009 (Aug.) 1960.

122. Sandler, H.C. - The detection of early cancer of the mouth by exfolintive cytology. Acta Cytologica, 5:3, 191-194 (May-June) 1961.

123. Sandler, H.C. - Cytological screening for early mouth cancer: Interim Report of the Veterans Administration Co-operative Study of Oral Exfoliative Cytology. Cancer, 15:6, 1119-1124. (Nov.-Dec.) 1962.

124. Sandler, H.C. - Reliability of oral exfoliative cytology for detection of oral cancer. J. Amer. Dent. Assoc. 68:4, 489-499 (April) 1964.

125. Sandler, H.C. - Errors of oral cytodiagnosis: Report of follow-up of 1,801 patients. J. Amer. Dent. Assoc., 72:4, 851-861 (April) 1966.

126. Sandler, H.C. - Oral cytology. C.A., 16:3, 97-101 (May-June) 1966.

127. Saxena, V.S. and Allt, W.E.C. - Cancer of the tongue. J. Canad. Assoc. Radiol., 18, 274-277 (Mar.) 1967.

128. Scheman, P. - The instrumentation and clinical application of exfoliative cytology in the prophylaxis of oral cancer. Oral Surg., 20:2, 226-235 (Aug.) 1965.

129. Scheman, P., Alltchuler, L. and Wilson, K. - Cytoplasm: Nuclear ratios of epithelial cells in oral aspirates. Oral Surg., 24:2, 231-239 (Aug.) 1967.

130. Schiller, W. - Early diagnosis of cancer of the cervix. Surg. Gynaec. Obstet., 56:210-222 (Jan.-June) 1933.

131. Selbach, G.J. and Haam, E. von - The clinical value of oral cytology. Acta Cytologica, 7, 337-345 (Nov.-Dec.) 1963.

132. Shafer, W.G., Hine, M.K. and Levy, B.M. - A textbook of oral pathology. Philadelphia and London, W.B. Saunders Co., 2nd ed., 1963.

133. Shapiro, B.L. and Gorlin, R.J. - An analysis of oral cytodiagnosis. Cancer, 17:11, 1477-1479 (Nov.) 1964.

134. Shapiro, B.L., Gorlin, R.J. and Jordan, W.A. - The role of exfoliative cytology in oral cancer detection. Oral Surg., 17:3, 327-330 (Mar.) 1964.

135. Shaw, H. - Treatment of carcinoma involving the floor of the mouth, inferior alveolus and mandible. J. Otolaryng. Soc. Aust., 2:2, 18-24 (Mar.) 1967.

136. Shedd, D.P., Hukill, P.B., Bahn, S. and Ferraro, R.H. - Further appraisal of in vivo staining properties of oral cancer. Arch. Surg. (Chicago), 95:1, 16-22 (July) 1967.

137. Shklar, G., Meyer, I., Cataldo, E. and Taylor, R. - Correlated study of oral cytology and histopathology. Oral Surg., 25:1, 61-70 (Jan.) 1968.

138. Sicher, H. and Orban, B.J. - Oral mucous membrane. (Chapter IX in Orban, B.J. Oral histology and embryology. St. Louis, C.V. Mosby Co., 5th ed., 1962).

139. Silverman, S., Jnr., Becks, H. and Farber, S.M. - The diagnostic value of intraoral cytology. J. Dent. Res., 37:2, 195-205 (April) 1958.

140. Silverman, S., Jnr., - Detection of intraoral carcinoma in situ with exfoliative cytology. J. Dent. Res., 38:4, 717 (Abstract) (July-Aug.) 1959.

141. Silverman, S., Jnr. and Ware, W.H. - Comparison of histologic, cytologic and clinical findings in intraoral leukoplakia and associated carcinoma. Oral Surg., 13:4, 412-422 (April) 1960.

142. Silverman, S., Jnr., and Sheline, G.E. - Effects of radiation on exfoliated normal and malignent oral cells: A preliminary study. Cancer, 14:3, 587-596.(May-June) 1961.

143. Silverman, S., Jnr. and Shklar, G. - The effects of carcinogen (DMBA) applied to the hamster's buccal pouch in combination with croton oil. Oral Surg., 16:11, 1344-1355 (Nov.) 1963.

144. Silverman, S., Jnr. - Cytology of benign oral lesions. Acta Cytologica, 9:4, 287-295 (July-Sept.) 1965.

145. Silverman, S., Jnr., Sheline, G.E. and Gillooly, C.J. - Radiation therapy and oral carcinoma: Radiation response and exfoliative cytology. Cancer, 20, 1297-1300 (Aug.) 1967.

146. Silverman, S., Jnr. - Ultrastructure of human buccal epithelium. J. Dent. Res., 46:6, 1433 (Nov.-Dec.) 1967.

147. Silverman, S., Jnr. and Rosen, R.D. - Observations on the clinical characteristics of natural history of oral leukoplakia. J. Amer. Dent. Assoc., 76:4, 772-777 (April) 1968.

148. Sirsat, M.V. and Doctor, V.M. - A histopathological study on the effects of tobacco chewing on the buccal mucosa in Indians in its relationship to cancer. Brit. J. Cancer, 21, 277-284 (June) 1967.

149. Slaughter, D.P. - Multicentric origin of intraoral carcinoma. Surg., 30, 133-146 (July) 1946.

150. Solomon, Bethel. - Lecture notes on dermatology. Oxford, Blackwell Scientific Publications, 1965.

151. Spengas, M.N. - Dental diagnosis of oral carcinoma: Early detection by cytology and stomatoscopy. Dent. Radiog. & Photog. (Athens, Greece), 40:3, 51-57, 1967.

152. Stahl, J. and Wiman, L.G. - Exfoliative cytology in oto-laryngology: Using sulfydryl activity, acridine-orange and morphologic staining procedures. Acta Oto-Laryng., 55, 377-392 (Nov.-Dec.) 1962.

153. Stahl, S.S. - Exfoliative cytology in an experimentally-induced carcinoma of the hamster cheek pouch. Acta Cytologica, 7:4, 262-267 (Aug.) 1963.

154. Stahl, S.S. - Correlation of cytodiagnosis and biopsy in the evolution of an experimentally induced carcinoma. Oral Surg., 16:8, 985-992 (Aug.) 1963.

155. Stahl, S.S., Sandler, H.C. and Cahn, L.R. - Significance of dyskaryotic cells in oral exfoliative cytology. Acta Ctyologica, 8:1, 73-79 (Jan.-Feb.) 1964.

156. Stahl, S.S., Koss, L.G., Brown, R.C. and Murray, D. - Oral cytologic screening in a large metropolitan area. J. Amer. Dent. Assoc., 75:6, 1385-1388 (Dec.) 1967.

157. Stevenson, J. - The accuracy of fluorescent microscopy for the diagnosis of cancer. Acta Cytologica, 8:3, 224 (May-June) 1964.

158. Stockard, C.R. and Papanicolaou, G.N. - Existence of a typical oestrus cycle in guinea pigs with a study of histological and physiological changes. Amer. J. Anat., 22, 225-283, 1917.

159. Strong, M.S., Vaughan, C.W. and Inoze, J.S. - Toluidine blue in the management of carcinoma of the oral cavity. Arch. Otolaryng., 87:5, 527-531 (May) 1968.

160. Suad-Al-Ani, Shklar, G. and Yurstas, A.A. - Effect of dentures on the exfoliative cytology of palatal and buccal oral mucosa. J. Prosth. Dent., 16:3, 513-521 (May-June) 1966.

161. Swan, A.G. - White lesions of the oral cavity. M.D.S. Thesis, Sydney University, 1965.

162. Swanbeck, G. - Macromolecular organisation of epidermal keratin: An X-ray diffraction study of the horny layer from normal ichthyotic and psoriatic skin. Acta Dermato-Venereol (Stockholm), 39 (Suppl. 43), 1-37, 1959.

163. Swanbeck, G. and Thyresson, N. - A study of the state of aggregation of the lipids in normal and psoriatic horny layer. Acta Dermato-Venereol (Stockholm), 42:445-457, 1962.

164. Swancar, J.R. - The role of oral cytology in dental practice. Oral Surg., 24:1, 52-58 (July) 1967.

165. Tan, K.N. - Oral cancer. M.D.S. Thesis, Sydney University, 1967.

166. Tieke, R.W., Calandra, J.C. and Kendrick, F.J. - Use of the Papanicolaou technique in the diagnosis of oral cancer. (Abstract). J. Dent. Res., 39:4, 702 (Aug.) 1960.

167. Tieke, R.W., Kendrick, F.J. and Calandra, J.C. - Smear techniques in the diagnosis of intraoral carcinoma. Dental Progress, 1:3, 192-198 (April) 1961.

168. Tieke, R.W. and Blozis, G.G. - Oral cytology. J. Amer. Dent. Assoc., 72:4, 855-861 (April) 1966.

169. Tolles, W.E., Horwath, W.J. and Bostrom, R.C. - A study of the quantitative characteristics of exfoliated cells from the female genital tract. Cancer, 14:3, 455-468 (May-June) 1961.

170. Tzank, A. and Aron-Brunetiere, R. - Le "cytodiagnostic immediat" des dermatoses bulleuses. Gaz. Med. Port., 2, 667-675, 1949.

171. Umiker, W. - Grading of squamous cell carcinoma by cytologic smears: A preliminary report. University of Michigan Medical Bulletin, 23, 353-355 (Oct.) 1957.

172. Umiker, W., Lampe, I., Rapp, R., Latourette, H. and Boblitt, D. - Irradiation effects on malignant cells in smears from oral cancers: A preliminary report. Cancer, 12:3, 614-619 (May-June) 1959.

173. Umiker, W., Lampe, I., Rapp, R. and Hiniker, J.J. - Oral smears in the diagnosis of carcinoma and premalignant lesions. Oral Surg., 13:8, 897-907 (Aug.) 1960.

174. Umiker, W. - Oral laryngeal exfoliative cytology. C.A., 10, 160-164 (Sept.-Oct.) 1960.

175. Umiker, W. - Fluorescent microscopy in exfoliative cytology in oral cancer. Oral Surg., 14:10, 1269-1274 (Oct.) 1961.

176. Van Scott, E.J. - Definition of epidermal cancer. (In Montagna, W. and Lobitz, W.C., Jnr., ed. The epidermis. New York and London, Academic Press, 1964).

177. Voegtlin, C. and Thompson, J.W. - Glutathione content of tumour animals. J. Biol. Chem. 70, 801-806, 1926.

178. Wahi, P.N., Path, F.C. and Kehar, U. - Morphological and cytochemical studies of cells of precancerous lesions of the oral cavity. Acta Cytologica, 10:3, 173-178 (May-June) 1966.

179. Wahi, P.N. and Gupta, K. - Exfoliative cytology in the diagnosis of carcinoma of the oral cavity. J. Indian Med. Assoc., 23:425-427 (July) 1954.

180. Walsh, W.H. - A practical treatise of the diseases of lung and heart. London, Blanchard and Lea, 1841.

181. Watanabe, Y. and Shiraishi, Y. - Exfoliative cytology of mouth washings and garglings. (Abstract). J. Dent. Res., 40:2, 228-229 (March-April) 1961.

182. Watanabe, Y., Ogawa, K., Kobayashi, T. and Komoto, T. - Clinico-statistical, histopathologic and cytologic observations of 101 cases of maxillo-oral malignant neoplasm. Bulletin of the Cancer Institute, Okayama University Medical School, 2, 123-142 (Dec. 31) 1962.

183. Weathers, D.R. and Griffin, J.W. - Oral exfoliative cytology. J. Oral Surg., Anaesth. Hosp. Dent. Service 21:5, 377-382 (Sept.) 1963.

184. Wellmann, K., McDermott, M. and Gray, E. - An evaluation of acridine-orange fluorescence microscopy in cytology. Acta Cytologica, 7:2, 111-117 (Mar.-April) 1963.

185. Weinman, J.P. - Keratinization of human oral mucosa. J. Dent. Res., 19:1, 57-71 (Feb.) 1940.

186. Whitten, J.B. - Cytologic examination of aspirated material from cysts and cyst-like lesions. Oral Surg., 25:5, 710-716 (May) 1968.

187. Wilgram, G., Caulfield, J.B. and Madgic, E.B. - A possible role of the desmosome in the process of keratinization; An electron microscopic study of acantholysis and dyskeratosis. (In Montagna, W. and Lobitz, W.C., Jnr., ed. The epidermis. New York and London, Academic Press, 1964).

188. Williamson, J.J. and Shapiro, B.L. - The use of mucosal smears in the detection of cancer of the buccal cavity. Aust. Dent. J., 9:5, 408-413 (Oct.) 1964.

189. Witkop, C.J., Shankle, C.H., Graham, G.B., Murray, M.R., Rucknagle, D.L. and Byerly, B.H. - Hereditary benign intra-epithelial dyskeratosis: II. Oral manifestations and hereditary transmission. Arch. Path., 70, 696-711 (Dec.) 1960.

190. Witkop, C.J. and Gorlin, R.J. - Four hereditary mucosal syndromes: Comparative histology and exfoliative cytology of Darrier-White's Disease, hereditary benign intraepithelial dyskeratosis, white sponge nevus, and pachyonchia congenita. Arch. Dermatol. (Chicago), 84:5, 762-771 (Nov.) 1961.