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FLOW CYTOMETRIC PLOIDY DETERMINATION OF ORAL PREMALIGNANT AND MALIGNANT LESIONS

by

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Submitted to the Graduate Faculty of the School of Dentistry in partial fulfillment of the requirements for the degree of Master of Science in Dentistry, Indiana University School of Dentistry, 1989.

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ABSTRACT

Nuclear DNA content was evaluated for use as an objective parameter of diagnostic value in oral premalignancy and malignancy. Fifty-three blocks of formalin-fixed and paraffin-embedded archival tissue were selected from 20 cases which had been diagnosed as premalignant epithelial lesions and subsequently diagnosed as having progressed to malignancy. A single cell suspension was prepared from each tissue block, stained with propidium iodide and subjected to flow cytometric analysis. This yielded histograms which depicted the ploidy status for each specimen. For five specimens, the tissue quantity was insufficient and for an additional six specimens, the coefficient of variation for the histogram exceeded the established limit of seven. The ploidy status was determined for all specimens in 13 of the 20 cases. The initial premalignant lesions in four cases were euploid and of these, three of the subsequent malignant lesions were euploid while one was aneuploid. Five cases had initial lesions which showed aneuploidy, two of which emerged as euploid in the subsequent carcinoma, while two showed aneuploid malignancies and one acquired a tetraploid malignant phenotype. The initial premalignant lesions of the remaining four cases were characterized by subpopulations of cells in the S phase of the cell cycle

which exceeded 10 percent of the total number of cells and thus were considered neoplastic. Of these, the subsequent malignancy was euploid in one case, aneuploid in one case and tetraploid in two cases.

This study of a limited number of cases affirms that, given an adequate tissue sample size, flow cytometric analysis of nuclear DNA content is a reproducible objective parameter of oral lesions which is applicable to formalin-fixed, paraffin-embedded tissue. The diagnostic value and the use of this parameter in predicting the biologic behavior of oral premalignant and malignant lesions must await further studies which are both retrospective and prospective in nature.

Thesis accepted by the faculty of the Department of Oral Pathology, Indiana University School of Dentistry, in partial fulfillment of the requirements for the degree of Master of Science in Dentistry.

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INTRODUCTION

When considering many premalignant lesions evaluated by many experienced observers, a predictable number of these lesions will undergo malignant transformation. However, when considering each individual lesion, the predictability of malignant transformation diminishes greatly and usually disappears. All methods of diagnosis and, in particular, prediction of malignant transformation are inherently altered by the observer's subjectivity which is a composite of his/her experiences and biases. These influence the importance one attaches to the classic diagnostic criteria for premalignancy.

The search for an objective method of predicting malignant transformation has a long history. This investigation is an assessment of an as yet untried method for providing an objective and reproducible parameter for predicting the potential for malignant transformation in oral premalignant lesions. The parameter under study is the nuclear deoxyribonucleic acid (DNA) content of lesions from a series of patients whose premalignant lesions were diagnosed by light microscopy and which subsequently developed epidermoid carcinoma in the same anatomic site.

Flow cytometric analysis provides an accurate and rapid means for measuring the amount of nuclear DNA in cells of premalignant and malignant lesional tissue. The DNA content is referred to as the ploidy of the cell with euploid being the normal state and aneuploid an abnormal state. A significant relationship between the ploidy of premalignant oral epithelial lesions and the subsequent malignant transformation would be an objective parameter of predictive value in assessing the potential of an individual lesion to undergo malignant transformation. Additionally, the ploidy status of malignant intraoral tumors may prove to be a valuable prognostic indicator and, as such, could advantageously influence patient management.

REVIEW OF LITERATURE

ORAL PRECANCER

As early as 1957 the World Health Organization[1] (WHO) established collaborating centers for the study of neoplasms with the objective of formulating an internationally accepted system of histologic classification of tumors. This objective was extended to precancerous lesions of the oral cavity in 1967 with the formation of the WHO Collaborating Center for Oral Precancerous Lesions.[1] This group hoped to identify and characterize those oral lesions which have an associated risk of becoming malignant and to foster uniformity in terms, definitions, and light microscopic diagnostic criteria.

The WHO[2] in 1971 described oral squamous cell carcinoma as a tumor consisting of irregular nests, columns or strands of malignant epithelial cells, infiltrating subepithelially. The tumor cells may resemble any or all of the layers of stratified squamous epithelium. In 1972 a WHO Meeting of Investigators on the Histological Definition of Precancerous Lesions[3] defined a precancerous lesion as "a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart."

This group also defined a precancerous condition as "a generalized state associated with a significantly increased risk of cancer."

The results of the WHO collaborating center were prepared by Kramer et al.[1] and published in 1978. They reported that while many carcinomas were not preceded by identifiable lesions, some were associated with oral white lesions, either concomitant or precedent. These white lesions were referred to as leukoplakia, a term originally proposed by the Hungarian dermatologist Erno Schwimmer[4] in 1877. The WHO collaborating center[1] states that:

> One of the most important factors influencing the reported prevalence of malignant transformation in leukoplakia has been variation in the definition of leukoplakia. Some authors have applied this term only to white patches that on histologic examination show epithelial dysplasia. Naturally, such cases carry a greater risk of malignant transformation. However, ...we recommend that the term leukoplakia should carry no histologic connotation and should be used in a clinical descriptive sense only.

Based on this view, they defined leukoplakia as a white patch or plaque that cannot be removed by scraping and that cannot be characterized clinically or pathologically as any other disease and emphasized that the term is unrelated to the presence or absence of dysplasia. The WHO center[1] extended its effort for uniformity to the cellular and morphologic changes which appear in some lesions that precede cancer. They enumerated the light microscopic changes, one or more of which occur in

epithelial dysplasia and may occasionally be seen in inflammatory and reactive conditions as well as in lichen planus and candidiasis. These changes are:

1) loss of polarity of the basal cells
2) the presence of more than one layer of cells having a basaloid appearance
3) an increased nuclear/cytoplasmic ratio
4) drop-shaped rete processes
5) irregular epithelial stratification
6) increased number of mitotic figures (a few abnormal mitoses may be present)
7) the presence of mitoses in the superficial half of the epithelium
8) cellular pleomorphism
9) nuclear hyperchromatism
10) enlarged nucleoli
11) reduction of cellular cohesion
12) keratinization of single cells or cell groups in the prickle layer.

The center also recognized erythroplakia as a premalignant lesion and defined it as a bright red velvety plaque that cannot be characterized clinically or pathologically as being due to any other condition. Shafer and Waldron[5] found through microscopic examination that erythroplakia is invariably associated with carcinoma, carcinoma-in-situ, or epithelial atrophy with a variable degree of epithelial dysplasia. Mashberg et al.[6] in 1973 studied early asymptomatic oral squamous cell carcinoma and found that 90.5 percent of 58 lesions had an erythroplastic component while only 62 percent had a white component. Seventy-one percent of the lesions were invasive as

determined by light microscopic examination and 90 percent occurred on the floor of the mouth, ventral or lateral tongue, or soft palate and anterior pillar complex. Oral submucous fibrosis and the Plummer-Vinson syndrome were considered to be premalignant conditions by the WHO center[1] while epithelial atrophy and infections of Candida albicans were variably found as components of premalignant lesions. The premalignant status of oral submucous fibrosis was later confirmed by Pindborg and associates.[7]

Regarding oral infections with Candida albicans, the center[1] often found an associated moderate epithelial dysplasia which variably regressed following successful treatment for the fungal infection. They concluded that the interrelationship between candidal infection, epithelial dysplasia and the risk of malignant transformation is uncertain. The study by Roed-Petersen et al.,[8] which found candidal infections in two-thirds of the dysplasias and cytologic atypia in 40 percent of the candidal infections, did not resolve the uncertain causal relationship between the two. Oral white lesions and conditions which the WHO center[1] found to be unassociated or unlikely to be premalignant were stomatitis nicotina, lichen planus, habitual cheek biting, discoid lupus erythematosus, and white sponge nevus.

Pindborg et al.[9] considered two factors to be the source of difficulty in evaluating precancerous lesions. These were a lack of sufficient knowledge about which criteria are important in predicting malignant transformation and a lack of objectivity in evaluating the established criteria. In 1984 they evaluated the extent of subjectivity in the diagnosis of dysplasia, carcinoma-in-situ and initial squamous cell carcinoma and concluded that there was a definite need for uniform criteria for diagnosis of epithelial dysplasia.

The light microscopic criteria as defined by the WHO reference center[1] are generally accepted today for use in the diagnosis of oral epithelial dysplasia. However, subjectivity remains an inherent part of the diagnostic process.[9] According to Dabelsteen,[10] subjectivity is most apparent in the determination of which criteria are more important in predicting which dysplastic lesions will progress to epidermoid carcinoma.

In 1961 Shafer and Waldron[11] emphasized the impossibility of determining the incidence of cases of leukoplakia which will undergo malignant transformation. In 1975 Waldron and Shafer[12] affirmed the general agreement that a disputed percentage of cases of leukoplakia undergo malignant transformation into epidermoid carcinoma. Their data show that attempts to distinguish clinically between those leukoplakias which

were innocuous and those which were potentially premalignant yielded widely variable results. These authors undertook a clinical-pathologic correlation of oral leukoplakia encountered in the biopsy services of two dental schools. They found an incidence of leukoplakia of 4 percent with 68 percent being male and 81 percent occurring in persons over 40 years of age. Of their series of biopsied clinical leukoplakia, 80 percent were innocuous benign keratoses, 3 percent were invasive carcinoma, and the remaining 17 percent were dysplasia or carcinoma-in-situ. Banoczy and Csiba[13] reported a slightly higher finding of 24 percent of leukoplakias which were dysplastic or malignant.

In the 1930s and early 1940s, malignant transformation was considered to occur in 10 percent to 70 percent of the cases of leukoplakia.[14] More recent studies have offered much lower figures. In 1967 Einhorn and Wersall[15] observed 782 cases of leukoplakia for 10 years and reported a 2.4 percent rate of progression to carcinoma while their observations over a 20-year period yielded a 4 percent rate. Silverman and Rosen[16] in 1968 followed 117 cases in the United States for one to 11 years and found a 6 percent transformation rate. Studies in India by Mehta et al.[17] and Silverman and associates[18] found malignant transformation occurred in .9 percent and .13 percent respectively. In 1977 Banoczy[19] published the results of

her study which found an overall rate of 6 percent malignant transformation in 670 Hungarian cases of leukoplakia observed over an average of 9.8 years. Dysplasia was present in 24 percent of the leukoplakias and 13 percent of these developed carcinoma.

Silverman and associates[20] studied 257 patients with oral leukoplakia for an average period of 7.2 years. All lesions were more than one centimeter in size and had been present and observed for a minimum of six months. Light microscopic examination showed dysplasia in the leukoplakic lesions of 22 of these patients while the remainder had benign keratoses. Epidermoid carcinoma developed in 36.4 percent of the dysplasias and in 15.7 percent of the benign keratoses for an overall rate of 17.5 percent. In addition to the presence of dysplasia, they identified additional high risk factors for malignant transformation. These factors included the presence of erythroplakia as a component of the lesion, a clinical verrucous-papillary pattern, and long duration of the lesion.

In other longitudinal studies of leukoplakia in which dysplasia was diagnosed by light microscopy, Mincer et al.[21] found an 11 percent rate of malignant transformation, Banoczy and Csiba[13] 13 percent, Pindborg et al.[22] 14 percent, and Banoczy[23] 5.9 percent. None of these authors, however, offered a means for predicting which specific lesions would progress to carcinoma and in

fact Silverman[20] noted that up to one-fourth of the lesions regressed entirely and unpredictably even with no treatment while Mincer et al.[21] found that 6.7 percent disappeared without surgery, 4.4 percent decreased in size, and 22.2 percent remained unchanged.

In a clinical context Kramer and colleagues[24] and Katz et al.[25] confirmed Mashberg's[6] findings that the floor of the mouth is a high risk site for progression of premalignant lesions to squamous cell carcinoma. In Waldron and Shafer's[12] series, of the 20 percent of the leukoplakic lesions which were dysplastic, carcinoma-in-situ, or invasive carcinoma, 43 percent of these were from the floor of the mouth. Mashberg[26] described color as an additional clinical feature which carried an increased risk of malignant transformation. The color with the highest risk was red followed by red with focal white areas. Banoczy[23] found that progression to carcinoma occurred more often in erosive leukoplakia than in any other type.

Despite the above findings of an overall incidence of malignant transformation in leukoplakic lesions of from .13 percent to 17.5 percent it is still, at present, not possible to predict the potential for an individual lesion to become malignant.[27] Shafer[28] interpreted these data as meaning that if 6 percent of clinical leukoplakias are actually epidermoid carcinoma and a conservative estimate of 4 percent will develop epidermoid carcinoma, then 10

percent of patients with leukoplakia either have invasive carcinoma or will develop carcinoma in the future. As Pindborg and associates[9] summarize, the ideal situation would be to establish an objective method of grading epithelial dysplasia and then follow these lesions and study which ones develop into carcinoma. However, professional ethics prevents this from being done and we are left with the search for alternatives which may accurately indicate which premalignant lesions will progress to oral epidermoid carcinoma.

QUANTITATION OF LIGHT MICROSCOPIC FEATURES OF DYSPLASIA AND NEOPLASIA

The desire to reduce diagnostic subjectivity[29] and to quantitate the parameters of dysplastic lesions[30] with the intent to predict their biologic behavior surfaced with the realization that some oral lesions, especially leukoplakia and erythroplakia, did indeed progress to invasive epidermoid carcinoma.[9] Barlogie[31] relates that the evolution of the identification of these parameters has been along two major lines. One is the quantitation of those characteristics seen by light microscopy and that have traditionally been used subjectively. The other is quantitation of parameters not observable by light microscopy such as nuclear deoxyribonucleic acid (DNA) content.

In 1969 Smith and Pindborg[29] used a weighted numerical scoring system based on photographic standards of dysplasia in an attempt to evaluate and reduce the extent of subjectivity involved in the diagnosis of dysplasia and to instill standardization in the relative importance attached to the diagnostic criteria of premalignancy. Kramer et al.[30,32] studied tissue specimens from lesions of keratosis, leukoplakia and lichen planus using discriminant analysis and cluster analysis and programmatically attached computer calculated values to histologic variables in each lesion. Though time-intensive and cumbersome, they found they could retrospectively separate leukoplakias that subsequently developed carcinoma from those that did not. This was significant at the 5 percent level. This technique was essentially computer manipulation of recorded data derived through subjective light microscopic interpretation by the investigators of the presence or absence of the dysplastic parameters. As an extension of their prior work, Kramer and associates[33] calculated the importance of each histologic variable. From these calculations they found it possible to depict the histologic characteristics of each group of lesions in diagrammatic form.

Expansion of the use of automated objectivity led to morphometric analysis of structural features of oral epithelium. Barry and Sharkey[34] investigated the

feasibility of quantifying histological differentiation of oral epithelium using morphometric point counting, the degree of keratinization and architectural features. They concluded that the method has a high degree of observer reproducibility and is sufficiently sensitive to be applied to practical biologic problems.

Franklin et al.[35] found stereological methods for evaluation of oral epithelium provided reliable and accurate quantitative information. Of all the stereological parameters studied at the light microscopic level, they found the nuclear/cytoplasmic ratio, nuclear density and interface ratios were best able to distinguish between benign and premalignant epithelia. The ratios included the volume of epithelium to keratin interface, the volume of epithelium to connective tissue interface, and the keratin interface to connective tissue interface. Keszler and Cabrini[36] thought it possible to differentiate between oral white lesions of leukoplakia, lichen planus, and carcinoma-in-situ based on histometric analysis of nuclear density divided into the categories of total and basal nuclear density and total and basal nuclear area. They found a greater total nuclear density in leukoplakia than in lichen planus and greater total and basal nuclear areas in carcinoma-in-situ than in leukoplakia or lichen planus. They also believed it

possible to make a differential diagnosis based on the numerical variables derived from these differences.

Shabana et al.[37] attempted to overcome the subjectivity involved in the evaluation of the nuclear/cytoplasmic ratio in dysplastic epithelium through the application of computer based image analysis techniques. This parameter of premalignancy receives great emphasis by many pathologists. Their results showed a steady increase in both cellular and nuclear dimensions through a spectrum of conditions and lesions including normal epithelium, reactive conditions of traumatic keratosis, inflammatory lesions of lichen planus, potentially premalignant lesions of leukoplakia, candidal leukoplakia, and dysplasia or carcinoma. However, the nuclear/cytoplasmic ratio did not change significantly. They felt the increase in nuclear size may be due to an increase in DNA synthesis.

QUANTITATION OF THE NUCLEAR DNA CONTENT OF DYSPLASIA AND NEOPLASIA

Focusing on the nuclei of cells in different oral tumors and epithelial lesions, several investigators[16,21,27,38-45] have studied the DNA content or degree of ploidy. These studies were based on the belief that a main advantage of DNA content analysis is the early detection of the emergence of cell stem lines

containing an abnormal amount of DNA. Diploid cells are those which contain the usual amount of DNA found in a normal somatic cell and is twice the haploid amount present in a normal germ cell. Euploidy is defined as an exact multiple of the normal haploid number. Polyploidy is the state of a cell nucleus containing a multiple of three or higher of the haploid number of chromosomes. Cells containing three, four, five, or six multiples are referred to, respectively, as triploid, tetraploid, pentaploid, or hexaploid. Aneuploidy is the state of having an abnormal number of chromosomes which is not an exact multiple of the haploid number and may be more or less than the diploid number.[46] Regardless of the method used for determining the ploidy status of a cell population, the data may be graphically represented as a histogram. Conventionally, the axis of the histogram reflects the DNA content per cell and the abscissa indicates the number of cells.[47] Thus, a peak in a histogram would indicate the presence and size of a subpopulation of cells containing a specific amount of DNA.

In 1966 Atkin et al.[40] compared the DNA content and chromosome number of 50 human tumors. One of their goals was to establish the validity of the Feulgen microspectrophotometry method for determining the DNA content of cells. Fairly close mutual agreement was found between the modal DNA content determined by the Feulgen

technique and the chromosome number as determined by karyotyping. However, a small but consistent discrepancy occurred with the expected chromosome number exceeding the actual chromosome number by an average of 4 percent.

Giminez and Conti[41] employed Feulgen microspectrophotometry to determine the DNA content of epithelial basal cells in radicular cysts, odontogenic keratocysts, benign keratoses, epithelial dysplasias, and oral epidermoid carcinomas. The inflammatory radicular cysts yielded histograms with a definite diploid peak with a slight deviation to the right (toward hyperdiploid) similar to that of normal oral epithelium[42] while keratocysts showed an additional cell population in the tetraploid range. Benign keratoses showed a diploid peak with a percentage of cells in the diploid to tetraploid range while dysplastic lesions showed shifts toward higher ploidy values which corresponded well with the light microscopic interpretation of the degree of dysplasia present, i.e., the more severe the dysplasia, the greater the shift to the right. Carcinomas had histograms with multiple peaks or ill-defined peaks ranging from near diploid to beyond octaploid. They postulated that the tetraploid peak in odontogenic keratocysts reflected an increased proliferative rate while the shift to the right in dysplastic lesions and carcinomas may have reflected an increased percentage of cells in the S phase (DNA synthesis phase of the cell cycle) or a possible karyotype variation.

Using similar techniques Doyle and Manhold[39] found that in 33 lesions studied, 50 percent of the carcinomas and 75 percent of the leukoplakias showed changes in nuclear DNA content. They felt that this represented the emergence of a cell line with a non-euploid karyotype and was an early change in the development of cancer. Additionally, some of the clinical leukoplakias with aneuploid peaks were innocuous in appearance by light microscopy. This supports the findings of Silverman[16] and Pindborg[43] and Mincer et al.[21] that light microscopic features of premalignancy are often not present in original biopsy specimens of lesions which subsequently develop into epidermoid carcinoma. However, as a routine method for predicting malignant transformation, Doyle and Manhold[39] state that ploidy determination is of minimal value since half of the carcinomas in their study showed diploid stem lines.

Abdel-Salam et al.[27] combined the techniques of morphometry and DNA cytofluorometry (using the azure A-Feulgen reaction for DNA) in the study of leukoplakia with and without light microscopic features of dysplasia. Five variables were analyzed: nuclear total staining, nuclear average stain, nuclear area, nuclear form factor and nuclear ellipticity. In this study a three-variable model was created which allowed discrimination between normal or hyperplastic epithelium and dysplasia with 81

percent accuracy. The three variables were those related to nuclear morphology alone. Applying this model to the prediction of malignant transformation in oral epithelial lesions, Abdel-Salam and colleagues[44] found they could predict the malignant potential of lesions with 87.5 percent accuracy.

Saku and Sato[38] in 1983 selected cytofluorometry and smears of cells isolated from paraffin blocks to investigate the possibility of predicting malignant transformation of oral precancerous lesions. In the DNA histograms of epithelial proliferations without malignant transformation there was a shift of the modal peak to the right of diploid as well as cell populations in the aneuploid range. The degree of aneuploidy was in proportion to the degree of dysplasia as determined by light microscopy. This was termed a "dysplastic pattern" of the DNA histogram. This is consistent with the findings of Giminez and Conti[41] and Pfitzer and Pape.[45] Saku and Sato's cases[38] which later developed into carcinoma were depicted by a dysplastic pattern of the DNA histogram irrespective of the histological degree of dysplasia. Additionally, these lesions displayed bimodal peaks which were interpreted as possibly representing proliferation of two separate stem lines.

DNA content as a valid predictive tool may not be limited to assessing the likelihood of malignant

transformation, but may also have relevance in predicting the prognosis of patients with cancer.[48] In this context, cytofluorometry and cytophotometry have been used to study both oral and non-oral carcinomas. Tytor et al.[49-51] related the DNA pattern in oral cavity carcinomas to the clinical stage and histological grading. Using cytofluorometric DNA analysis, they found 48 percent of the carcinomas studied to be aneuploid. Aneuploidy correlated with increasing tumor size, decreasing histologic grading and the presence of lymph node metastases. In their most recent study,[51] they stated "tumor DNA ploidy may be a complement to clinical and morphologic parameters as a prognostic predictor in squamous cell carcinoma of the oral cavity." Cytophotometric DNA analysis of esophageal carcinoma was carried out by Sugimachi and associates.[52] They classified the tumors as type I, II, III, and IV based on the DNA histograms. Type I was least divergent from the diploid state and type IV had multiple peaks and broad dispersion. In all cases of types I and II esophageal carcinoma, the patients showed no recurrence while 20 percent of the type III and 55 percent of patients with type IV died following a recurrence. They felt that tumor ploidy was as valid a prognostic indicator as clinical stage. Stressing the importance of light microscopic interpretation of nuclear morphology as a prognostic

indicator in prostatic cancer, Diamond and associates[53] developed a nuclear roundness factor which they applied to excised prostatic cancers. Their calculated nuclear shape factor, based on computerized image analysis, appeared to differentiate prostatic tumors with a high metastatic potential from those that were less aggressive. They considered the accuracy, reproducibility, and quantitative nature of this method to be its great advantages.

SIGNIFICANCE OF NUCLEAR CHANGES IN THE MALIGNANT TRANSFORMATION PROCESS

Boyd and Reade[54] recognize that a number of changes occur in preneoplastic and neoplastic cells as they progress to a greater degree of malignancy and that some of these are contributory to the pathologic process while others are reactive. In some instances, the relationship between the detected changes and the disease process are not understood. Among genetic alterations which may be associated with carcinogenesis are the classic concept of mutation as a single base pair substitution, DNA rearrangement, gene duplication, activation or suppression of genes by neighboring base sequences, and alteration in the degree of DNA methylation. Nowell[48] believes that regardless of the mechanism, it appears that chromosomal alteration occurs in the evolution of many mammalian solid tumors, and in sequential experimental neoplasms, the trend

is toward an increased chromosome number. This confers potential survival advantages upon the cell at the expense of other subpopulations. He also supports the concept of a "cascading" effect so that with tumor evolution the hyperdiploid cells are increasingly genetically unstable. He believes this may account for the increasing numbers of abnormal mitoses in advanced malignancies.

The available data are interpreted by Nowell[48] as indicating that most mammalian neoplasms have demonstrable cytogenetic abnormalities and that many carcinogenic agents and precancerous conditions are associated with chromosome damage. Demonstrable chromosome changes may appear early or late in the disease process.[48] This is consistent with the clonal evolution concept[55] that tumors progress on the basis of genetic instability within the neoplastic population leading to sequential emergence of mutant subpopulations with increasingly malignant properties.[48]

True genetic alteration and mutations are considered to be permanent and irreversible. Epigenetic mechanisms for acquisition of the malignant phenotype result in alteration of the expression of an essentially normal cellular genome and are potentially reversible. Theories of acquisition of the malignant phenotype which would result in deviation from the normal amount of DNA in the malignant cells include chromosome truncation or deletion, incorporation of viral DNA into the host genome, gene

amplification, and formation of double minutes.[48] Others[55] suggest that inherent in the production of subpopulations is an increased mitotic activity which carries an increased risk of genetic variation which may become more pronounced as the tumor develops. Such an increase in the proliferation rate may cause a greater than normal number of cells to be in the S phase and tetraploid range.

However, there are other theories for acquisition of the malignant phenotype which do not involve a change in the DNA content of the cells.[48] These include alteration of cell surface expression, suppression or enhancement of gene expression, cell surface antigen shedding, point mutation, translocation, transactivation and ectopic gene expression. Therefore, while the finding of cells with aneuploid DNA strongly suggests the acquisition of the malignant phenotype, the presence of euploidy does not rule it out. So the ploidy status of premalignant and malignant lesions may be more important in the prognosis of disease progression rather than in the predictive value of malignant transformation.[48]

Boyd and Reade[54] feel that by investigating underlying genetic alterations in oral carcinogenesis it may be possible to predict which dysplasias will remain as such and which will evolve into carcinoma. They further believe that oral cancer results from exposure to one or

more etiologic agents which may include chemical, physical and viral agents. Exposure alone, however, is rarely enough to cause cancer. Nowell[48] relates that although the exact mechanism is not understood, there appears to be a clear correlation between the capacity to produce chromosomal aberrations and the capacity to induce neoplasia. Furthermore, the malignant transformation process is modulated by such factors as familial, dietary, hormonal, gender and age influences, but their precise role has not been determined.[56]

It has been found by Monier et al.[57] that three groups of DNA viruses cause cancer in animals and humans: papovaviruses, adenoviruses and herpesviruses. At least a portion of the viral DNA must be incorporated into the host cell genome and be expressed in the form of proteins to maintain the transformed phenotype. It has been postulated[57] that an important aspect of the oncogenic function of the DNA virus is the ability to induce host cell replication as a component of their transforming potential. This would possibly account for the finding of an increased population of cells in the S phase in some tumors which may be of viral etiology. Similarly, Neel and colleagues[58] found that retroviruses must incorporate into the host genome to effect their transforming capabilities.

Boyd and Reade[59] state that although there is evidence that some oral carcinomas may be related to viral or physical agents, the majority of oral mucosal carcinogens are likely a result of exposure to chemical carcinogens. Rous and Kidd,[60] Berenblum and Shubik,[61] and Friedewald and Rous[62] developed the concept of initiation and promotion as the two stages in the acquisition of the malignant phenotype. It is accepted[63] that the initiation step is a permanent alteration of the DNA of a cell which may occur following a single exposure to an initiating agent. The nature of the change is uncertain and may involve point mutation, deletion, translocation, and altered gene expression. Some of these avenues would result in a change from the normal DNA content of the cell while others would not.

Foulds'[64] concept of progression is similar to promotion and involves sequential selection of variant subpopulations of an initiated cell population. The selected subpopulations are believed to have acquired, through initiation, a survival advantage as a result of the qualitative change in one or more of the cellular components.

The second step in the classic concept of carcinogenesis is promotion[63] and results in the permanent alteration of the cells. Initiated cells may revert to normal through excision repair mechanisms after

one or more generations of cell division. Promotion, however, may result in permanent alteration of the genotype. This can be viewed as selection of initiated cells that would create cell lines that have acquired the malignant phenotype. Once these cell lines form a critical mass they may be detected by various methods including staining procedures, light microscopy, clinical alterations, and specialized techniques of cytofluorometry and flow cytometry.

APPLICATION OF FLOW CYTOMETRIC PLOIDY DETERMINATION TO HUMAN TUMORS

Barlogie and colleagues[31] purport that the management of neoplastic disease may be advanced through application of tumor determinants not appreciated at the light microscopic level. In their report, the significance and applicability of flow cytometry and DNA content to clinical cancer research is aptly stated:

> Quantitative cytology in the form of flow cytometry has greatly advanced the objective elucidation of tumor cell heterogeneity by using probes that discriminate tumor and normal cells and assess differentiative as well as proliferative tumor cell properties. Abnormal nuclear DNA content is a conclusive marker of malignancy and is found with increasing frequency in leukemia, in lymphoma, and in myeloma, as well as in solid tumors for an overall rate of 67% in 4941 patients. The degree of DNA content abnormality varies according to disease type....From a patient management perspective, a role for flow cytometry is emerging as a tool for diagnosis of cancer (abnormal DNA content)...,prognosis (adverse impact of aneuploidy and high S percentage), and treatment (cytokinetically oriented, monoclonal antibodies, drug pharmacology).

> The pace of past progress justifies the hope that cytometry may soon provide "fingerprint-type" information of an individual patient's tumor which, if proven prognostically relevant, may provide the basis for treatment selection in the future.

In their review Kute and Muss[47] explain that the technique of laser-based flow cytometry, which was developed in 1972, can rapidly count individual particles or cells introduced into a stream of fluid that passes through a laser beam. The stream restricts the passage of cells to a single file. The function of the laser is to excite a fluorescent probe which has been attached to a component of the cells being analyzed. Depending on the specific probe used, the flow cytometer can deliver information about DNA, RNA, protein and antigen presence and density per cell. Integrated analytical devices such as digital, optical and other electronic instruments can rapidly manipulate the data and provide analyses appropriate to the area of investigation. Prior to the advent of flow cytometry, the common methods for analyzing DNA were the Feulgen stain and tritiated thymidine. Flow cytometric analysis is less time-consuming and tedious and allows for the study of a much larger population of cells.

Thornthwaite et al.[65] propose three reasons why the application of flow cytometry as a diagnostic and prognostic technique has been slow in developing. First, is inadequate resolution to measure DNA within acceptable coefficients of variation. Second, development of, and

more importantly, standardization of methods and materials for tissue preparation has only recently been established. Third, the diagnostic and prognostic significance of DNA measurements is just recently being appreciated. Taylor and Milthrope[66] discussed several combinations of sample preparation techniques and DNA stains which fulfill the above criteria, but concluded that the wide variation of techniques and stains in use makes comparison impossible and suggest that it is appropriate to base the selection of techniques and stains on available instrumentation, laboratory facilities and investigator experience.

Herman and associates[67] concluded that ploidy determination is useful in two separate circumstances: the diagnosis of malignancy and in the determination of the prognosis for patients with clearly malignant tumors. In the first situation, the presence of aneuploidy provides support for the diagnosis of malignancy. The second circumstance presents an entirely different application of the information derived from flow cytometric determination of ploidy in that aneuploidy in a tumor generally has been proven to connote a poorer prognosis than lesions with the same light microscopic features but with no aneuploid cell populations.

Flow cytometric study of non-oral tumors has contributed to improved diagnostic, prognostic, and treatment parameters for patients with malignant disease.

Investigation has been performed on fresh tissue specimens and, with the development by Hedley and associates[68] of techniques for analysis of DNA content of paraffin-embedded pathological material, retrospective studies using archival tissue are now possible.

Van Bodegom et al.[69] followed 52 patients with stage 1 squamous cell lung cancer for a minimum of six years. They found that within the 56 percent with aneuploid tumors, those with greater than 10 percent aneuploid cells showed a 35 percent, six-year survival, while those with less than 10 percent aneuploidy had a 78 percent, six-year survival. They concluded that within the bounds of their staging criteria, the percentage of aneuploid tumor cells is correlated with prognosis. Volm and colleagues[70] investigated a series of patients with previously untreated non-small cell lung carcinoma using flow cytometry. Patients with aneuploid tumors had significantly shorter survival times than those with diploid tumors. The results were identical with those obtained through predicting prognosis by clinical staging factors. This demonstrated two independent groups of prognostic factors for patients with non-small cell lung carcinoma: clinical factors and flow cytometric factors. Giullen and associates[71] found aneuploidy in one of 20 nevi and 68 of 162 primary melanomas studied. The ploidy varied significantly among the melanomas. Sixteen percent of those classified as

Clark's levels I and II were aneuploid, while 66 percent of levels IV and V were aneuploid. Thirty-three percent of the superficial spreading melanomas were aneuploid and 65 percent of the nodular melanomas were aneuploid. Aneuploidy also correlated proportionately to an increase in the number of mitoses. They felt there was a significant correlation between conventional morphologic parameters and ploidy and planned to evaluate the clinical progress of these patients in hopes of attaching prognostic significance. Flotte et al.,[72] in comparing ploidy in formalin-fixed paraffin embedded tissue from mycosis fungoides, epithelioid sarcoma, normal skin and inflammatory conditions, found the S phase fraction to be an unreliable diagnostic or prognostic factor. However, ploidy determination by flow cytometry was a useful diagnostic adjunct for mycosis fungoides and epithelioid sarcoma but was not a prognostic indicator.

Hanselaar and colleagues[73] found age to have a significant relationship with ploidy in grade III cervical intraepithelial neoplasia with and without synchronous invasive squamous cell carcinoma. Eighty percent of the women over 50 had aneuploid lesions while 60 percent of those under age 35 had a diploid pattern. In the cases with adjacent invasive carcinoma, both lesions generally displayed similar ploidy patterns indicating that the two were related. Lage and associates[74] applied flow

cytometry to the study of hydatidiform moles and found complete agreement between cytometer derived ploidy and ploidy determined by karyotypic analysis. They concluded that this is an accurate supplement to histological interpretation of hydropic placentas.

Sasaki et al.[75] investigated regional differences in DNA ploidy of gastrointestinal carcinomas by flow cytometric analysis. They found intratumoral differences in the ploidy status in 40 percent of their cases and concluded that proper ploidy determination, especially in gastric carcinomas, requires sampling multiple sites and therefore multiple subpopulations within the same tumor. El-Naggar et al.[76] studied Hurthle cell tumors of the thyroid and found that nuclear DNA ploidy did not distinguish benign from malignant tumors, that diploid carcinomas behaved much less aggressively than aneuploid Hurthle cell carcinomas, and that all patients with aneuploid carcinomas died of their disease or were alive with persistent disease.

Lampe et al.[77] found that flow cytometry proved to be a practical method of analysis for quantitative measurement of DNA content in squamous cell carcinomas of the upper aerodigestive tract. Their admittedly preliminary findings suggested a capability for identification of a DNA histogram characteristic of a more aggressive squamous cell carcinoma which tended to recur

more quickly and frequently than other squamous cell carcinomas in the study. These histograms included a discrete hyperdiploid peak and no corresponding diploid peak.

Very few of the above studies addressed the difficulty encountered in interpretation of histograms. This aspect of flow cytometric measurement of DNA was thoroughly discussed by Koss et al.[78] They stressed that although the machine-generated data are reproducible and independent of observer bias (even though tissue preparation techniques may introduce a degree of variability), the interpretation of the data varies among investigators. This is particularly true for histograms which deviate from the normal or euploid state. They also found that the results from fresh tissue did not always mirror those derived from the use of archival tissue. Additionally, most, if not all, ploidy information is organ or tissue-specific. Even then, the tumors do not always follow the pattern discerned by flow cytometric analysis for that tissue or organ.

In his extrapolation of the potential of flow cytometry, Barlogie[31] expressed the opinion that it may become possible to quantitate the genotypic and phenotypic properties of a patient's tumor analogous to an SMA profile in laboratory chemistry. Furthermore, the identification of quantitative features associated with malignant

transformation may provide information useful in the prevention of cancer.

METHODS AND MATERIALS

CASE SELECTION

Tissue used in this study was retrieved from the archives of the Indiana University School of Dentistry Departmant of Oral Pathology using the automated data storage and retrieval system. The case selection process began with the identification of all accessions from January 1, 1984 through September 14, 1988 which were diagnosed and coded as epidermoid carcinoma. For each of these accessions, the system was queried for previous specimens which had been received from the patient and which were on file. The anatomic site of each previous biopsy was compared to the anatomic site of the epidermoid carcinoma. A case was considered for inclusion in the study if the anatomic sites were the same. Nineteen cases met these criteria and hematoxylin and eosin stained sections of all specimens were reviewed. Concurrence with the original diagnoses resulted in acceptance of all 19 cases. All biopsy tissue was originally fixed in 10 percent formalin and embedded in paraffin. The blocks were retrieved from storage and delivered for processing and flow cytometric analysis.

Additional clinical and demographic information, when provided, was retrieved for each case and included date of

birth (DOB), sex, date of each biopsy, and the clinical appearance of the lesion. All information for the cases under study is provided in Table I and Table II.

TISSUE PREPARATION

Preparation of the formalin-fixed and paraffin-embedded tissue for flow cytometric analysis was performed using the technique originally developed and described by Hedley et al.,[68] modified by Bauer,[79] and adapted by Kotylo.[80] The process is logically divided into three phases separated by steps requiring overnight incubation or soaking in distilled water and propidium iodide to enhance peak resolution and analysis.

Phase 1

The tissue blocks were visually compared with the stained sections and scored to outline the epithelial component and/or the neoplasm. Sections were then cut at a thickness of 50 microns and tissue outside the scored area was discarded. This procedure grossly eliminated most of the keratin, lamina propria, and other non-epithelial, inflammatory and necrotic tissue. Sufficient sections were obtained to yield a bulk of epithelium estimated to contain a minimum of 10^5 epithelial cells for analysis. The tissue sections were placed in glass test tubes which were

appropriately labeled for identification (as were all subsequent containers into which tissue was transferred).

Under a fume hood, the tissue was deparaffinized with two changes of 15 ml of xylene for 10 minutes each. Rehydration was then accomplished by adding 15 ml each of 100% ethyl alcohol (two changes), 95%, 70%, and 50% ethyl alcohol. The duration for each change of alcohol was 10 minutes. The tissue was then washed twice in 15 ml of distilled water, placed in fresh distilled water and allowed to sit overnight. This ensured the removal of as much formalin as possible since formalin forms cross-links with DNA and would block the subsequent reactions between nuclear DNA and DNA specific stains or intercalating dyes.

Phase 2

With a wooden stick, the tissue sediment was removed, transferred to a watch glass, and minced with dissecting scissors to form a paste which was then placed in a clean plastic test tube. Pepsin digestion for tissue disassociation was then accomplished by the addition of 1 ml of 0.5% pepsin in saline with 3% PEG 6000 (a detergent) adjusted to a pH of 1.5 with HCl. The tube was then placed in a water bath at 37 degrees centigrade for 45 minutes to one hour until the pepsin solution turned cloudy. The tube was agitated using a vortex mixer at five-minute intervals during pepsin digestion. The pepsin proteolysis reaction

was halted with the addition of 15 ml of buffer. The digested material was then centrifuged at 1500 rpm for 10 minutes to produce a pellet. The supernatant was discarded and the pellet resuspended in 2 ml of Hanks' balanced salt solution with 10 mM of HEPES (4-[2 hydroxyethyl]-1-peperazine ethanesulfonic acid) buffer and filtered through a 44 micron nylon mesh filter to remove all non-nuclear tissue including large tissue fragments and debris.

The number of nuclei obtained was determined using a Coulter S + IV (Coulter Company, Hialeah, Florida) counter. If the number was much greater than 10^6, the tissue mass was adjusted downward to approximate this amount. If less than 10^6 nuclei were harvested, the sample was processed as a means of assessing the adequacy of small tissue samples for future studies. The nuclei were recentrifuged at 1500 rpm for 10 minutes, the supernatant discarded and 1 ml of 0.1% Triton X-100 detergent (Sigma Chemical Company, St. Louis, Missouri) in phosphate buffered saline (PBS), (NaCl, 7.6 g/L; Na_2HPO_4, 1.27 g/L; KH_2PO_4 g/L), was added to the resultant pellet. This was then incubated in ice for three to five minutes. The purpose of the detergent was to remove cytoplasm from the nuclei and to damage nuclear membranes, thereby increasing nuclear membrane permeability and allowing access to the nuclear DNA by subsequent stains. Centrifugation was again performed, the supernatant removed, and the pellet was

gently resuspended in 1 ml of RNAase (180 U/ml in PBS). The RNAase (Worthington Biochemicals, Freehold, New Jersey) was chromatographically pure and was heated during reagent preparation to remove any remaining DNAase activity. This enzymatic process destroyed all nucleic acid except double-stranded DNA. The test tube containing this solution was placed in a water bath at 37 degrees centigrade for 20 minutes, centrifuged and the supernatant poured off.

DNA staining was accomplished using fluorescent propidium iodide in a concentration of 50 micrograms per milliliter in PBS. For each 10^6 cells in the pellet, 1 ml of stain was added. The test tube was capped, covered with foil, since the propidium iodide is light sensitive, and placed in a refrigerator overnight to allow the stain to intercalate into the DNA base pairs.

Phase 3

The next day, the solution was again filtered through 44 micron nylon mesh and was then ready for flow cytometric analysis.

FLOW CYTOMETRIC ANALYSIS

The instrument used was an Epics Profile flow cytometer (Coulter Company) equipped with a 125 mw argon ion air cooled laser. It was calibrated prior to each

session using beads with a known fluorescent potential. Normal oral mucosa was processed in parallel with the study material as a control. The principle employed by the instrument is one of a stoichiometric relationship between the propidium iodide and the nuclear DNA. As the amount of nuclear DNA increases, the amount of the bound stain and, therefore, the degree of fluorescence of the bound stain increase proportionately. The instrument is sensitive to plus or minus two chromosomes and will not detect deletions or additions of chromosomal material below this limit. The cytometer captures data on the number of cells which fluoresce at specific values corresponding to the amount of DNA they contain. This is represented as a histogram with the X axis representing the intensity of fluorescence and the Y axis representing the number of nuclei (Figure 1).

The data were transferred to a double-sided, double-density floppy disk and analyzed off-line on an Intel SYP301 microcomputer using Cytologic, a proprietary software program from the Coulter Company. Each histogram was evaluated for the presence of background debris and, if present, it was subtracted. All subsequent analyses were then considered to be dealing with only bare nuclei from lesional tissue.

Criteria were set for what were acceptable data. Ill-defined and wide histogram peaks with a high coefficient of variance (CV) give imprecise information as to the ploidy

of the cell populations and may mask underlying smaller peaks or shoulders which would indicate the presence of a subpopulation of cells with a different ploidy status. The CV was calculated for each histogram and the data were considered acceptable if the CV was less than seven.

Analysis was performed using the method of Baisch[81] which gives the relative number of nuclei in each cell cycle phase of $G_{1/0}$, S, and G_2/M. This is accomplished by fitting a rectangular polygon to the base portion of the histogram curve and mathematically calculating the areas under appropriate portions of the curve thus partitioned (Figure 2). Intralaboratory standards were applied to differentiate normal from abnormal subpopulation sizes in the growth phases of the cell cycle. To distinguish a tetraploid tumor from tissue having a prominent G_2/M subpopulation, the figure of 15 percent was used. That is, if the tetraploid peak contained more than 15 percent of the entire sample of nuclei, it was considered to be a tetraploid tumor subpopulation. Conversely, if this portion of the curve contained 15 percent or less of the nuclei, it was considered to represent a G_2/M peak. The possibility that this area of the curve was caused either in whole or in part by the presence of doublets was eliminated by syringing, filtering and visually examining the population. In this manner, less than 5 percent doublets was assured. An abnormally high subpopulation in

the S cell cycle phase was defined as an S phase which contained greater than 10 percent of the total number of test nuclei.

The standards employed for assigning ploidy status to cell subpopulations were based on the DNA index (DI). The DI is a ratio derived by dividing the mean fluorescence channel of a subpopulation of test nuclei by the mean fluorescence channel of the diploid subpopulation of nuclei in the sample. Diploid status was assigned if the DI was .95 to 1.05 and near diploid when the DI was 1.05 to 1.15. A cell population was defined as hypodiploid if the DI was less than .95, hyperdiploid in the 1.15 to 1.80 range, tetraploid when the DI was 1.80 to 2.20, and a DI greater than 2.20 was labeled hypertetraploid.

Data generated by the flow cytometer and analyzed with the Cytologic program were subjected to statistical analysis using the chi-square test. Due to the small sample size, only statistically significant associations between the ploidy status and the light microscopic diagnosis and the clinical features were sought.

RESULTS

Anatomic sites varied widely as shown in Figure 3. Seven cases, which comprised 35 percent of the total, were located in the floor of the mouth (FOM). Three cases each were located on the lateral tongue and buccal mucosa, two on the ventral tongue, two from the ridge or tuberosity and one each were located on the lower lip and palate.

The period of time from the initial biopsy to diagnosis of carcinoma ranged from 10 days, which probably represented a sampling phenomenon, to a disease process which lasted 12 years and three months with an average of 14.5 months. The median time was six months.

Thirteen (68.4 percent) of the 19 cases were female. At the time of diagnosis of the carcinoma, five (26.3 percent) were in their eighth decade, three (15.8 percent) were in their sixth decade and one each were in their fifth, ninth and tenth decades.

Thirty-nine of the biopsied lesions were described clinically based on their color. Sixteen were white, nine were red, and 14 were red and white. Five of the white lesions were euploid, five were aneuploid and six showed diploid peaks with an S phase fraction exceeding 10 percent. Only two of the red lesions were euploid while four were aneuploid, one had a diploid peak with an S phase

fraction greater than 10 percent, and two had tetraploid neoplastic subpopulations. Of the red and white lesions, three were euploid, six had an aneuploid component, four contained an S phase fraction exceeding 10 percent, and one had a tetraploid subpopulation. This is depicted in Figure 4.

Of all the lesions for which a color was specified, 25.64 percent were euploid, and of these, half were white, 20 percent were red, and 30 percent were red and white. White, and red and white lesions each comprised 37.93 percent of the noneuploid lesions while 24.14 percent of these lesions were red.

For each case under study, data were gathered and presented as histograms by the flow cytometer as a routine function performed by this instrument. An example is shown in Figure 1. Analytic results of the raw data of each tissue specimen using the Cytologic program and the method of Baisch were printed in graphic form with numeric results tabulated below the graph as shown in Figure 2.

The numeric values for each case were extracted from the printed products described above and are presented in Table III through Table XXII. Listed separately within each case are the data for each biopsy specimen. In several instances, the quantity of tissue was not sufficient (QNS) for analysis and for other specimens, the coefficient of variation (CV) was greater than seven which

rendered the data unreliable for use in analysis. These situations are so indicated. Juxtaposition or overlap of a diploid peak and a near diploid aneuploid peak compromised the reliability of the calculated S phase fraction. Cases for which this situation existed are identified in the appropriate table footnote.

Euploid cell populations were identified in all analyzable specimens of cases 1, 2 and 12. Separate aneuploid peaks were present in one or more of the biopsies in cases 4, 5, 8A, 9, 13, 14, 15, 18, and 19. Cases 3, 7, and 17 had diploid peaks but with S phase fractions exceeding 10 percent of the total curve thus qualifying for categorization as neoplastic. Additionally, cases 8B and 17 had a G_2/M phase fraction exceeding 15 percent of the total histogram curve and were classified as having neoplastic tetraploid subpopulations.

Of the 15 cases with adequate sample size and acceptable CVs throughout the disease process, three began as euploid. Of these, one (case 2) remained euploid in the carcinoma phase, one (case 8A) progressed through a tetraploid neoplasm to an aneuploid carcinoma and one (case 12) developed into a diploid carcinoma with a high hyperdiploid fraction ($G_2/M + S$).

Six cases began as proliferative lesions characterized by a diploid peak with a high S phase fraction and three of these (cases 9, 13, and 14) progressed to aneuploid

carcinomas. One (case 3) retained the diploid peak and high S phase fraction and one reverted to euploidy (case 7) with an S phase fraction which was within normal limits. The sixth (case 17) developed into a tetraploid neoplasm with a high S phase fraction.

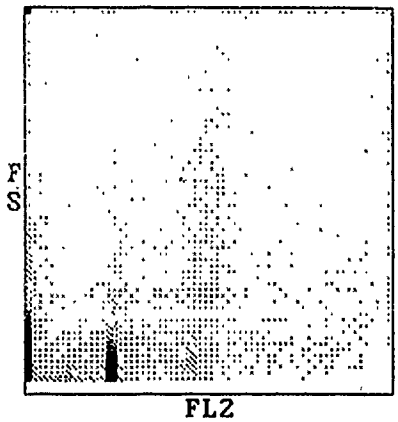
The remaining six analyzable cases began as aneuploid and three remained aneuploid (cases 5, 8B, and 19). One of these, case 8B, showed a tetraploid neoplastic subpopulation in the carcinoma, while case 19 showed an intermediate lesion that had a diploid peak and a high S phase fraction. Two of the cases that were initially aneuploid showed subsequent euploid carcinomas (cases 4 and 15). One case (case 18), though the initial biopsies were unacceptable for analysis, showed intermediate aneuploidy and a carcinoma with a diploid peak and high S phase fraction.

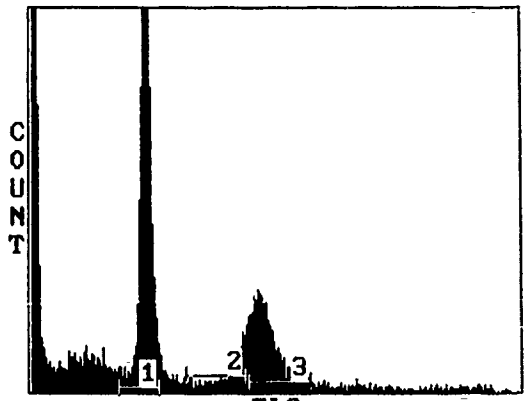
Chi-square analyses of ploidy status versus the parameters of light microscopic diagnoses and clinical color of the lesions were statistically not significant and had p values of .55 and .23 respectively.

FIGURES AND TABLES

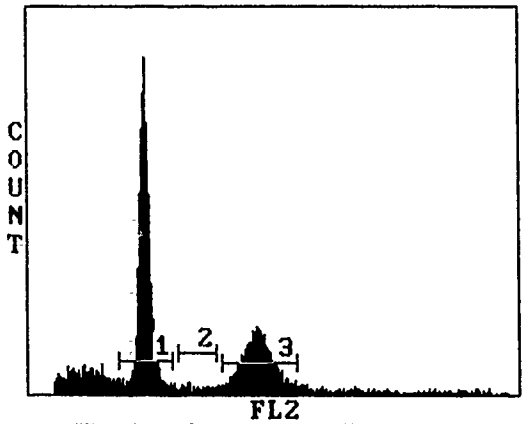
FIGURE 1. Sample histogram and scattergram produced by the Epics Profile Analyzer flow cytometer.

EPICS® Profile Analyzer
COULTER CYTOMETRY TEST RESULTS





	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	46	67	4422	24.2	59.2	3.5	4.12
2	81	112	928	5.1	102.0	9.4	8.54
3	114	146	2034	11.1	123.7	7.8	6.09



	MIN	MAX	COUNT	PERCENT	MEAN	SD	% HPCV
1	47	75	4581	45.8	59.8	4.1	4.12
2	78	98	344	3.4	87.9	6.3	3.19
3	101	140	2633	26.3	119.0	8.4	6.09

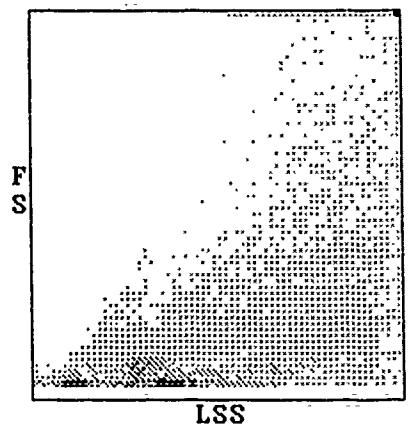


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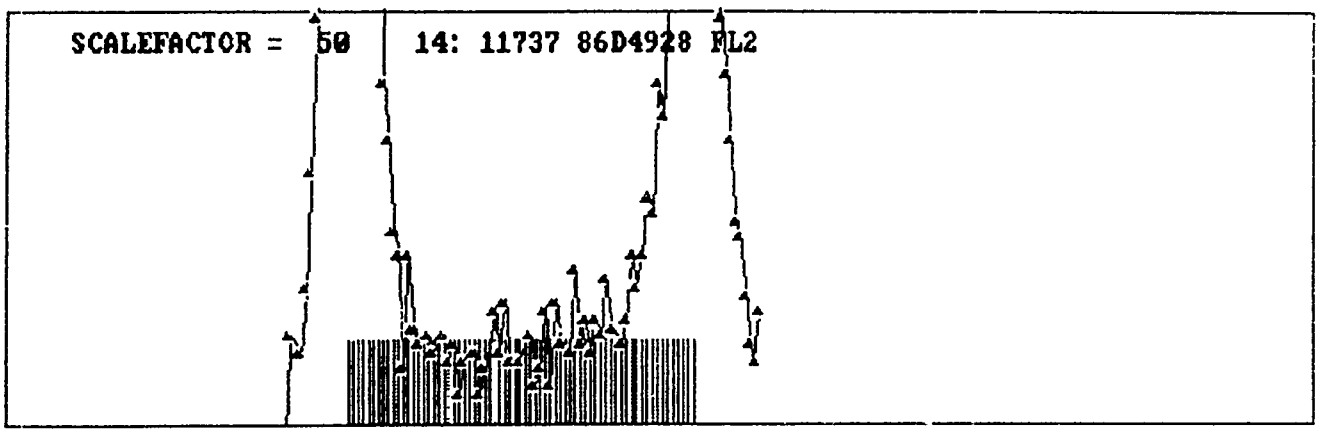
FIGURE 2. Sample analysis of flow cytometric data using the Cytologic program and the method of Baisch.



	G0-G1	G2-M
AREA:	6674	1188
%:	78.04	13.89
MEAN:	66.58	133.87
CV:	5.00	3.30

HISTOGRAM CV
3.30

G2/G1 RATIO
2.01

S-PHASE FRACTION
BETWEEN:
G0/G1 - G2/M : 8.1

FIGURE 3. Anatomic site of the 20 cases under study.

SITE vs. NUMBER OF CASES

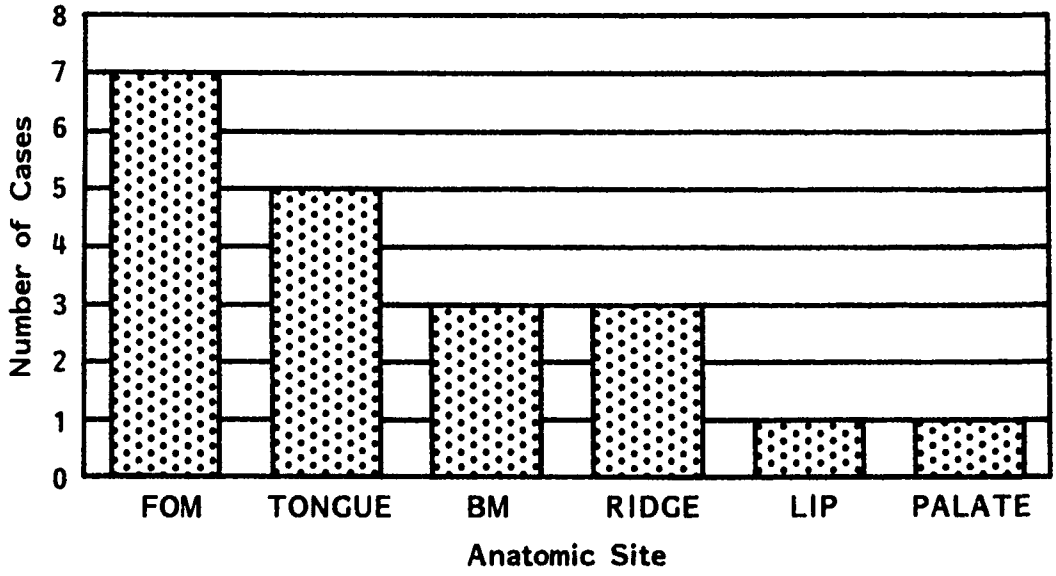


FIGURE 4. Color of the lesions versus their ploidy status.

COLOR OF LESION VERSUS PLOIDY

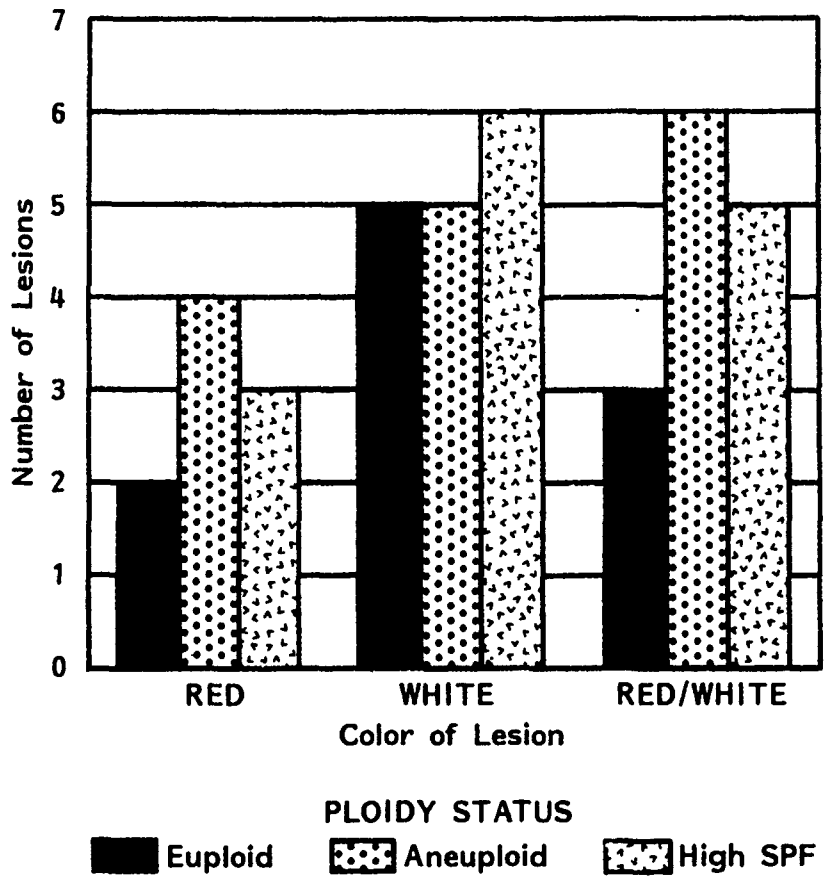
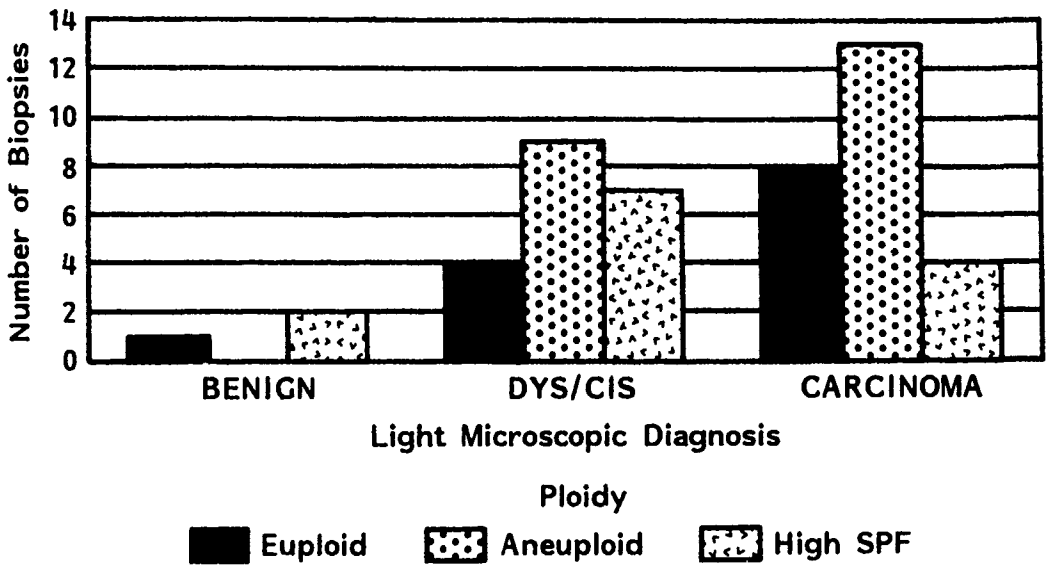


FIGURE 5. Light microscopic diagnosis of the lesions versus ploidy status.

LM DIAGNOSIS vs. PLOIDY



LM = light microscopic;
DYS = dysplasia
CIS = carcinoma-in-situ

FIGURE 6. Ploidy status of the initial premalignant lesion and the subsequent epidermoid carcinoma for each analyzable case.

PROGRESSION OF LESION'S PLOIDY STATUS

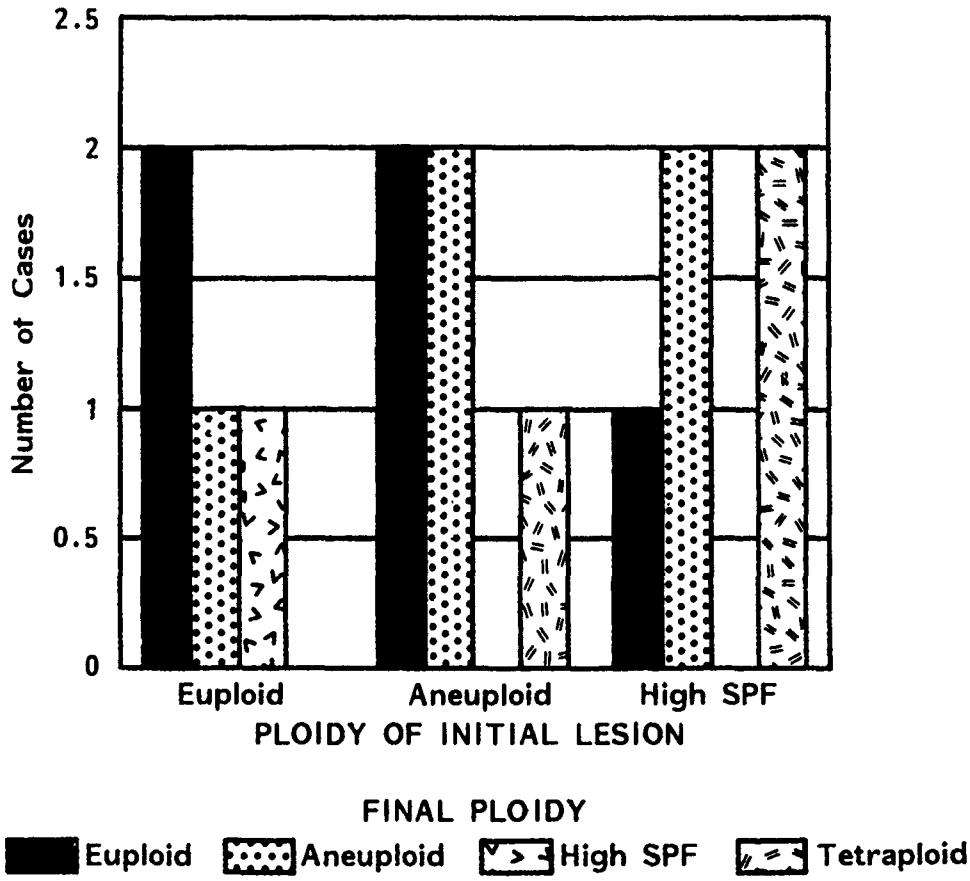


FIGURE 7. Light microscopic appearance of the euploid carcinoma-in-situ from case 2 (hematoxylin and eosin stain, original magnification X100).

FIGURE 8. Light microscopic appearance of the euploid well differentiated epidermoid carcinoma from case 2 (hematoxylin and eosin stain, original magnification X100).

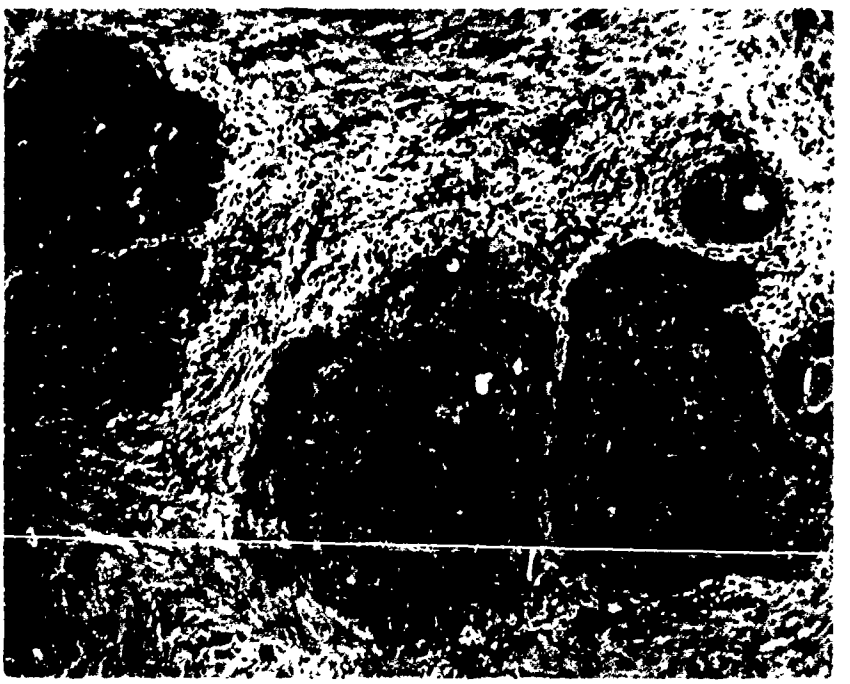


FIGURE 9. Light microscopic appearance of the euploid severe epithelial dysplasia from case 8A (hematoxylin and eosin stain, original magnification X100).

FIGURE 10. Light microscopic appearance of the tetraploid carcinoma-in-situ from case 8A (hematoxylin and eosin stain, original magnification X100).

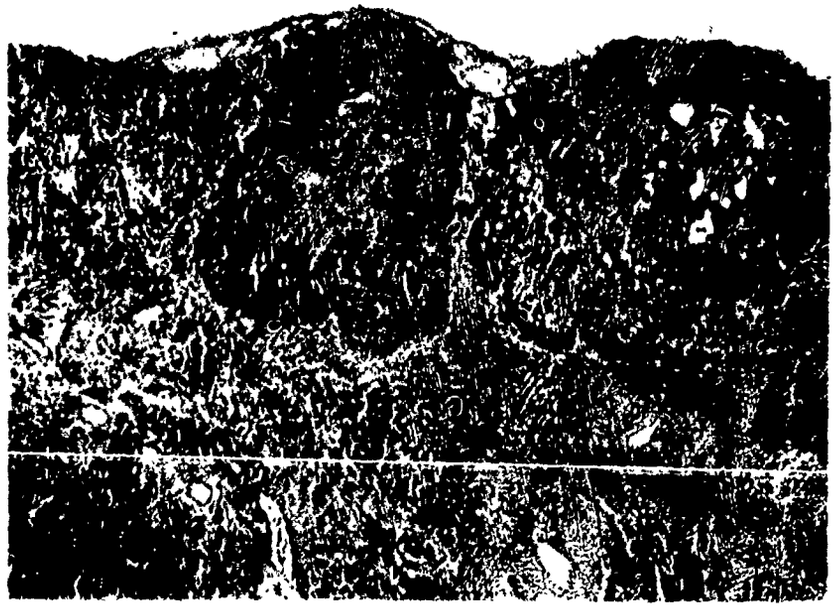


FIGURE 11. Light microscopic appearance of the aneuploid superficially invasive epidermoid carcinoma from case 8A (hematoxylin and eosin stain, original magnification X400).

FIGURE 12. Light microscopic appearance of the aneuploid carcinoma-in-situ from case 8B (hematoxylin and eosin stain, original magnification X100).

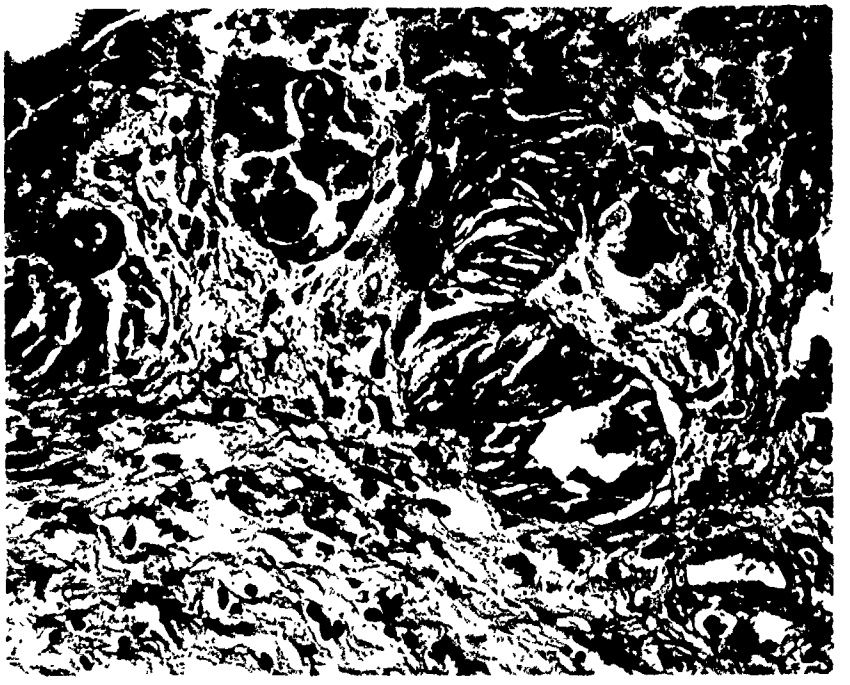
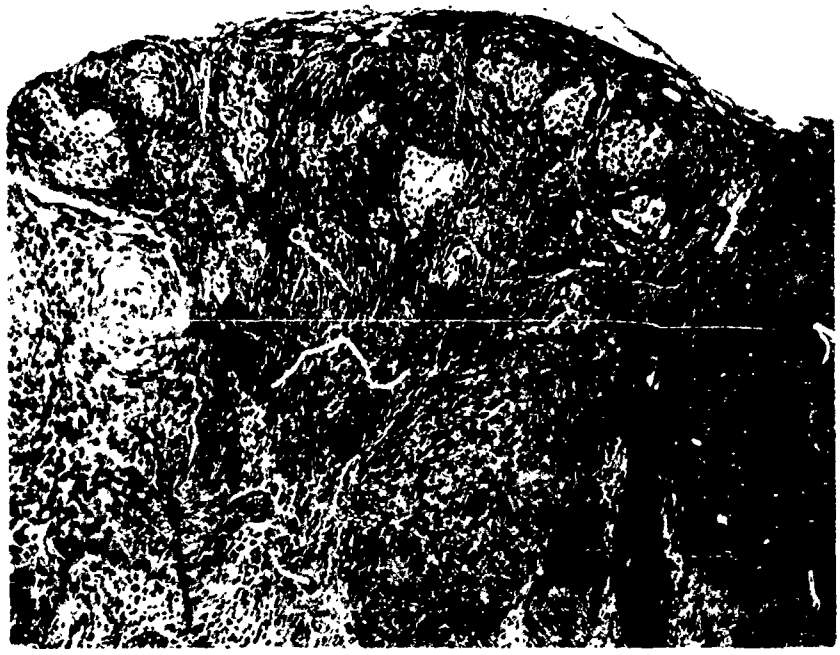


FIGURE 13. Light microscopic appearance of the aneuploid superficially invasive carcinoma from case 8B (hematoxylin and eosin stain, original magnification X100).

FIGURE 14. Light microscopic appearance of the aneuploid verrucous carcinoma from case 15 (hematoxylin and eosin stain, original magnification X40).



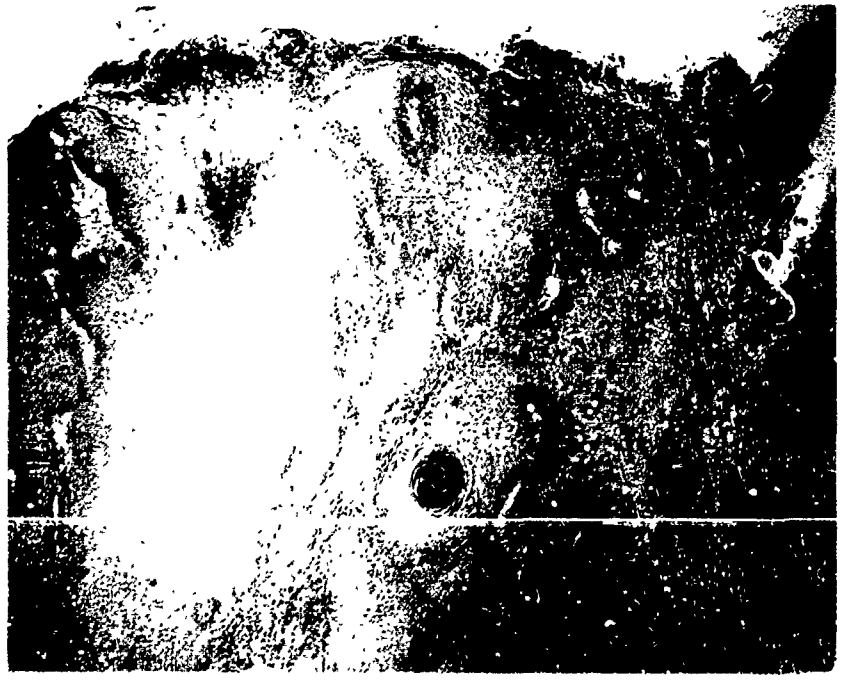


FIGURE 15. Light microscopic appearance of the verrucous carcinoma exhibiting a high S phase ıraction from case 15 (hematoxylin and eosin stain, original magnification X40).

FIGURE 16. Light microscopic appearance of the euploid epidermoid carcinoma from case 15 (hematoxylin and eosin stain, original magnification X100).

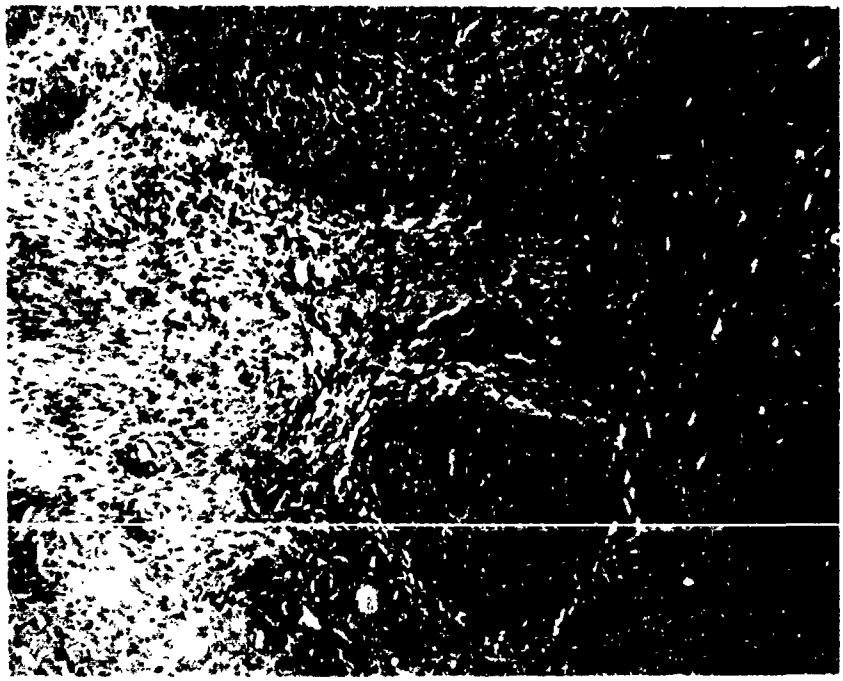


TABLE I

Case information including age at initial diagnosis, sex, and anatomic site

Case	Age	Sex	Anatomic Site
1	55	F	Left lateral surface of tongue
2	68	F	Right posterior floor of mouth
3	54	F	Left ventral surface of tongue
4	94	F	Right floor of mouth
5	64	M	Left anterior floor of mouth
6	77	F	Left posterior buccal mucosa
7	42	M	Right lateral surface of tongue
8A	61	F	Left posterior buccal mucosa
8B	63	F	Left anterior buccal mucosa
9	69	F	Anterior floor of mouth
10	54	F	Right lateral surface of tongue
11	67	M	Left mandibular alveolar mucosa
12	54	F	Left anterior floor of mouth
13	77	F	Left posterior mandibular ridge
14	72	F	Lower lip, right side
15	79	F	Right maxillary tuberosity
16	58	M	Right anterior floor of mouth
17	51	M	Left lateral surface of tongue
18	81	M	Left posterior hard palate
19	67	M	Right posterior floor of mouth

TABLE II

Case information including the date of biopsy, histologic diagnosis, clinical color of the lesion, and the ploidy status

Case	Date	Diagnosis	Color	Ploidy Status
1	5/6/69	Carcinoma-in-situ	NS	QNS
	12/10/81	Epidermoid ca, well differentiated	NS	CV > 10
	7/19/84	Epidermoid ca, mod differentiated	R,W	Euploid
2	7/9/84	Carcinoma-in-situ	W	Euploid
	7/27/84	Epidermoid ca, well differentiated	R	Euploid
3	8/6/84	Mod dysplasia, atrophy, HOK	W	Euploid
	8/16/84	Epidermoid ca, mod-well differentiated	W	-1 High SPF -2 Euploid
4	5/14/84	Mod dysplasia, HOK, ulceration	W	Aneuploid
	5/30/84	Carcinoma-in-situ	W	Euploid
	8/21/84	Epidermoid ca, superficially invasive, lichen planus	W	Euploid
5	12/28/84	Carcinoma-in-situ	R,W	Aneuploid
	1/25/85	Epidermoid ca	W	Aneuploid
6	5/22/83	Carcinoma-in-situ	NS	Excess debris
	8/24/84	Sev lichenoid mucositis, eosinophilia	R,W	Euploid
	3/12/85	Epidermoid ca, well differentiated	R	QNS

(continued)

TABLE II (continued)

Case information including the date of biopsy, histologic diagnosis, clinical color of the lesion, and the ploidy status

Case	Date	Diagnosis	Color	Ploidy Status
7	11/4/85	Mod dysplasia, HOK, ulceration	W	High SPF
	12/9/85	Epidermoid ca, mod differentiated	NS	-1 Euploid -2 Euploid
8A	12/18/84	Sev dysplasia, carcinoma-in-situ	R	Euploid
	9/5/85	Carcinoma-in-situ	R	Tetraploid
	1/10/86	Epidermoid ca, superficially invasive	R,W	Aneuploid
8B	10/28/86	Carcinoma-in-situ	R	-1 Aneuploid -2 Tetraploid
	5/18/88	Epidermoid ca, superficially invasive	R,W	-1 Tetraploid -2 Tetraploid
9	10/22/85	Sev dysplasia	W	High SPF
	3/6/86	Epidermoid ca, superficially invasive	W	-1 Aneuploid -2 Aneuploid
10	2/19/85	Mod dysplasia, HOK	W	QNS
	6/20/86	Epidermoid ca, mod-well differentiated	R	Aneuploid
11	1/20/86	Mild dysplasia	W	CV > 22
	7/29/86	Epidermoid ca, mod differentiated	W	CV > 20
12	8/6/86	Mod dysplasia	W	Euploid
	8/25/86	Epidermoid ca, mod differentiated	R,W	Euploid

(continued)

TABLE II (continued)

Case information including the date of biopsy, histologic diagnosis, clinical color of the lesion, and the ploidy status

Case	Date	Diagnosis	Color	Ploidy Status
13	7/9/87	Mod dysplasia	R,W	High SPF
	7/17/87	Epidermoid ca, mod-well differentiated	R,W	Tetraploid
14	11/5/85	Carcinoma-in-situ	R,W	High SPF
	11/14/85	Sev dysplasia	R,W	Aneuploid
	6/12/87	Sev dysplasia, ulceration	R	QNS
	9/25/87	Papillary epidermoid ca	R	Aneuploid
	10/12/87	Epidermoid ca	R	Aneuploid
15	8/25/86	Verrucous ca	R,W	Aneuploid
	9/4/86	Verrucous ca	R	High SPF
	11/3/86	Epidermoid ca, mod-well differentiated	W	Euploid
16	4/30/86	Mild dysplasia	W	CV > 12
	4/30/86	Mod dysplasia	W	CV > 7
	1/22/88	Mod dysplasia	W	High SPF
	1/22/88	Epidermoid ca, superficially invasive	W	High SPF
17	8/9/84	Lichen planus	W	High SPF
	7/14/86	HPK, lichenoid mucositis	R	High SPF
	2/2/88	Epidermoid ca, mod differentiated	NS	Tetraploid

(continued)

TABLE II (continued)

Case information including the date of biopsy, histologic diagnosis, clinical color of the lesion, and the ploidy status

Case	Date	Diagnosis	Color	Ploidy Status
18	4/18/86	Carcinoma-in-situ	R	CV > 15
	2/17/87	Atypical epith hyperplasia, candida	R,W	Aneuploid
	4/25/88	Epidermoid ca, superficially invasive	R	High SPF
19	12/14/87	Sev dysplasia	W	Aneuploid
	2/25/88	Sev dysplasia	NS	High SPF
	6/16/88	Epidermoid ca, mod differentiated	W	Aneuploid

Abbreviations: QNS = quantity not sufficient
CV = coefficient of variation
SPF = S phase fraction

-1 and -2 indicate separate portions of the same biopsy specimen

TABLE III

Case 1 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 69D-27123	QNS			
2) 81D-87810		>10 - not analyzable		
3) 84D-3854	$G_{0/1}$	5.31		85.40
	G_2/M		2.02	11.46
	SPF			3.00
	$G_2/M + S$			14.46

Biopsies #1 and #2 were unacceptable for analysis. #3 is euploid with a good CV and low S phase.

TABLE IV

Case 2 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 84D-3613	$G_{0/1}$	4.51		96.12
	G_2/M		2.03	2.06
	SPF			2.00
	$G_2/M + S$			4.06
2) 84D-4034	$G_{0/1}$	6.10		88.67
	G_2/M		2.04	4.62
	SPF			7.00
	$G_2/M + S$			11.62

Both specimens are euploid with good CVs. The SPF for #2 rose to the high end of the normal range while the G_2/M fraction remained steady.

TABLE V

Case 3 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 84D-4177	$G_{0/1}$	4.39		61.78
	G_2/M		2.01	10.05
	SPF			28.00
	$G_2/M + S$			38.05
2) 84D-4398-6	$G_{0/1}$	4.75		83.12
	G_2/M		2.06	5.94
	SPF			11.00
	$G_2/M + S$			16.94
3) 84D-4398-7	$G_{0/1}$	6.58		92.69
	G_2/M		2.04	5.70
	SPF			2.00
	$G_2/M + S$			7.70

The sample size for #1 was small which rendered the SPF value unreliable. #2 is diploid but has a high S phase. #3 is diploid with a low S phase but has a suspicious notch in the incline of the diploid peak. #2 and #3 represent different portions of the same lesion.

TABLE VI

.Case 4 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 84D-2596	$G_{0/1}$	5.86		76.03
	G_2/M		2.04	0.83
	Aneu 1		1.19	20.58
	Aneu 2		2.29	1.00
	SPF			3.00
2) 84D-2925-2	$G_{0/1}$	4.21		92.05
	G_2/M		2.01	2.39
	SPF			6.00
	$G_2/M + S$			8.39
3) 84D-4485-4	$G_{0/1}$	4.10		99.66
	G_2/M		2.01	0.45
	SPF			0.00
	$G_2/M + S$			0.45

#1 has an adequate CV and an aneuploid peak in the hyperdiploid range. #2 is diploid with a moderate SPF. #3 is euploid but the scant sample size makes the SPF unreliable.

TABLE VII

Case 5 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 84D-6968	$G_{0/1}$	2.74		24.32
	G_2/M		1.96	0.96
	Aneu 1		1.18	42.38
	Aneu 2		2.29	2.74
	SPF			30.00
2) 85D-0383-3	$G_{0/1}$	3.82		76.81
	G_2/M		1.87	7.59
	Aneu 1		1.10	9.56
	Aneu 2		2.05	4.70
	SPF			3.00

Biopsy #1 was a scant sample which renders the SPF unreliable but still shows a near diploid aneuploid peak which is retained in specimen #2 as a slight shoulder on the incline of the diploid peak.

TABLE VIII

Case 6 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 83D-96451	$G_{0/1}$	5.26		3.28
	G_2/M		2.02	0.12
	Aneu 1		1.46	66.65
	Aneu 2		3.06	3.95
	SPF			26.00
2) 84D-4558	$G_{0/1}$	3.60		92.72
	G_2/M		2.08	0.55
	SPF			6.77
	$G_2/M + S$			7.32
3) 85D-1263	QNS			

The results for #1 are thought to be due to debris in the sample and #3 had an insufficient number of cells for analysis. Specimen #2 is diploid and represents a lesion diagnosed as chronic lichenoid mucositis with eosinophilia and no dysplasia or carcinoma.

TABLE IX

Case 7 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 85D-6194	$G_{0/1}$	3.01		73.87
	G_2/M		2.01	13.15
	SPF			13.00
	$G_2/M + S$			26.15
2) 85D-6972-5	$G_{0/1}$	4.61		84.93
	G_2/M		1.97	7.31
	SPF			7.80
	$G_2/M + S$			15.11
3) 85D-6972-6	$G_{0/1}$	5.60		88.40
	G_2/M		2.01	6.73
	SPF			4.90
	$G_2/M + S$			11.63

#1 has a moderately high SPF which did not persist in specimens #2 or #3 which are euploid.

TABLE X

Case 8A flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 84D-6784	$G_{0/1}$	4.81		87.22
	G_2/M		2.04	5.09
	SPF			7.70
	G_2/M + S			12.79
2) 85D-4881	$G_{0/1}$	4.83		63.00
	G_2/M		1.90	29.28
	SPF			7.70
	G_2/M +S			36.98
3) 86D-0110	$G_{0/1}$	3.44		72.86
	G_2/M		2.07	0.69
	Aneu 1		1.13	16.02
	Aneu 2		2.26	3.52
	SPF			6.90

Specimen #1 has a good CV and an SPF within the normal range and is euploid. In #2, the SPF remains similar but the G_2/M fraction increases suggesting the emergence of a tetraploid subpopulation. #3 does not reflect the presence of the tetraploid component but does show a near diploid aneuploid peak as well as a small hypertetraploid subpopulation.

TABLE XI

Case 8B flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 86D-6239	$G_{0/1}$	2.44		76.01
	G_2/M		2.01	5.63
	Aneu 1		1.09	11.53
	SPF			6.80
2) 86D-6240	$G_{0/1}$	5.54		27.38
	G_2/M		2.03	56.79
	SPF			15.80
	$G_2/M + S$			72.59
3) 88D-3211-1	$G_{0/1}$	4.84		52.20
	G_2/M		2.05	33.45
	SPF			14.30
	$G_2/M + S$			47.75
3211-2	$G_{0/1}$	4.12		56.16
	G_2/M		1.99	30.70
	SPF			13.10
	$G_2/M + S$			43.80

Biopsy #1 has an aneuploid peak similar to #3 of case 8A, while #2, #3, and #4 show persistent tetraploid aneuploid subpopulations. This was confirmed by syringing and visual

(continued)

TABLE XI (continued)

Case 8B flow cytometric results

examination to rule out the presence of doublets. Though anatomically separate sites, cases 8A and 8B show the possibility of multiple malignant cell lines emerging in the same disease process and underscores the differences that may be found in sampling different areas of the same tumor or premalignant lesion.

TABLE XII

Case 9 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 85D-5882	$G_{0/1}$	4.57		84.41
	G_2/M		2.00	5.38
	SPF			10.20
	G_2/M + S			15.58
2) 86D-1356-1	$G_{0/1}$	4.02		20.94
	G_2/M		2.03	3.66
	Aneu 1		1.11	75.39
	SPF			0.00
3) 86D-1356-2	$G_{0/1}$	5.86		29.50
	G_2/M		2.05	1.57
	Aneu 1		1.21	55.40
	SPF			16.70
4) 86D-1356-4		>12 - not analyzable		

Specimen #1 shows a high S phase and #2, although the sample was scant, shows a near diploid aneuploid peak which persists in #3 in which the sample size was adequate. #4 was not analyzable due to a CV greater than 12 and the SPF in #2 is unreliable due to the scant sample size.

TABLE XIII

Case 10 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 85D-0837	QNS			
2) 86D-3707	$G_{0/1}$	5.55		81.22
	G_2/M		2.02	13.09
	Aneu 1		1.59	5.69
	SPF			0.00

Biopsy #1 had an insufficient quantity for analysis and #2 was aneuploid and characterized by a small near triploid peak.

TABLE XIV

Case 11 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 86D-0309		>22 - not analyzable		
2) 86D-4501		>20 - not analyzable		

Both specimens were not suitable for analysis due to high CVs which were thought to be due to debris in the sample.

TABLE XV

Case 12 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 86D-4583	$G_{0/1}$	7.00		86.49
	G_2/M		2.02	6.31
	SPF			7.20
	$G_2/M + S$			13.51
2) 86D-4928	$G_{0/1}$	3.37		78.04
	G_2/M		2.01	3.37
	SPF			8.10
	$G_2/M + S$			11.47

Both specimens, #1 and #2, are euploid and have S phase fractions in the high range of normal.

TABLE XVI

Case 13 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 87D-4070	$G_{0/1}$	3.72		79.22
	G_2/M		2.01	8.92
	SPF			11.90
	$G_2/M + S$			20.82
2) 87D-4247	$G_{0/1}$	5.66		70.45
	G_2/M		2.00	19.96
	Aneu 1		1.53	0.40
	SPF			9.20

Specimen #1 has a diploid peak with a high S phase fraction suspicious of an emerging malignant cell line. Biopsy #2 contains an aneuploid peak which is too small to be considered a true subpopulation. However, the G_2/M population exceeds 15 percent and represents a tetraploid neoplastic subpopulation.

TABLE XVII

Case 14 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 85D-6207	$G_{0/1}$	3.54		72.43
	G_2/M		1.93	12.74
	SPF			14.80
	$G_2/M + S$			27.54
2) 85D-6405-2	$G_{0/1}$	3.96		47.91
	G_2/M		1.96	16.25
	Aneu 1		1.11	18.90
	SPF			16.90
3) 87D-3516	QNS			
4) 87D-5708	$G_{0/1}$	5.47		48.75
	G_2/M		2.01	6.56
	Aneu 1		1.09	36.58
	SPF			8.10
5) 87D-6125	$G_{0/1}$	3.64		60.70
	G_2/M		1.98	3.39
	Aneu 1		1.09	26.21
	Aneu 2		2.09	2.84
	SPF			6.90

Progression is seen from specimen #1 with a high SPF through biopsies with increasingly prominent near diploid aneuploid peaks to a lesion with two aneuploid peaks, one of which is near diploid and the other hypertetraploid.

TABLE XVIII

Case 15 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 86D-4915	$G_{0/1}$	4.60		62.69
	G_2/M		2.06	2.63
	Aneu 1		1.12	31.81
	SPF			3.00
2) 86D-5137-4	$G_{0/1}$	4.82		84.85
	G_2/M		2.05	3.34
	SPF			11.80
	G_2/M + S			15.14
3) 87D-6369	$G_{0/1}$	4.85		83.31
	G_2/M		2.00	7.77
	SPF			8.90
	G_2/M + S			16.67

#1 shows a near diploid aneuploid peak while #2 has a diploid peak and a high S phases which indicates the presence of neoplastic activity. #3 is a euploid neoplasm.

TABLE XIX

Case 16 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 86D-2581		>12 - not analyzable		
2) 86D-2586		>7 - not analyzable		
3) 88D-0441	$G_{0/1}$	4.04		78.51
	G_2/M		2.01	10.54
	SPF			10.90
	$G_2/M + S$			21.44
4) 88D-0442	$G_{0/1}$	6.11		76.52
	G_2/M		2.00	10.80
	SPF			12.70
	$G_2/M + S$			23.50

The CV is too high in #1 and #2 for analysis. #3 and #4 show diploid peaks with high G_2/M and S fractions indicating a high proliferation rate.

TABLE XX

Case 17 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 84D-4265	$G_{0/1}$	4.13		83.07
	G_2/M		2.00	6.50
	SPF			10.30
	$G_2/M + S$			16.80
2) 86D-4132	$G_{0/1}$	4.60		78.39
	G_2/M		2.01	10.34
	SPF			11.30
	$G_2/M + S$			21.64
3) 88D-0764	$G_{0/1}$	3.35		64.85
	G_2/M		1.93	21.22
	SPF			13.90
	$G_2/M + S$			35.12

Specimen #1 has a minimally elevated S phase fraction which increased progressively through #2 and #3 until #3 additionally shows a tetraploid neoplastic subpopulation.

TABLE XXI

Case 18 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 86D-2323-4		>15 - not analyzable		
2) 86D-2323-5		>9 - not analyzable		
3) 87D-0792-2	$G_{0/1}$	4.02		46.85
	G_2/M		1.99	0.18
	Aneu 1		1.31	27.03
	SPF			25.90
4) 88D-2534	$G_0/G1$	3.54		72.70
	G_2/M		2.03	0.09
	SPF			27.20
	G_2/M + S			27.29

Biopsies #1 and #2 are not suitable for analysis. #3 and #4 have triploid peaks and #5 has a diploid peak with a high SPF.

TABLE XXII

Case 19 flow cytometric results

Accession #	Phase Fraction	CV	DI	% of Curve
1) 87D-7590-2	$G_{0/1}$	3.34		64.23
	G_2/M		1.97	12.62
	Aneu 1		1.75	12.07
	SPF			11.10
2) 88D-1405	$G_{0/1}$	4.57		82.94
	G_2/M		2.03	5.57
	SPF			11.50
	$G_2/M + S$			17.07
3) 88D-3920	$G_{0/1}$	3.77		69.92
	G_2/M		1.93	0.52
	Aneu 1		1.33	10.55
	SPF			19.00

Specimen #1 shows a near tetraploid aneuploid peak while #2 is euploid but with a slightly elevated S phase fraction. #3 contains a near triploid aneuploid subpopulation and a high SPF.

DISCUSSION

This study incorporated methodologies which took into account the criteria enumerated by Thornthwaite et al.[65] for meaningful DNA flow cytometric analysis. These criteria included proper nuclear isolation procedures, judicious selection of a DNA specific stain, adequate sampling, the use of a stable DNA standard, and sharp resolution of measurements. Due to the retrospective nature of the study and the use of archival material, the only criterion over which there was no control was sample size.

Adequate sampling is difficult to ensure given the often small size of oral biopsy specimens and the need to first use what tissue may be available to procure sections for light microscopic examination. Of the 58 blocks retrieved for examination, four contained an insufficient quantity of tissue from which too few cells were retrieved for flow cytometric analysis. One contained excessive debris, and six rendered CVs so large that the data were not interpretable. Where possible, multiple tissue samples were processed as suggested by Sasaki et al.[75] in order to obtain accurate ploidy determinations. In five cases, where they were available, multiple samplings from a single

lesion were processed. In case 3, one sample contained a diploid cell population and a high S phase fraction while the other sample was euploid. Within each of the other four cases (7, 8B, 9, and 16), the respective samples rendered very similar ploidy analyses.

The opportunity for multiple sampling from anatomically separate areas of a single, but widespread, disease process presented in cases 8A and 8B. These cases were from the posterior and anterior buccal mucosa respectively of the same patient. These two sites produced lesions which varied from euploid to aneuploid to a neoplasm with a tetraploid subpopulation. This emphasizes the need to analyze as many samples as possible in studies designed to investigate the clinical usefulness of tissue ploidy status.

Stability of DNA in formalin-fixed paraffin-embedded tissue and the suitability of archival tissue for retrospective study is now firmly established.[68] Additionally, the use of paraffin-embedded tissue allows for light microscopic viewing of hematoxylin and eosin stained sections as a means for selection of specific portions of a lesion for analysis. This is an advantage in helping to ensure appropriate qualitative and quantitative sampling since cell masses of interest can actually be dissected macroscopically from the tissue block.

In their discussion of fluorescent materials for use in flow cytometry, Taylor and Milthrope[66] ranked DNA-specific stains such as 4,6-diaminidino-2-phenylindole (DAPI) higher than DNA intercalating dyes such as propidium iodide (PI), which was used in this study. Their ranking was based on CV values and relative fluorescence. Also, DNA-specific stains are not affected by the presence of RNA. However, the relatively low power argon laser of the instrument employed required the use of an intercalating dye and, therefore, RNAase treatment of the sample was mandatory. Of the intercalating dyes, PI was selected due to its being suited to the argon laser excitation system of the Coulter Epics Profile Analyzer. A technique which results in significantly higher fluorescence intensities in all preparation techniques,[66] and was used in this investigation, is the treatment of the sample with detergent. This is thought to be possibly due to removal of histones resulting in a greater availability of dye binding sites.

In order to apply a completely objective parameter of excised tissue to the diagnostic, prognostic and patient management aspects of a disease process, it must first be determined that such an objective parameter exists, that it is reproducible, scientifically accurate and clinically applicable. This study measured the ploidy status of oral premalignant and malignant lesions by flow cytometric

analysis as a means to assess its potential as an objective diagnostic and/or prognostic parameter. Acceptance of any new diagnostic or prognostic modality requires substantial long-term supporting cases as evidence that it fulfills the above criteria. Flow cytometric analysis of oral lesions gives reproducible machine generated results which are seemingly easy to interpret and readily lend themselves to computer program manipulation. This apparent objectivity is contradicted when the human aspect of interpretation of histograms is introduced. Difficulties similar to those encountered by Koss et al.[78] were experienced in the interpretation of S phases in situations where peaks were closely juxtaposed. Subjectivity is also an unavoidable component of other interpretative parameters such as determining how large an aneuploid cell count must be before it is accepted as a subpopulation and what determines adequate lesion sampling. The answers to these and other questions differ among pathologists and are, in fact, just as subjective as the interpretation of the significance of various light microscopic features of dysplasia and malignancy. It is likely that, even after the long time which will be necessary to establish standards for use of ploidy status in diagnosis and prognosis, it will take an equally long time to educate both pathologists and clinicians in the indications, contraindications, advantages, and

disadvantages of this potential indicator of the biologic behavior of oral premalignant and malignant lesions.

Research has shown that there are practical applications of tumor ploidy status with clinical significance to many nonoral tumors. Herman et al.[67] state that these applications include the diagnosis of malignancy and the determination of the prognosis for patients with clearly malignant tumors. Aneuploid subpopulations have been found in premalignant lesions of the uterine cervix, skin, larynx, esophagus, stomach, and colon, but the prognostic significance of these changes is unknown.[78] Application to oral premalignant and malignant conditions and lesions must await the lengthy but necessary process of thorough investigation.

According to Baisch and colleagues,[81] the determination of the fraction of cells in the $G_{1/0}$, G_2/M and S phases of the cell cycle could have treatment implications since radiation therapy and chemotherapy vary in their influence on cells in different phases. Therefore, cell proliferation parameters may influence the selection of a phase-specific therapeutic agent or regimen.

Clinical presentation of the lesions included in this study shared findings similar to those of past investigations. The floor of the mouth as a high risk location for malignant transformation of premalignant lesions, as reported by Mashberg,[6] Shafer and

Waldron,[12] Kramer and associates,[24] and Katz and colleagues[24] is consistent with the findings in this study in which 35 percent of the oral premalignant lesions which progressed to epidermoid carcinoma were from this site (Figure 3). A total of 19 biopsy specimens were examined from these seven cases and 16 gave interpretable results following flow cytometric analysis. Six (37.5 percent) were euploid, an equal number were aneuploid, and four (25 percent) had an S phase fraction exceeding 10 percent. The next most frequent site was the tongue, and of the 11 biopsies adequate for analysis, five were euploid, two were aneuploid, and four had high S phase fractions. Considering these two anatomic sites together, which accounted for 60 percent of the cases and 58.7 percent of the analyzable specimens, 40.7 percent of the biopsies were euploid and 59.3 percent were noneuploid (Figure 4).

In only one instance, the second biopsy of case 18, which was diagnosed as atypical epithelial hyperplasia and was an aneuploid lesion, were pseudohyphae of Candida albicans present. This finding sheds no further light on the possibility of a causal relationship existing between premalignancy and the presence of fungal organisms.

Lichen planus was diagnosed as coexisting with the euploid superficially invasive carcinoma in case 4. A euploid lichenoid mucositis was combined with eosinophilia

as the diagnosis of the intermediate lesion in case 6 and occurred a year after the diagnosis of carcinoma-in-situ and a year prior to the diagnosis of well differentiated carcinoma. Lichen planus was the initial diagnosis in case 17 and lichenoid mucositis with hyperparakeratosis was the diagnosis for the second biopsy of this case. Both of these lesions contained high S phase fractions. Nineteen months later, an aneuploid moderately differentiated epidermoid carcinoma arose in the same site. These occurrences do not challenge the reports of the WHO which found lichen planus to be unassociated with malignant transformation or unlikely to be premalignant.[1]

In some cases, particularly those with the shorter time periods between initial biopsy and diagnosis of carcinoma, the premalignant and malignant lesions may have actually been coexistent and the appearance of malignant transformation merely a function of lesion sampling during the biopsy procedure, or of the biopsy procedure itself, i.e., excision versus incision. However, the possibility also exists that malignant transformation did occur. Therefore, judgmental influences as to the presence of coexistent lesions as opposed to malignant transformation did not result in the exclusion of any cases from study. Nor did any case receive special consideration or handling based on these aspects of clinical behavior and patient management.

Two-thirds of the clinically white lesions were noneuploid and these were divided nearly evenly between aneuploid and those with a high S phase fraction. In contrast, 77.7 percent of the red lesions and 78.6 percent of the red and white lesions were noneuploid. Noneuploid cell populations were seen 3.6 times more frequently in lesions with a red component than was euploidy (Figure 4). Thus, a red component seems to confer a greater likelihood that the lesion will be noneuploid.

Epithelial dysplasia and carcinoma-in-situ are four times more likely to be noneuploid than euploid and the ploidy status of epidermoid carcinoma is 2.13 times more likely to be aneuploid (Figure 5).

These findings, however, do not allow diagnostic prediction based on the ploidy status, since any individual premalignant or malignant lesion may be composed of euploid cell populations. This corroborates Doyle and Manhold's[39] findings in which half of their carcinomas showed diploid stem lines. Figures 6 through 15 illustrate the lack of a trend or pattern for the ploidy status to change from euploidy to aneuploidy as the degree of dysplasia worsens or as the carcinoma becomes less differentiated. Figure 6 depicts the lack of a trend or progression of ploidy from the premalignant lesion to the subsequent malignant lesion. Thus, agreement was not reached with Saku and Sato,[38] Giminez and Conti[41] or

Pfitzer and Pape[45] who found a "dysplastic pattern" in the premalignant lesions they studied.

The preliminary results along this course of investigation, as reported in this study, do not suggest the appropriateness of ploidy status as a predictive tool in the transformation of oral premalignancy to malignancy, although it would be premature to discount this possibility.

In support of the probability that ploidy status may be more promising as a prognostic indicator for malignant lesions rather than a predictor of malignant transformation are the reversals which occurred in the disease processes under study. Transition from euploid lesions to aneuploid lesions and vice versa are in keeping with the report by Miller and Miller[63] that repair mechanisms may result in reversion to normal in cells which have acquired the malignant phenotype. Underscoring the potential for ploidy status to be of important prognostic significance is the presence of euploid tumors with normal S phase fractions in seven of the cases included in this study. This may be due to the occurrence of events other than alteration in the DNA content of the cells, as summarized by Nowell,[48] which may lead to the acquisition of a malignant phenotype. The reasons for differences in biologic behavior of euploid and aneuploid malignancies is obscure. Therefore, while the finding of an aneuploid subpopulation

may strongly suggest malignancy, the presence of euploidy does not rule it out.

Some aspects of patient management persist which will thwart any effort to establish a predictive parameter which is 100 percent subjective or reliable. The oral and paraoral regions are particularly susceptible to events which alter the biologic behavior of premalignant conditions and lesions as well as malignancies. These include patient habits and lifestyles which may have been contributory to the disease process and which, after initiation of diagnostic and/or treatment procedures, may be altered through reduction in frequency, quantity, both frequency and quantity, or by cessation.

Perhaps the single greatest factor altering the natural progression of a lesion is conventional surgical biopsy intervention. These interventions may or may not have an effect on the biologic behavior of the lesion, but most certainly have an effect on the evaluation of the ploidy progression of cell subpopulations within the disease process and the subsequent application of ploidy findings to diagnosis, prognosis and patient management. It cannot be determined whether lesions initially biopsied and found to be euploid or aneuploid and subsequently biopsied and found to contain cell populations with the same or different DNA content would have behaved differently had the surgical procedure not been performed.

This creates a situation where ethics and the professionally compelling provision of appropriate and prudent care precludes the observation of undisturbed premalignant lesions and, thus, documentation of the natural progress of individual lesions.

Subsequent studies of this nature would benefit from the ability to have available more information concerning eventual patient management and outcome including treatment modalities (surgical, radiation, etc.), recurrences, clinical stage, or death from disease. This would be particularly helpful in studying the prognostic validity of the ploidy status of oral carcinoma. For clinically relevant studies in the diagnostic and predictive aspects of oral leukoplakias and erythroplakias, similar information must be available to be able to identify a group of patients which had an oral lesion fitting the clinical criteria and/or histologic criteria for premalignancy but which, by history, did not develop a carcinoma or which regressed. Thus, a valid long-term study should have both retrospective and prospective components.

SUMMARY AND CONCLUSIONS

All methods of light microscopic diagnosis are inherently flawed by the unavoidable incorporation of observer subjectivity. Additionally, the clinical assessment of whether a lesion is premalignant or malignant bears a large degree of subjectivity.

When considering thousands of oral leukoplakic lesions evaluated by many experienced clinicians and histopathologists, it can be predicted that 6 to 14 percent will progress to a malignant lesion. However, when considering each individual lesion on its own merits, the predictive accuracy greatly diminishes or disappears.

An expeditious and affordable method for measurement of an objective diagnostic or prognostic parameter may instill a level of confidence in predicting the biologic behavior of oral premalignant lesions and/or may prove to be a meaningful prognostic indicator with clinical relevance to patient management.

To my knowledge, flow cytometric determination of the DNA content of oral premalignant and malignant lesions had not been previously reported. Other means of establishing an objective diagnostic parameter of oral lesions have been attempted. These include photographic standardization of the classic light microscopic features of dysplasia,

computer analysis of the presence or absence of light microscopic features, stereoscopic analysis of dysplastic features including nuclear and cellular size and ratio, and static cytofluorometry. Some authors report the ability to diagnose dysplasia with 85 percent accuracy while others disclaim a diagnostic advantage and feel the major benefit will lie in the prognostic potential of identified objectively determined cell parameters, specifically tumor ploidy.

Many nonoral solid tumors as well as hematopoietic and lymphoproliferative disorders benefit from the determination of the ploidy status of the cell populations. This parameter becomes a useful adjunct to diagnosis, prognosis and patient management. Therapeutic treatment modalities such as radiation therapy and chemotherapy are frequently cell phase-specific and the determination of the size of cell subpopulations in the various cell cycle phases may impact the selection of treatment regimens.

Acquisition of the malignant phenotype by a cell population may be accompanied by a change in the amount of nuclear DNA. However, malignant transformation may occur by means which do not involve an alteration in nuclear DNA. Therefore, while aneuploidy suggests the presence of malignancy, the absence of aneuploidy does not rule it out.

Nineteen cases were selected from the tissue archives of the Indiana University School of Dentistry Department of Oral Pathology which fulfilled the criteria of diagnosed premalignant lesions which underwent documented malignant transformation into epidermoid carcinoma. The paraffin-embedded tissue blocks were retrieved and processed to yield a monocellular suspension. This was treated with RNAase and stained with propidium iodide, a DNA intercalating dye. The cell suspension was subjected to flow cytometric analysis using an Epics Profile analyzer and the data were subjected to computer analysis using the proprietary software package, Cytologic. Both the flow cytometer and the software are from the Coulter Company, Hialeah, Florida.

The initial biopsies of the 19 cases studied were determined to have ploidy states which were variously euploid, aneuploid and diploid with high S phase fractions. Likewise, the intermediate lesions and the carcinomas resulting from malignant transformation of the premalignant lesions were found to contain cell populations with DNA contents seemingly unrelated to that of the precedent lesions. No pattern was revealed as the lesions acquired the malignant phenotype and there was no correlation between the severity of dysplasia or the degree of differentiation of epidermoid carcinoma and the ploidy status.

Lesions with a red component, i.e., erythroplakia or erythroleukoplakia, were more likely to be aneuploid or to contain an S phase fraction in excess of 10 percent. This is thought to indicate neoplastic growth and the emergence of a malignant stem cell line.

In order to confirm or reject DNA ploidy as a diagnostic tool or as a prognostic indicator, extensive retrospective and prospective studies will be required. These studies will require close patient follow-up with detailed clinical history, treatment and patient outcome in order to provide data that are clinically relevant, scientifically accurate, reproducible, easily taught and readily learned.

Flow cytometric analysis of formalin-fixed and paraffin-embedded tissue is considered to be technically practical, using one of several tissue preparation and staining protocols in use today. Its feasibility is thought to be worthy of continued use in search for a potentially objective parameter in the cells which comprise premalignant and malignant oral lesions.

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CURRICULUM VITAE

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ABSTRACT

FLOW CYTOMETRIC PLOIDY DETERMINATION OF ORAL PREMALIGNANT AND MALIGNANT LESIONS

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Nuclear DNA content was evaluated for use as an objective parameter of diagnostic value in oral premalignancy and malignancy. Fifty-three blocks of formalin-fixed and paraffin-embedded archival tissue were selected from 20 cases which had been diagnosed as premalignant epithelial lesions and subsequently diagnosed as having progressed to malignancy. A single cell suspension was prepared from each tissue block, stained with propidium iodide and subjected to flow cytometric analysis. This yielded histograms which depicted the ploidy status for each specimen. For five specimens, the tissue quantity was insufficient and for an additional six specimens, the coefficient of variation for the histogram exceeded the established limit of seven. The ploidy status

was determined for all specimens in 13 of the 20 cases. The initial premalignant lesions in four cases were euploid and of these, three of the subsequent malignant lesions were euploid while one was aneuploid. Five cases had initial lesions which showed aneuploidy, two of which emerged as euploid in the subsequent carcinoma, while two showed aneuploid malignancies and one acquired a tetraploid malignant phenotype. The initial premalignant lesions of the remaining four cases were characterized by subpopulations of cells in the S phase of the cell cycle which exceeded 10 percent of the total number of cells and thus were considered neoplastic. Of these, the subsequent malignancy was euploid in one case, aneuploid in one case and tetraploid in two cases.

This study of a limited number of cases affirms that, given an adequate tissue sample size, flow cytometric analysis of nuclear DNA content is a reproducible objective parameter of oral lesions which is applicable to formalin-fixed, paraffin-embedded tissue. The diagnostic value and the use of this parameter in predicting the biologic behavior of oral premalignant and malignant lesions must await further studies which are both retrospective and prospective in nature.

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