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## Product Identification of Normal Oral Mucosa and Oral Squamous Cell Carcinoma by Immunohistochemical Techniques

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PRODUCT IDENTIFICATION OF NORMAL ORAL MUCOSA  
AND ORAL SQUAMOUS CELL CARCINOMA  
BY IMMUNOHISTOCHEMICAL TECHNIQUES

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by

YING-TAI JIN

A Thesis Submitted to the Faculty of the Graduate  
School of Loyola University of Chicago in Partial  
Fulfillment of the Requirements for the Degree of  
MASTER OF SCIENCE

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1984

## DEDICATION

To my parents and my wife,  
whose loving devotion, encouragment, and sacrifices  
made possible the completion of this phase of my education.

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## VITA

The author, Ying-Tai Jin, B.D.S., is the son of Kuo-Ping Jin and Fen-Ju (Chang) Jin. He was born in Taipei, Taiwan on November 9, 1953.

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## CHAPTER I

### INTRODUCTION

The detection and diagnosis of cancer is dependent upon the ability to discriminate between normal and neoplastic tissues. At present, this determination relies upon the recognition of abnormal morphologic features associated with neoplasia. The utilization of specific biochemical products differences between normal and neoplastic tissues in the development of highly sensitive assays might yield definitive methods for early detection of tumors. Furthermore, specific biochemical products differences may be exploited for preventive and/or curative measures.

Considerable evidence indicates that changes in cell products occur during transformation of a normal cell into malignant cell and that these changes determine some of the important properties of tumor cells. These properties include loss of contact inhibition, decrease in cell adhesion, increased growth, prolonged survival, expression of new antigens, and escape from immune destruction by the host. Cell surface carbohydrates, glycoproteins and intracytoplasmic proteins are believed to be involved in determining many of these properties.

The purpose of this study is to identify products in oral squamous cell carcinoma and normal oral mucosa by using specific antibodies to identify glycoproteins and using high affinity lectins to identify cell

surface carbohydrates. It is expected that there is difference of products between normal tissue and malignant transformation. Furthermore, such product definition may significantly improve the utility of the current histomorphologic standards employed for criteria in establishing the diagnosis of oral neoplasm.

## CHAPTER II

### REVIEW OF THE LITERATURE

#### KERATIN

Matoltsy (1969,1975) studied the cytoskeletal components of epidermis by using electron microscopic and biochemical methods. He reported that the main component of tonofilaments, keratin, is a low-sulfur  $\alpha$ - protein composed of a pair of three-chain subunits with non- $\alpha$ -helical segments. The studies by electron microscopy showed that 60- to 80 A keratin filaments assemble into bundles which run in all directions throughout the cytoplasm of epidermal cells. Presumably keratin forms a network within living epidermal cells which serves as a stabilizing cytoskeletal system.

Sun and Green (1978) first used immunofluorescent staining to identify keratin in sectioned tissue and cultured cells. They used antibody prepared against a group a keratins purified from human stratum corneum. In sectioned tissue and in culture, keratinocytes of skin and other stratified squamous epithelium stained strongly. In all cases, the antibody revealed a dense cytoplasmic network of discrete fibers probably consisting of aggregated tonofilaments. No keratins were detected in cultured cells of mesenchymal origin or in connective tissues.

Oberle et al. (1979) investigated the anti-keratin antibody reac-

tivity in human oral epithelium of normal and pathological conditions. They found that reactivity to antiserum was seen throughout the epithelium in normal oral mucosa and in lichen planus. However, reactivity of epithelial cells was variable in erythema multiforme, pemphigoid and pemphigus.

Löning et al. (1980) used the immunoperoxidase method to studied the distribution of total keratin (TK) and three different cytokeratin polypeptides of molecular weight 67,000 dalton (67K), 62,000 dalton (62K), and 55,000 dalton (55K), in 82 cases of normal epidermis and oral mucosa, and in various inflammatory, premalignant and malignant lesions. This study showed that the antisera against TK and 55K labeled all keratinocytes in normal and pathological conditions. The 62K and 67K keratin were identified only in the spinous, granular and keratinized layer of normal epidermis and oral mucosa. No labelling of basal layer was achieved with these antisera.

Viac et al (1980) studied the keratin distribution in human epidermis by using anti-keratin sera which was prepared by immunizing guinea pigs with the polypeptide (molecular weight: 67,000 dalton) of keratin of normal human stratum corneum. The keratin filaments were shown to react in immunofluorescence only to cell cytoplasmic antigen of the upper layers of the epidermis. They thought these findings confirmed two steps (basal and malpighian layers) in epidermal differentiation defined by antigenic markers and, especially, by the keratin component of molecular weight 67,000 dalton present in normal stratum corneum.

Schlegel et al. (1980a) used immunoperoxidase technique to localize the intracellular keratin in normal human tissue. They reported that keratin is not only a major cytoplasmic element in squamous cells but is found also in the epithelium of human intestinal, urinary, female genital, and respiratory tracts as well as bile duct and ducts of salivary glands, pancreas, breast, and prostate. These same studies indicate that keratin is not present in melanocytic cells, neuroendocrine cells, mesenchymal cells or cells of the lymphoreticular system.

Schlegel et al. (1980b) identified the keratin in human neoplasms by immunoperoxidase method. They reported the localization of keratin in squamous cell carcinomas, transitional cell tumors, and mesotheliomas. No keratin can be detected in undifferentiated lung carcinomas, lung adenocarcinomas, sarcomas, lymphomas, and neural tumors. According to the results, they thought that the analysis of keratin by immunoperoxidase technique appears helpful in establishing the epithelial nature of primary or metastatic poorly differentiated neoplasms.

Sieinski et al. (1981) supported the studies of Schlegel (1980b) by using immunofluorescence staining to identify keratin in various tumors. The keratin was identified in all epithelial cells of other histologic types. Cells of lymphoid, melanotic, neural, and connective tissue origin were not stained.

Caselitz et al. (1981) used immunofluorescence staining to show that squamous cell carcinoma in the parotid gland was positive for keratin. The intensity of the staining, however, was different in individual cells: some were stained strongly, others only moderately. The



surrounding connective tissue was negative for keratin.

Viac et al (1982) used both biochemical and immunohistochemical methods to compare the keratin in human basal cell carcinoma, squamous cell carcinoma and in normal epithelium. They found both types of tumor showed either an absence or a very low amount of the major protein band (molecular weight 67,000) present in normal human epidermis. This correlated well with results of immunolabelling showing that the 67,000 keratin antisera only reacted with some dyskeratotic cells in sections of these tumors.

Recently studies used immunohistochemical method to identify keratin proteins in the differential diagnosis of tumors all showed keratin positive in epithelial tissues. However, neoplasms of mesenchymal, lymphoreticular, or neural crest origin did not contain intracellular keratin (Altmannsberger et al., 1982; Madri and Barwick, 1982; Bejui-Thivolet et al., 1982; Kahn et al., 1983; Nagle et al., 1983; Said et al., 1983).

#### CARCINOEMBRYONIC ANTIGEN (CEA)

Carcinoembryonic antigen (CEA) as originally described by Gold and Freedman (1965, 1968) was defined as an endodermally derived tumor-associated antigen that was also present in embryonic and fetal gut, pancreas and liver but not in normal adult gastrointestinal tissues. However, Reynoso et al. (1972) found this antigenic material also had been demonstrated in the blood of a large number of patients with other than gastrointestinal neoplasms.

Primus and Wang (1975) used the immunoperoxidase method for

detecting and localizing CEA in tissue sections. By the technique they used, a cell-surface localization of CEA in colonic carcinoma and ovarian mucinous cystadenocarcinoma cells could be visualized. In the case of the colonic carcinoma, both the tumor from the descending colon and metastasis to the skin gave positive peroxidase reactions for CEA.

Goldenberg (1976) used the peroxidase-antiperoxidase (PAP) method for demonstration of CEA in formalin-fixed, paraffin-embedded specimens. They found CEA can be detected in carcinomas of stomach, colon, rectum, pancreas, lung, and cervix. Furthermore, the formalin-fixed, paraffin embedded sections as old as 10 years still had demonstrable CEA.

Pascal et al. (1977) also used the PAP method to examine pulmonary lesions for CEA. They found that CEA was present in the majority of pulmonary adenocarcinomas and generally absent in the squamous cancers. The major exception was in the well-differentiated squamous lesions where CEA was occasionally found in the keratinizing areas.

Goldenberg et al. (1978) used the peroxidase-antiperoxidase (PAP) method for staining CEA in conventional histopathologic sections to evaluate the presence of CEA in 950 different specimens; the vast majority of which were available as paraffin-embedded tissues fixed in formalin. The sensitivity of this method clearly discriminated between normal and nonmalignant, diseased tissues and neoplasms having increased quantities of CEA.

Lindgren and Seppala (1979) studied the tissue CEA in premalignant epithelial lesions and squamous cell carcinoma of the uterine cervix. The frequency of tissue CEA positivity was found to increase with

advancing clinical disease in the following manner: mild dysplasia, 25%; severe dysplasia, 37%; carcinoma in situ, 60%; and invasive carcinoma, 60%-80%. The increasing occurrence of CEA from premalignant lesions to advancing malignant growth suggests that CEA reflects an aggressive potential in premalignant lesions.

Toto (1979), reported the intercellular localization of CEA of the squamous epithelium both in human and hamster pouch squamous cell carcinoma by using goat anti-CEA antibody in immunofluorescence technique.

O'Brien et al. (1981) used the PAP method to localize CEA in benign and malignant colorectal tissues. Their conclusions were as follows: (1) Localization of CEA to the surface epithelial cells is a normal finding in the colon and is similar in normal colon and mucosa distant and adjacent to infiltrating carcinoma. (2) Poorly defferentiated colorectal carcinoma usually contains much less demonstrable surface CEA, but may occasionally stain cytoplasm strongly.

van Nagell et al. (1982) studied the CEA in carcinoma of uterine cervix by the PAP method. Antigen staining was present in 82% of keratinizing squamous carcinoma as compared with 50% in nonkeratinizing tumors, and was present both in the cytoplasm and cell membrane of the tumor cell.

Bychkov et al. (1983) studied 61 cases of invasive cervical carcinoma and 40 cases of dysplasia and carcinoma in situ by the PAP method for the presence of CEA. They reported CEA was not detected in normal cervical epithelium but was present in 90% of the neoplastic lesions. Not only the mere presence of CEA, but also a pattern of CEA tissue dis-

tribution emerged as the main differential point between noninvasive and invasive lesions. Twenty-nine of 51 invasive squamous cell carcinoma (57%) contained CEA-positive cells at the stromal edge of epithelium, affecting basal and suprabasal cell while this feature was not found in dysplasia and carcinoma in situ.

### β2-MICROGLOBULIN

β2-Microglobulin is a low molecular weight protein (MW 11,800) originally isolated by Berggård and Bearn (1968) from the urine of patients with tubular proteinurias. It was also found in human serum, cerebrospinal fluid, and saliva.

Peterson et al. (1972) used biochemical methods to determine the amino acid sequence of β2-microglobulin. The β2-microglobulin was isolated from the urine of patients with chronic cadmium poisoning, as described by Beggard and Bearn (1968). They found that this human protein is homologous in sequence to the constant domains ( $C_L$ ,  $C_{H1}$ ,  $C_{H2}$ , and  $C_{H3}$ ) of the immunoglobulin G (IgG), particularly to  $C_{H3}$ . The protein also contains a disulfide loop similar in size to those found in each of IgG domains.

Evrin and Wibell (1972) studied the serum levels and urinary excretion of β2-microglobulin in 119 healthy persons, aged 17-76. They found that β2-microglobulin exist in normal, healthy people. No obvious differences are found between man and women. However, serum levels increased with age.

Bernier and Fanger (1972) studied on synthesis of β 2-microglobulin in by stimulated lymphocytes. Their observation indi-

cated that the  $\beta$ 2-microglobulin is elaborated by lymphocytes in vitro.

Fanger and Bernier (1973) used fluorescent anti- $\beta$ 2-microglobulin antibody to stain normal human lymphocytes.  $\beta$ 2-Microglobulin was detected on the surface of all peripheral lymphocytes.

Evrin and Wibell (1973) reported the serum level of  $\beta$ 2-microglobulin in patients with various disorders, as determined by a radioimmunoassay. While such patients had normal serum levels, high values of serum  $\beta$ 2-microglobulin were often found in patients with malignant neoplasm.

Cresswell et al. (1973) studied the human histocompatibility antigens (HL-A) by solubilizing them with papain from cultured human lymphocytes and then purifying them. It has been shown that the material obtained consisted of two noncovalently bound peptide fragments. One fragment is a peptide of molecular weight 11,000-12,000 dalton. Grey et al. (1973) tried to determine if any relationship existed between the 11,000 dalton peptide chain found in HL-A antigens and  $\beta$ 2-microglobulin. According to their findings on : (1) immunoprecipitation of surface-radioiodinated lymphocyte protein with anti- $\beta$ 2-microglobulin serum, and (2) formation of immune complexes with radioactive papain digests of human lymphocytes; they made the conclusion that the small subunits of HL-A antigens and  $\beta$ 2-microglobulin are identical, or so closely related that they cannot be distinguished by immunological means.

Peterson et al. (1974) also reported  $\beta$ 2-microglobulin can be found in highly purified papain-solubilized HL-A antigens. Two polypeptide chains were obtained by purification; the larger subunit carried the

antigenic specificity whereas the small polypeptide chain was very similar if not identical to  $\beta$ 2-microglobulin.

Evrin and Nilsson (1974) studied the capacity of normal and malignant human cells to synthesize  $\beta$ 2-microglobulin in vitro. Quantitative determinations of  $\beta$ 2-microglobulin by a radioimmunoassay were performed on culture media harvested from seven freshly explanted lymphoma cells and 37 cell lines of hematopoietic, mesenchymal, or epithelial origin.  $\beta$ 2-Microglobulin was detected in all but one lymphoma cell culture. Lymphoma cells, freshly explanted, as well as those of continuously growing lines seem to have a low capacity to synthesize  $\beta$ 2-microglobulin while permanent lymphoblastoid lines secrete the protein at approximately the same rate as mesenchymal cells. The highest production rate of  $\beta$ 2-microglobulin was observed in some epithelial carcinoma cell lines. No correlation was found between the capacity to synthesize immunoglobulins and  $\beta$ 2-microglobulin.

Governa and Biguzzi (1976) investigated the distribution of  $\beta$ 2-microglobulin in human normal tissues by the indirect immunofluorescent antibody method. Lymphoid, macrophage and endothelial cells were consistently positive in every organ studied, whereas the other cell types were generally negative. In addition, some tracts of the columnar epithelium of the digestive system and some endometrial tubular glands showed a specific fluorescence.

However, Forsum and Tjernlund (1977) used the immunofluorescence technique to identify the  $\beta$ 2-microglobulin on the epidermal cell surface. Thirty-four normal skin biopsies (from healthy medical students

and staff members) showed a strong intercellular fluorescence with anti- $\beta$ 2-microglobulin from the basal layer upwards to keratin layer. The results indicate that  $\beta$ 2-microglobulin is a constant feature of the human epidermis.

Tjernlund and Forsum (1977) studied basal cell carcinoma and basal cell papillomas by using anti- $\beta$ 2-microglobulin antibody in the immunofluorescence method. In 15 of the 20 cases of solid and superficial basal cell carcinoma in their study, immunoreactive  $\beta$ 2-microglobulin appeared to be absent, while it was readily detected in all basal cell papillomas examined. They thought the absence or alteration of the cell surface  $\beta$ 2-microglobulin might render these tumors increasingly unresponsive to controlling mechanisms involved in cell interactions and might reflect a disturbance of the gene regulation of  $\beta$ 2-microglobulin on the cell surface of solid and superficial basal cell carcinomas.

Turbitt and Mackie (1981) examined skin biopsies from various skin malignancies as well as premalignant and benign conditions, to investigate further the possible loss of expression of  $\beta$ 2-microglobulin from the surface of malignant cells by using the immunoperoxidase technique. In the benign conditions studied, six cases of keratoacanthoma and six of basal cell papilloma, strong intercellular staining was observed both in normal and in abnormal areas of ten out of twelve cases. Of twenty-three cases of skin malignancy, comprising six squamous cell carcinomas, six basal cell carcinomas, six Bowen's disease and five primary malignant melanomas, nineteen showed no intercellular staining. One case of Bowen's disease showed strongly positive staining on the surface mem-

brane of the tumor cells and three of the squamous cell carcinomas showed some very faint and patchy intercellular staining. This sparse, usually weakly positive, staining of lesional skin was also observed in seven out of ten cases of actinic keratosis, a premalignant condition. Of the remaining three cases, two were strongly positive and one was negative. The result showed a significant loss of demonstrable surface  $\beta$ 2-microglobulin from the surface of malignant cells compared to benign, and a partial loss in premalignant cases.

#### BASEMENT MEMBRANE

Ashwoth et al. (1961) reported a comparative study of normal cervical epithelium, carcinoma in situ, and invasive cervical carcinoma, using electron microscopy and histochemical procedures. They found that the basement membrane was absent around at least some of the invading cell groups, whereas it was present in normal epithelium and carcinoma in situ. This observation emphasize a correlation between invasive properties and the absence of basement membrane, as well as a correlation of noninvasion with the presence of basement membrane.

Rubio and Biberfeld (1975) reported a study on normal epithelium, dysplasia, carcinoma in situ, and squamous invasive carcinoma of human cervix by the immunofluorescence method. They used human sera containing antibodies against the basement membrane (in specimens taken from patients with bullous pemphigoid) in order to demonstrate the basement membrane.

They reported a continuous basement membrane was observed in normal epithelium, dysplasia, and carcinoma in situ, but was faintly stained,



discontinuous or even absent in specimens with invasive squamous carcinoma.

Moragas et al. (1970) also used antibodies against basement membrane taken from the sera of patients with bullous pemphigoid to study the basement membrane in epithelial tumors. By using the indirect immunofluorescence methods, mono-specific fluorescein conjugate of IgG was used to detect the serum antibody binding sites. In seborrheic keratoses and keratoacanthomas, the basement membrane appeared to be of uniform thickness and strongly fluorescent; viral warts showed, a thicker basement membrane than that observed in normal skin; basal cell carcinoma showed a thin basement membrane that contrasted with the thicker basement membrane of the overlying normal epidermis; squamous cell carcinoma, including those of deeply invasive well differentiated squamous cell carcinoma, both showed clusters of malignant cells surrounded by a well-defined basement membrane. However, those poorly differentiated squamous cell carcinomas, even located in the subepidermal area, showed complete lack of basement membrane antigen. These findings indicate the fact that basement membrane antigen could be elaborated by basal cells of the epidermis and, that this capacity is maintained in epidermal tumors whose differentiation maintains this cell line.

Tosca et al. (1980) also studied tumors of epidermal origin by means of immunofluorescence technique with sera from patients with proven bullous pemphigoid. They found that in one hundred tumors of epidermal origin, there was a loss of antigenicity of the basement membrane; this was minimal for benign tumors, greater for premalignant

tumors, and very clear for malignant tumors. Those findings were thought to be consistent with the hypothesis that the basement membrane acts as a barrier between epithelial cells and their connective tissue, and that loss of basement membrane antigens is accompanied by invasion of cells.

Ingber et al. (1981) studied a transplantable carcinoma of the rat pancreas that was composed of cytologically differentiated acinar cells which had lost their epithelial orientation and did not form acini. Light microscopy showed, however, consistent palisading, reorientation, and polarization of these cells in areas of contact with the vasculature. Electron microscopy revealed a normal basal lamina along the basal portions of repolarized tumor cells that is physically separate from the endothelial basal lamina. They also used immunofluorescence to examine the distribution of basal lamina constituents, laminin, and type IV collagen, within the different microenvironments of this tumor. They found that the nonmetastatic tumor has lost the ability to produce or maintain a complete basement membrane within its disorganized parenchyma, while its cells retain the capacity to produce and reorganize along linear basement membrane, when in contact with vascular adventitia. This study suggested that failure to maintain a complete basement membrane may be involved in the neoplastic disorganization of normal tissue architecture as well as in the breakdown of boundaries during the development of invasive carcinoma.

The basement membrane provides a structural substrate for the epithelial cells and serves as a barrier limiting access of macromolec-

ules to the intercellular clefts (Fawcett, 1981). Some macromolecules had been identified as restricted only to certain areas of basement membrane. For example, type IV collagen is restricted to lamina densa, while laminin and pemphigoid antigen are found only in lamina lucida (Courtoy et al., 1982; Stanley et al., 1982a).

Stanely et al. (1982b) studied the basement membrane of basal cell carcinoma by the immunofluorescence method, using antibodies to laminin, type IV collagen, and bullous pemphigoid antigen. They found the basal cell carcinoma has defects in bullous pemphigoid antigen but is intact in laminin and type IV collagen.

#### LAMININ

Laminin is a large noncollagenous glycoprotein, first isolated from the mouse Engelbreth-Holm-Swarm (EHS) sarcoma, which produces an extracellular matrix of basement membrane. It contains two polypeptide chains of molecular weight 220,000 and 440,000 daltons which are linked by disulfide bonds. Immunological studies using purified antibody against this protein showed that it is produced by a variety of cultured cells. In addition, these antibodies react with the basement membrane of normal tissues, suggesting that laminin is a constituent of the basement membranes of these tissues (Timpl and Rohde, 1979; Rohde et al., 1979).

Foidart et al. (1980) studied the distribution and immunoelectron microscopic localization of laminin. By using immunofluorescence, laminin was localized in the basement membrane zones of those human, chick, guinea pig, bovine, monkey, rat, and mouse tissues examined. Epithelial

and endothelial cells in culture synthesize laminin while mesenchymal cells do not. By immunoelectron microscopy, laminin was localized to the lamina lucida of human epidermal basement membrane. The wide distribution of laminin among diverse tissues and species, and its production in early stages of embryonic development suggests that laminin is an ubiquitous component of basement membrane.

Ekblom et al. (1980) used immunofluorescence method to study the distribution of laminin during the early formation of kidney tubules in mouse embryos. The first laminin spots were found after 12 hr. of culture, 24 hr. before overt morphogenesis. As the mesenchymal cells began to aggregate and elongate (at 36 hr.), laminin was detected in those cells destined to become epithelial. In more mature tubules (at 72 hr.), laminin was seen as a sharp band in the basement membranes. It is suggested that laminin is involved in the increased cell adhesiveness during the early aggregation of nephrogenic mesenchyme.

The role of laminin in the attachment of PAM 212 (epithelial) cells to basement membrane collagen had been studied by Terranova et al. (1980). They found that measurements of the attachment of PAM 212 line of mouse epithelial cells to various collagen substrates showed that these cells adhere preferentially to type IV basement membrane collagen. When laminin was preincubated on plates coated with either type I, II, III, IV, or V collagen, and plates subsequently washed, high levels of attachment were seen only on type IV collagen substrate. Thus, laminin appears to be a specific attachment protein for epithelial cells, since it did not stimulate the attachment of fibroblasts to type IV collagen

substrate. This data suggest that laminin is produced and utilized by these epithelial cells to attach to basement membrane collagen.

Hayman et al. (1981) reported loss of cell surface laminin from transformed rat kidney cells by immunofluorescence methods. Their studies suggested one of the characteristics of cells which become malignant is that laminin is lost from the cell surface.

Albrechtsen et al. (1981) used anti-laminin antibody to detect basement membrane changes in breast carcinoma by immunoperoxidase technique. They found that the neoplastic cells in malignant breast tissues showed strongly cytoplasmic staining for laminin, and a positive reaction was also found in lymph node metastases. In nonmalignant breast tissues, the epithelial cells of the ducts were positive for laminin, but the staining was weaker than in the carcinoma. In normal breast tissue and benign lesions, the laminin staining delineated continuous basement membranes. In well-differentiated carcinoma the basement membrane could be revealed by laminin staining, but they were thinner and discontinuous. The poorly differentiated carcinomas lack organized basement membranes detectable by laminin staining. This suggests that staining for laminin may be a useful adjunct test for detection of disintegration of the laminin-containing basement membranes of anaplastic tumors and supports the notion that basement membranes may play a role in tumor invasion.

Eklom et al. (1982) reported laminin can be demonstrated by immunoperoxidase staining in sections of normal human tissues fixed in formalin and routinely processed with paraffin. Sections from paraffin

blocks stored for years at room temperature could be stained with this procedure. Developing fetal tissues and tumors also could be stained with this method.

Miettinen et al. (1982) use PAP method to demonstrate the laminin in soft tissue tumors. The results showed intense laminin positivity in schwannomas and neurofibromas and less intense positivity in leiomyomas and leiomyosarcomas, whereas fibrous histiocytomas and fibrosarcomas generally were negative. It is suggested that immunohistochemical demonstration of laminin is a valuable aid in the differential diagnosis between tumors derived from fibroblasts and schwann cells. In addition to the expression seen in the tumors, all vascular walls were positive for laminin. Therefore, demonstration of laminin also can be used to examine the vascular pattern of tumors.

Terranova et al. (1983) studied the laminin receptor on human breast carcinoma cells. They found that human breast carcinoma cells possess a receptor-like moiety on their surface that has a high affinity for laminin. The whole laminin molecule has the configuration of a four-armed cross with three short arms and one long arm (Timpl et al., 1983). A major cell-binding domain was found to reside near the intersection point of the short arms; and the typeIV collagen-binding domain was associated with the globular end regions of the short arms. The receptor for laminin on the surface of these tumor cells may be involved in the initial interaction of tumor cells via laminin with the vascular basement membrane to facilitate invasion and subsequent promotion of metastasis.

## FIBRONECTIN

Fibronectin, a high molecular weight glycoprotein, was first isolated by Morrison et al. (1948) from a partially purified fraction of human plasma, which they termed "cold-insoluble globulin" (CIG). Since then, other workers independently described the various proteins or factors which were named according to sources or biological activities, such as long external transformation-sensitive (LETS) protein by Hynes and Bye (1974) or cell-surface protein (CSP) by Yamada and Weston (1978). However, in the review by Yamada and Olden (1978), these proteins reportedly are closely related; there are only two specific proteins: cell surface (cellular) fibronectin; and plasma fibronectin. The cellular fibronectin has an apparent subunit molecular weight of between 200,000 and 250,000, and plasma fibronectin is a dimeric glycoprotein with subunit polypeptides of 200,000-220,000 that circulates in blood.

Chen et al. (1976) used immunofluorescent staining to study the distribution of fibronectin on the cell surface of normal, and transformed cells. In cells expressing a transformed phenotype in vitro, fibronectin was detected only in cell-cell contact areas, whereas in "untransformed" cells fibronectin was distributed over the entire cell surface. Transformed cells capable of inducing invasive tumors, and the cells of established tumor lines, have low or undetectable levels of fibronectin. These results indicated that fibronectin has a role in cell-cell adhesion and that reduced expression of this protein at the cell surface is related to the oncogenic phenotype.

Yamada (1978) used anti-fibronectin antibody to investigate fibro-

nectin's localization, and its transfer from intracellular to extracellular pools by using immunofluorescence method. They found anti-fibronectin antibody localized to extracellular fibrils located under and between sparse cells, and to a dense matrix that surrounds confluent cells. Cellular fibronectin is also present in granular intracytoplasmic structures containing newly synthesized fibronectin before secretion. Transformation of chick fibroblasts results in a decrease in both extracellular and intracellular fibronectin, and is associated with altered cell shapes.

Experiments performed in cell culture suggest that the function of cell-associated fibronectin is to attach cells to the extracellular matrix (Chen et al., 1978; Culp and Bensusan, 1978)

Culp and Bensusan (1978) reported the search for collagen in substrate adhesion site of two murine cell lines by biochemical methods. They found that collagen does not mediate the cell-substrate adhesion process of these two murine cell lines, and that fibronectin may bind to some other component in substrate-attached material, to generate adhesive bonds between the cell surface and substrate-bound serum receptor molecules.

The studies reported by Chen et al. (1978), also supported the fact that one of the functions of fibronectin is cell-cell attachment. They used immunofluorescence and electron microscopic method to study the intercellular fibronectin matrices secreted by chick embryo fibroblasts in culture. Under scanning and transmission electro microscopy, the smallest fibers found in the matrix were 5-10 nm in diameter; these



fiber tend to cluster to form bundles. Immunofluorescence and immunoferritin methods revealed that fibronectin is one of the components of the matrices. The matrices are isolated from other cellular organelles by detergent treatment. More than 90% of the proteins in cell free matrices are fibronectin protein and suggesting that the matrices are probably made of only one component, namely, fibronectin.

Gallimore et al. (1979) reported their findings on examination of three adenovirus-transformed rat embryo brain cell lines and their sub-clones for fibronectin expression. The sub-clones were considerably more malignant than the parent cell lines, as measured by invasion and metastasis in nude mice (Gallimore et al., 1977). They found without exception the sub-clones contained significantly fewer fibronectin-positive cells than the parent lines; a number of sub-clones even contained no fibronectin-positive cell.

Smith et al. (1979) studied the production of fibronectin by human epithelial cells in culture. Human epithelial cell lines derived from both carcinomatous and nonmalignant tissues were characterized with respect to the presence and distribution of fibronectin by immunofluorescence microscopy. In cell lines derived from nonmalignant tissues or from primary carcinomas, fibronectin was found predominantly in an extracellular matrix. In contrast, cell lines derived from metastatic carcinomas displayed very little or no fibronectin. Metabolic labeling studies indicated that a positive synthesized fibronectin was de novo rather than absorbing the protein from the media. Negative lines neither synthesized fibronectin nor secreted it into the culture fluid,

suggesting that they were not producing fibronectin.

Burns et al. (1980) first used immunoperoxidase techniques to demonstrate fibronectin in sections of routine formalin fixed paraffin embedded renal tissue. Previous exposure of the sections to a solution of pepsin in 0.01N HCl for 2 hours at 37°C was essential, in order to demonstrate antigenicity of fibronectin previously masked by fixation and embedding procedures.

Stenman and Vaheri (1981) studied the presence of fibronectin in human solid tumors by immunofluorescence staining. In all sarcomas the individual tumor cells were surrounded by a network of fibronectin which was continuous with the stroma. The distribution of fibronectin was similar in benign soft-tissue tumors. In contrast, no fibronectin was detected in the individual carcinoma cells or on their periphery. These results show that, contrary to the situation in cell culture, in vivo sarcoma cells and benign soft-tissue tumor cells contain fibronectin in their pericellular matrix. On the other hand, fibronectin can be used to distinguish carcinomas from sarcomas in vivo.

Hayman et al. (1981) reported the loss of cell surface fibronectin from transformed rat kidney cells by radioimmunoassay technique. Soluble fibronectin was detected in the culture media of the transformed as well as of the normal cells. Culture supernates of the transformed cells contained even more fibronectin than the supernates of the normal cells. The authors suggested that this result showed that loss of fibronectin from the surface of the transformed cells is caused by failure of the cells to deposit this protein into an insoluble matrix and

that it is not caused by inadequate production.

Clark et al. (1982) reported that fibronectin provides a provisional matrix for epidermal cell migration during wound reepithelialization. In their studies of guinea pig skin wounds using immunofluorescence methods, the epidermis migrated over an irregularly thickened provisional matrix containing fibrin and fibronectin. However, the provisional matrix lacked laminin and type IV collagen, two major components of the normal basement membrane. Upon completion of wound reepithelialization at 7-9 days after wounding, the basement membrane zone lost its thickened appearance, type IV collagen and laminin reappeared. These findings demonstrate that epidermal cells do move over such a fibronectin matrix during in vivo wound repair.

However, Niemczuk et al. (1982) used an immunoperoxidase method to localize the fibronectin in human rectal carcinomas. Fibronectin was present in 29 out of 38 cases, in connective tissue stroma, and was not in direct association with the tumor cells. They found no correlation between the presence or absence of stromal fibronectin and (1) the degree of cellular differentiation within the tumor; (2) the subsequent development of metastases; and (3) patient longevity. These results do not support the conclusions from in vitro studies (Smith et al. 1979) that suggested the metastatic potential of carcinomas may be partly determined by the ability of tumor cells to synthesize pericellular fibronectin.

## LECTIN

The study of lectins was initiated by Stillmark (1888), who was the first to describe the phenomenon of blood hemagglutination by plant extracts.

The term "lectin" was first reported by Boyd and Shapleigh (1954a, 1954b), based on their observation that some seed extracts could distinguish among human blood groups as determined by agglutination. The lectins are multivalent glycoproteins obtained from plants or other sources, which can bind noncovalently to cell receptors containing specific carbohydrate groups. Because of the abundance of carbohydrates on the cell surface (glycocalyx), the multivalent lectins are able to cause agglutination of erythrocytes and other cells sometimes, this can occur after prior enzymatically catalyzed modification of the cells (Gordon et al., 1972).

The finding that the lectin wheat germ agglutinin caused differential agglutination of normal and malignantly transformed cells in vitro stimulated a great deal of research on lectins (Aub et al., 1965; Burger and Goldberg, 1967). Sharon and Lis (1972) found that in normal cells, lectin receptor sites are uniformly distributed on the cell surface. In contrast, the receptor sites in malignant cells become clustered in the plasma membrane (Rapin and Burger, 1974; Sharon and Lis, 1975). These studies suggest that the glycoprotein receptors on the surface of tumor cells differ from those on the normal cell surface. Such research, subsequently led to the finding of several other lectins that agglutinate to transformed malignant cells at much lower concentrations than normal

cells (Brown and Hunt, 1978).

Biochemical studies of the specificity of lectins for carbohydrates, have been determined by comparisons of hemagglutination and glycoprotein precipitation inhibition by various mono- and oligosaccharides, as well as by direct measurements of sugar-binding activities by equilibrium dialysis or spectrofluorometric titrations (Lotan et al., 1974; Goldstein and Hayes, 1978).

Debray et al. (1981) used numerous oligosaccharides and glycopeptides derived from N-glycosylproteins to define the specificities of twelve lectins, by determining their ability to inhibit the agglutination of human red blood cells induced by these lectins. It was found that *Canavalia ensiformis* agglutinin (Con A) and Pea lectins contain binding sites for  $\alpha$ -manno- and glucopyranosides, *Ricinus communis* agglutinin I (RCA I) binds  $\beta$ -galactose and *Ulex europeus* agglutinin I (UEA I) binds L-fucose.

#### CONCAVALIN A (CON A)

Concanavalin A (Con A), a carbohydrate binding protein of the Jack bean (*Canavalia ensiformis*) was first isolated by Sumner and Howell (1936).

This protein binds to substance containing mannose and glucose.

Moscona (1971), studied the Con A receptors on embryonic and neoplastic cell surfaces and found that embryonic tissue cells dissociated with ethylenediaminetetraacetate (EDTA) are readily agglutinated by the Con A. In this property, they resemble transformed, neoplastic cells; and they differ from untransformed adult cells, which are agglutinated

by Con A only after their receptors are unmasked by proteolytic treatment. He thought that Con A binding sites on embryonic cells may function in cell contact and cell organization during embryonic morphogenesis and differentiation, and later become masked in adult cells. The unmasking of these sites in neoplastic cells may represent a return, in this respect, to a condition resembling that of embryonic cells and may be related to cell mobility associated with infiltration and metastasis.

Martinez-Palomo et al. (1972) studied the ultrastructural distribution of Con A receptor sites on the surface of normal hamster embryo cells and transformed cells by means of the Con A-peroxidase method. They found that in vertical sections of normal cells, the precipitate appeared as a uniform surface layer, which gave the appearance of a homogeneous coat when seen on surface view. The reacting layer on the surface of transformed cells was similar, except that the precipitate was seen only in approximately one-third of the transformed cells. The latter showed a more discontinuous cell surface layer line in vertical sections, while in tangential sections, the cell surface coat was found to be formed by isolated clusters of the reacting precipitate. These results indicate that the surface of both normal and transformed cells equally possess exposed binding sites to Con A. However, the receptors tend to be more irregularly distributed on the surface of some transformed cells.

Louis et al. (1981) examined various epidermal lesions with fluorescein isothiocyanate-labeled Con A. The results showed lectin binding

in all malignant tumors. However, normal and hyperplastic tissues either failed to stain or showed a grossly diminished level of fluorescence.

#### RICINUS COMMUNIS AGGLUTININ I (RCA I)

Nicolson and Blaustein (1972) used Ricinus communis fraction I (RCA I) to specifically agglutinate several normal and transformed cell lines. The agglutinin RCA I consistently agglutinated transformed cells at much lower concentrations than those required to agglutinate normal cell lines, unless the normal cells were first treated with low concentrations of trypsin.

However, Kim et al. (1974, 1975) had used biochemical methods to study the membrane glycopeptides in human colonic adenocarcinoma and normal colonic mucosa. The carbohydrates of membrane glycopeptides were found to be markedly reduced in tumor tissue and relative proportions of the various sugars were altered. Although all of the sugars were lower in tumor tissue when compared to the adjacent normal mucosa, galactosamine, fucose, and sialic acid were more significantly reduced. The results showed that an alteration in glycoprotein biosynthesis occurred during tumorigenesis that results in a decrease in RCA binding sites. This was supported by Dabelsteen and Mackenzie (1978). They studied the histological distribution of receptors for RCA I in oral carcinoma by use of fluorescein-labeled RCA I. Their study showed that RCA I receptors are located at the cell membranes in the basal and spinous layer of the normal epithelium, whereas receptors could not be demonstrated in invading islets of the neoplastic cell.

ULEX EUROPEUS AGGLUTININ I (UEA I)

Since Cazal and Lalaurie's (1952) discovery of anti-H(0) hemagglutinating activity in extracts of three *Ulex* species, *Ulex europaeus* extract has become a standard serological reagent, used in typing O blood, and in distinguishing A from A blood types (Boyd and Shapheigh, 1954c).

Osawa and Matsumoto (1969, 1972) purified two lectins of distinctly different specificities from *Ulex europaeus* extracts; an L-fucose-binding protein, UEA I, and a D-glucose-binding protein, UEA II. They isolated and studied UEA I, which precipitated between 0% and 40% saturation of ammonium sulfate, and was purified by CM-cellulose chromatography followed by gel filtration. Homogeneity of the preparation was assessed by ultracentrifugation and gel filtration. Hemagglutination of O erythrocytes by UEA I was completely inhibited by L-fucose.

Holthöfer et al. (1982) used fluorescein isothiocyanate (FICT)- or tetramethylrhodamine isothiocyanate (TRICT)-labeled UEA I in fluorescence microscopy to stain cryostat sections of human tissues. They found that the endothelium of vessels of all sizes was stained ubiquitously with UEA I in all tissues studies, and the staining was not affected by the blood group type of the tissue donor.

SIALIC ACID

Sialic acids are important terminal oligosaccharide constituents of cell surface glycoproteins, and they have been shown to be implicated in several biologic functions. In malignantly transformed cells, one function is the invasive characteristics of tumor cells (Yarnell and



Ambrose, 1969). Therefore, this biologically important molecule deserves special consideration.

The relationship between the sialic acid content of cells and neoplastic transformation has been examined in a number of tumor systems. Studies of sialic acid on transformed and nontransformed cell lines in vitro have revealed either an increase in the total, surface-exposed, and surface-membrane-bound (van Beek et al., 1973), decrease (Perdue, et al., 1972), or no change (Hartmann et al., 1973) in the concentration of the transformed cell lines, as compared to nontransformed controls. In contrast to these observations, studies with malignant cells in vivo have consistently revealed that both total and plasma-membrane-bound sialic acid are elevated relative to normal cells (Merritt et al., 1978).

Warren et al. (1973) and van Beek et al. (1973, 1975) have demonstrated an increase in sialic acid in cell surface glycoprotein oligosaccharides in a wide variety of in vivo and in vitro transformed cells as compared to control cells.

The relationship between the sialic acid content of neoplastic cells and their metastatic behavior had been less extensively explored. An increase of total sialic acid has been reported in human liver metastatic deposits compared to the levels found in primary pancreatic adenocarcinoma (Mabry and Carubelli, 1972).

Yogeeswaran and Salk (1981) reported the ability of malignantly transformed cells cultured in vivo, when injected into mice, were found to metastasize spontaneously from subcutaneous sites. The metastatic

potential of such injected cells is positively correlated with the total sialic acid content of the cells in culture. The degree to which the sialic acid is exposed on the tumor cell surface, and, most strongly, with the degree of sialylation of galactosyl and N-acetylgalactosaminyl residues in cell surface glycoconjugates. These findings suggest that sialic acid on the cell surface may play a role in tumor cell metastasis.

#### IMMUNOHISTOCHEMICAL METHOD

By virtue of its unique ability to correlate the location of antigenic cellular constituents with anatomical structure, immunocytology has provided many significant contributions to biological and medical science, since its inception over forty years ago when Coons et al. (1942) first introduced his immunocytological technique. Although immunofluorescence remains a widely used immunocytological technique, the development over the past decade of immunoenzyme procedures in which the site of immunological reactivity is detected by a histochemical reaction has facilitated and expanded upon those studies, and has definite medical interest (Taylor and Mason, 1974).

Nakane and Pierce (1966) first suggested using immunohistochemical staining as a means of identifying antigenic markers in tumor tissue. They prepared peroxidase-labeled antibodies. The site of immunologic reactivity was identified by the histochemical reaction.

Graham and Karnovsky (1966) first used the diaminobenzidine (DAB) reaction at pH 7.2-7.6 for optimal localization of marker peroxidase-antiperoxidase (PAP) complex.

Weir et al. (1974) modified the methods for the immunohistochemical localization of peroxidase-labeled antibody without loss of specificity, by taking advantage of the optimum pH of horseradish peroxidase. They determined empirically, the appropriate buffer salts, hydrogen peroxide concentration, time of staining, and fixation after incubation with labeled antibody and counterstains .

Huang et al. (1976) further improved the immunohistochemical staining by treating formalin-fixed paraffin-embedded sections with trypsin prior to indirect staining. The trypsin treatment was found to decrease the nonspecific background fluorescence through partial digestion of the tissue, and also unmasked the immunoreactive sites of antigens.

Curran and Gregory (1977) also used trypsin based on Huang's finding (1976) to unmask the antigens in paraffin sections of tissue. When trypsin treatment was optimal, the preservation of the histological structure was unaffected and the sections reacted to high titers of antibody with the immunohistochemical method with very little nonspecific staining.

Most recent studies have reported the use of the immunoperoxidase technique to detect cellular antigens. Certain features of this procedure suggest that it may rapidly replace the use of immunofluorescence in many immunocytological applications, particularly in the diagnostic pathology laboratory (Knowles et al., 1977). Unlike immunofluorescence, specimen stained by immunoperoxidase are permanent and can be counterstained with routine histology stains. This procedure provides for

direct comparison of immunologic reactions with morphologic detail, and does not require special tissue manipulations if the antigen activity is preserved following formalin fixation and paraffin embedding. Thus, these properties of immunoperoxidase staining have the necessary convenience that promises to make immunohistochemistry more versatile in its use and to have greater utility in diagnostic pathology than does immunofluorescence (Mesa-Tejada et al., 1977).

Vacca et al. (1978) found maximal staining of erythrocyte pseudoperoxidase by DBA occurs at pH 7.2-7.6, the pH range ordinarily used to localize the PAP complex during immunological staining. They thought that by lowering the pH of the DAB solutions, it would be possible to selectively suppress the reactivity of pseudoperoxidase while maintaining optimal reactions in neutrophils and PAP complex. For this purpose, they recommend ammonium acetate-citric acid buffer at pH 5.5 (pH 5.0-6.0), containing 44 mg. DAB per 100 ml. buffer and 0.003%-0.03% H<sub>2</sub>O<sub>2</sub>.

Brandtzaeg (1981), also improved the immunohistochemical staining by prolonging the incubation time of conjugated antibody. He found that by prolonging the incubation time from 30 minutes to 20 hours at room temperature, the conjugates may be applied at about ten times higher dilution and yet produce specific staining of enhanced intensity.

Hsu et al. (1981) first used the antiavidin and avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. This method involved four sequential staining procedures: (1) primary antibody (goat anti-human antigen); (2) secondary antibody (rabbit anti-goat IgG) added

in relative excess; (3) goat anti-avidin antibody; (4) avidin-biotin-peroxidase complex. They found that the staining method using the ABC technique was found to be highly sensitive and easy to perform. However, they also found that not every biotin-labeled substance can be detected by the ABC, as it depends upon the molecular size and the amount of biotin attached or available in the biotinylated substances.

Hsu and Raine (1982) extended their studies in 1981 to include biotin-labeled lectin to demonstrate specific carbohydrate moieties in formalin-fixed tissue. They also compared the ABC method with the PAP method. The results show that the ABC method is superior in terms of its sensitivity and specificity for the localization of carbohydrate in the tissue section.

In a review of immunoenzyme histochemistry by Primus and Goldenberg (1982), the selected application with immunohistochemical methods were used to identify many tissue antigens. Both immunofluorescence and immunoperoxidase methods were used to identify the following: typing lymphoid cell markers (Siegal and Good, 1977; Koziner et al., 1978); localization of human blood group antigens (Davidsohn, 1979; Weinstein et al., 1981); localized carcinoembryonic antigen (CEA) in a variety of gastrointestinal and extragastrointestinal epithelial malignancies (review by Goldenberg et al., 1978; Primus et al., 1981); monitoring therapy and prognosis of human chorionic gonadotropin in gestational choriocarcinomas and  $\alpha$ -fetoprotein in hepatocellular carcinoma (Braunstein, 1979; Waldmann and McIntire, 1979); identifying prostatic acid

phosphatase in occult cases in for discriminating between metastatic prostatic carcinoma and adenocarcinomas of different origin (Stegéhuis et al., 1979; Manley et al., 1981); detection of intermediate filaments (1. keratin filaments in epithelial cells, 2. desmin filaments in muscle cells, 3. vimentin filaments in mesenchymal cells, 4. neurofilaments in neurons, and 5. glial filaments in glial cells.)(Schlegel et al. 1980a, 1980b).

According to their review of immunoenzyme histochemistry, Primus and Goldenburg (1982) found that immunohistochemical methods applied to the diagnostic pathology of cancer can offer the following advantages: (1) the cellular origin of a product can be identified; (2) the distribution of the product among certain or different cells within a heterogeneous population of a tumor and normal cells can be demonstrated; (3) the presence of a new, modified, or cellular constituent during or after the development of a neoplasm can be followed; and (5) the changes and interrelationship of multiple cellular products and substances can be assessed.

## CHAPTER III

### MATERIALS AND METHODS

#### MATERIALS

Formalin fixed, paraffin embedded oral mucosal specimens from sixty patients with histologically diagnosed squamous cell carcinoma, and five specimens of normal oral mucosa were obtained from those accessioned from the files of the Department of Oral and General Pathology, School of Dentistry, Loyola University of Chicago.

#### METHODS

Two immunohistochemical techniques were used:

1. Peroxidase-antiperoxidase (PAP) technique using specific antibodies for identifying glycoproteins.
2. Biotinylated lectin, avidin-biotin-horseradish peroxidase technique (ABC) using high affinity lectins for identifying carbohydrates.

The antisera used to identify specific antigens in this study were purchased from the following commercial sources:

<u>Antigen</u>	<u>Commercial Sources</u>
1. Keratin	Dako Corp. (a)
2. Carcinoembryonic antigen (CEA)	Dako Corp.
3. $\beta$ 2-Microglobulin	Dako Corp.
4. Laminin	Bethesda Research Lab. (b)

5. Fibronectin

Cappel Lab. (c)

Notes on Commercial Sources:

- a. Dako Corp., Santa Barbara, CA.
- b. Bethesda Research Lab., Gaithersburg, MD.
- c. Cappel Lab., West Chester, PA.

The lectins to identify specific sugar residues used in this study were purchased from the following commercial sources:

<u>Biotinylated Lectins</u>	<u>Commercial Source</u>
1. Concanavalin A (Con A) for mannose/glucose	Vector Lab. (a)
2. Ricinus communis agglutinin I (RCA I) for galactose	Vector Lab.
3. Ulex europaeus agglutinin I (UEA I) for fucose	Vector Lab.
4. Limax flavus agglutinin (LFA I) for sialic acid	Calbiochem-Behring (b)

Notes on commercial sources:

- a. Vector Lab., Burlingame, CA.
- b. Calbiochem-Behring., San Diego, CA.



PROCEDURES

## PEROXIDASE-ANTIPEROXIDASE (PAP) TECHNIQUE

- A. Sections of paraffin-embedded formalin-fixed tissue were cut at 2-3  $\mu$ m and mounted on glass slides that were pretreated with 2% aqueous Elmer's Glue-All.
- B. After drying at 60°C, the slides were deparaffinized in two changes of xylene. Xylene was then removed from the sections by absolute alcohol.
- C. Endogenous peroxidase and pseudoperoxidase were inactivated by treatment with 0.075% hydrochloric acid in absolute ethanol for 15 minutes. (Weir et al., 1974)
- D. The sections were then placed in water. For trypsinization, the tissue sections were placed in prewarmed pancreatic trypsin (General Biochemicals., Chagrin Falls, OH.), 0.1% in 0.01 M Tris-HCl buffer, pH 7.6, containing 0.1% CaCl<sub>2</sub>, at 37°C for 15 minutes. Tissues which were insufficiently fixed were given less time in trypsin. Trypsinized sections were washed in cold Tris buffer. All sections were washed in deionized water and pH 7.4 phosphate-buffer saline (PBS) (Curran and Gregory, 1977; Mepham et al., 1979). Enzymatic digestion for laminin or fibronectin was performed with 0.1% pepsin (Sigma., St. Louis, MO.) in M/100 HCl for 15-60 minutes at 37°C, followed by washing in distilled water. (Holund et al. 1981)
- E. In a moist chamber, sections were covered with a 3% solution of

normal serum (from the same species used to produce link antibody) in PBS with 0.2% bovine serum albumin (PBS/BSA). This was accomplished in order to block nonspecific tissue binding of subsequent reagent immunoglobulins. Time: 30 minutes. (Sternberger, 1979)

F. The sections were then blotted quickly on filter paper and covered with a solution of the primary antibody in PBS/BSA, containing 1% normal sheep serum. The following dilution of primary antibody were used: keratin 1:500, CEA 1:500,  $\beta$ 2-microglobulin 1:100, laminin 1:500, fibronectin 1:500. The incubation time required was 30-60 minutes for most antibodies, however, some (i.e., laminin and fibronectin) seem to benefit from prolonged exposure (1-2 days) at 4°C. (Sternberger, 1979; Brandtzaeg, 1981)

G. Incubation in antibody solutions at this and subsequent stages was followed by washing in three changes of PBS, 5 minutes each, to remove unreacted antibody.

H. Link antibody against rabbit IgG (whole molecule) was diluted 1:100 in PBS and applied to the sections for 30 minutes. This reagent consisted of whole antiserum from which anti-human IgG cross-reactivity had been removed by absorption onto CN Br-Sepharose linked human gamma globulins. (March et al., 1974)

I. After washing in three changes of PBS, sections were treated with PAP (sheep), diluted 1:200 in PBS/BSA with 1% normal serum, for 30 minutes.

J. The slides were then washed in three changes of PBS, then rinsed with deionized water and placed in peroxidase substrate solution for 2.5 minutes. This solution consists of a 0.05M ammonium acetate-citric acid buffer, pH 5.5, containing 0.04% 3,3'-diaminobenzidine tetrahydrochloride. It was prepared just prior to its use, filtered, and 0.0075% hydrogen peroxide added. (Weir et al., 1974; Vacca et al., 1978)

K. After thorough washing in water, the sections were counterstained with hematoxylin, dehydrated, cleared and mounted in Permount.

L. Representative sections were incubated with a 1:1,000 dilution of normal rabbit serum instead of the primary antibody as a control of non-specific staining.

#### AVIDIN-BIOTIN-PEROXIDASE COMPLEX (ABC) TECHNIQUE

A. Sections were deparaffinized and Treated with absolute and 95% Ethanol.

B. Treated 15 minutes in 0.0075% HCl in Ethanol to remove endogenous peroxidase.

C. Washed in water. Washed in 0.2% BSA in 0.05M Tris-buffered saline (TBS/BSA) for 20 minutes.

D. The sections were then incubated for 30 minutes in the appropriate dilution of biotinylated lectin (10ug/ml for Con A and LFA; 50ug/ml for RCA I, AND UEA I) in 10% albumin in Tris-buffered saline. (in the case of Con A , 1mM each of CaCl<sub>2</sub> and MnCl<sub>2</sub> were added.) Sections were then

washed in TBS/BSA, 3 changes. Biotin-labelled LFA was prepared from LFA (Calbiochem) and biotinyl-N-hydroxysuccinimide (Sigma) in the method of Bayer and Wilchek (1980).

E. Avidin-biotin-peroxidase complex was prepared according to the manufacturer's instructions 30 minutes before use, and applied to the sections for 1 hour (Vectastain ABC Kit #PK-4000), and then washed in 3 changes of TBS/BSA.

F. Treated with diaminobenzidine tetrahydrochloride, 0.04% in 0.05M ammonium acetate-citrate buffer, pH 5.5, containing 0.0075% hydrogen peroxide, for 2 minutes.

G. Washed in 5 changes of water.

H. Counterstained in hematoxylin for 3 minutes, washed, dehydrated, cleared and mounted in Permount.

I. The controls were prepared by treating serial sections with biotinyl-lectin solution of Con A, RCA I, UEA I, and LFA, to which specific glycosides (mannose, galactose, fucose, and sialic acid) were added, and then centrifuged, to assess negative reactions.

## CHAPTER IV

### RESULTS

The histologic examination of sections of normal mucosa and squamous carcinoma stained by the PAP and ABC methods both resulted in positive reactions as indicated by a reddish-brown deposit decorating the cells. Such reddish-brown deposits were found in the cytoplasm or the intercellular-cell surface area of the cells and indicated that the antibody defined proteins and lectin defined sugars were in the cytoplasm or on the surface of the cells. (Table 1)

### KERATIN

The keratin stain gave a positive deposition in the cytoplasm of the cells; and variably positive or negative on the cell surface of squamous epithelial cells both in the normal mucosa and in the squamous carcinoma. (Fig. 1,2).

In the normal mucosa, the reaction for keratin is weak in the basal cell layer. However, it increased in intensity in the stratum granulosum of the epithelium.

The isolated clusters of invasive tumor cells, however, all showed varying degrees of positive staining for keratin, even if they were located in areas of heavy infiltration of inflammatory cells. (Fig. 3)

In some cases, examination of the underlying supporting tissues, clearly, demonstrate how deeply the tumor cells can infiltrate into the

connective tissue.(Fig. 4)

### CEA

In the normal squamous epithelium, CEA was positively localized to the cell surface of the upper stratum spinosum, stratum granulosum, and stratum corneum. However, the surface of lower stratum spinosum and the stratum basale were negative. Also, the cytoplasm of the epithelial cells was negative.(Fig. 5)

In the squamous cell carcinoma, in contrast to normal cells, CEA was present both in the cytoplasm and on the surface of the cells.(Fig. 6) Furthermore, the continuity of the CEA staining of the epithelium was disrupted at those sites where cells appeared to become malignant as observed immunohistochemically.(Fig. 7)

In the well differentiated squamous carcinoma, the cell staining pattern was similar to that seen in the normal mucosa.(Fig. 8) In the moderately and poorly differentiated squamous cell carcinoma, the CEA was either lost or not synthesized, as less positive staining and an uneven distribution of stained cells occurred between the tumor cells, even in the same specimen.(Fig. 9)

Eighty-nine percent (32 out of 36 cases) of well differentiated squamous cell carcinoma, 69% (11 out of 16 cases) of the moderately differentiated type, and 37% (3 out of 8 cases) of the poorly differentiated type showed a cytoplasmic deposit of CEA. (Table 2 ) This atypical cytoplasmic localization of CEA differentiates the malignant from benign squamous cells.

### β2-MICROGLOBULIN

β2-microglobulin was shown in the normal mucosa with a positive deposition on the cell surface and intercellularly in the stratum basale and stratum spinosum, it was negative in the stratum granulosum and stratum corneum.(Fig. 10)

Similar to CEA, at the junctional area where the epithelium apparently began its malignant change, the tumor cells markedly show a decrease of the staining reaction on their surfaces.(Fig. 11) In the well differentiated squamous carcinoma, the deposits were on the cell surface predominantly and, like normal mucosa, the stratum basale stained more strongly.(Fig. 12) Positive β2-microglobulin staining was seen in the cytoplasm of a few tumor cells, where it usually was located in a perinuclear pattern or at one pole of the nucleus. This localization differs from that of the moderately differentiated squamous carcinoma, where the staining tends to be more cytoplasmic than intercellular for β2-microglobulin.(Fig. 13) Similar to the atypical distribution of CEA, this pattern of cytoplasmic staining reaction in the poorly differentiated squamous cell carcinoma was reduced, appearing less reactive than the well differentiated and moderately differentiated types, and it was predominant to the cytoplasm of the cells.(Fig. 14)

Another interesting finding was that about half of the mitotic figures in squamous cell carcinoma showed a strongly positive reaction for β2-microglobulin in the cytoplasm.(Fig. 13)

The β2-microglobulin, was observed in the cytoplasm in 100% (36/36) of the well differentiated type of squamous carcinoma, 94%

(15/16) of the moderately differentiated squamous carcinoma, and 88% (7/8) of the poorly differentiated squamous cell carcinoma. Also, a positive reaction for  $\beta$ 2-microglobulin was observed on the cell surface in 97%, 94%, and 88% of the well differentiated, moderately differentiated, and poorly differentiated squamous carcinomas, respectively. (Table 2)

#### LAMININ AND FIBRONECTIN

Laminin and fibronectin, two of the glycoprotein components of basement membrane, were positive in clearly defining the basement membranes both in the normal mucosa, (Fig. 15, 19) in the well differentiated squamous carcinoma, (Fig. 16, 20) the moderately differentiated squamous cell carcinoma, (Fig. 17, 21) and the poorly differentiated squamous cell carcinoma. (Fig. 18, 22) In addition to this, the basement membrane of the blood vessels in the supporting tissues also showed positive reactions. (Fig. 15, 19)

#### CON A

The Con A lectin binding showed positive staining for glucosyl-mannosyl sugar residues in the cytoplasm; and apparently variably positive or negative staining on the cell surface both in the normal mucosa (Fig. 23) and squamous carcinoma. (Fig. 24)

Again, the staining pattern with Con A of well differentiated squamous cell carcinoma was similar to the pattern observed in the normal mucosa. (Fig. 24) However, the positive deposits were more unevenly distributed in the less differentiated squamous cell carcinoma. (Fig. 25)



Variability of staining was noted in that the reaction was negative in some cells, and strongly positive in others, localized as perinuclear deposits. This uneven staining reaction with Con A can be specifically detected in those invasive small islets of squamous carcinoma and clearly differentiates them from normal appearing squamous cells (Fig. 26)

#### RCA I

RCA I receptors for galactosyl residues are shown to be present on the cell surfaces by the ABC method in well defferentiated squamous carcinoma,(Fig. 28) as it was observed in the normal mucosa.(Fig. 27) However, in the less differentiated squamous carcinoma, reduced staining was observed on the cell surface, but a more strongly positive staining for RCA I receptors were seen in the cytoplasm.(Fig. 29, 30)

The other interesting finding was that the generative population of cells in the less differentiated squamous cell carcinomas showed a decrease in the RCA I surface binding as compared to those in the well differentiated squamous cell carcinoma or the stratum basale of the normal mucosa.(Fig. 29 30)

#### UEA I

UEA I, defined fucosyl residues, was indicated by a positive staining reaction were present on the cell surfaces both, in the normal mucosa (Fig. 31) and squamous cell carcinoma.(Fig. 32, 33) However, the generative population of the cells in stratum basale of normal mucosa and those of squamous cell carcinoma both showed negative staining for

UEA I receptors. However, while such fucosyl residue receptors were defined and localized in the cytoplasm of the tumor cells only, the cytoplasm of the normal squamous cells of the mucosa did not show any positive reaction.

Another interesting finding is that there was an evenly distributed positive cytoplasmic reaction in the poorly differentiated squamous cell carcinoma.(Fig. 34)

#### LFA

LFA defined sialyl residues were found as positive stain on the cell surfaces, but negative in the cytoplasm, of the cells both in the normal mucosa (Fig. 35) and in the squamous cell carcinoma.(Fig. 36,37,38) However, the positive staining was reduced on cells in the squamous cell carcinoma as compared with those in the normal mucosa.(Fig. 37)

Interestingly, the sialic acid also can be detected by stain in the generative population of cells in normal mucosa (Fig. 35) and in well differentiated squamous cell carcinoma.(Fig. 36) However, in the invasive islets of tumor cells, the surface positive reaction to LFA was restricted only to the cell contact surfaces; but it was negative in the basement membrane surface area of the cells.(Fig. 38)

TABLE 1

Distribution of products defined by specific antibodies and lectins.

Antibodies	Normal Mucosa		Tumor Cells	
	cell surface	cytoplasm	cell surface	cytoplasm
keratin	-+	+	-+	+
CEA	+	-	+	+
$\beta$ 2-micro-globulin	+	-	+	+
	basement membranes		basement membranes	
Laminin		+		+
Fibronectin		+		+
Lectins	Normal Mucosa		Tumor Cells	
	cell surface	cytoplasm	cell surface	cytoplasm
Con A	-+	+	-+	+
RCA I	+	-	+	+
UEA I	+	-	+	+
LFA	+	-	+	-

-+= Variably positive or negative reaction

+ = Positive reaction

- = Negative reaction

TABLE 2

Positive reaction for CEA and  $\beta$ 2-microglobulin in well differentiated, moderately differentiated, and poorly differentiated squamous cell carcinoma.

		Cell surface	Cytoplasm
CEA	Well differentiated	100% (36/36)	89% (32/36)
	Moderately differentiated	100% (16/16)	69% (11/16)
	Poorly differentiated	63% (5/8)	37% (3/8)
		Cell surface	Cytoplasm
$\beta$ 2-micro- globulin	Well differentiated	97% (35/36)	100% (36/36)
	Moderately differentiated	94% (15/16)	94% (15/16)
	Poorly differentiated	88% (7/8)	88% (7/8)

Fig. 1

Positive deposition for keratin in the cytoplasm of the cells; and variably positive or negative on the cell surface of the squamous epithelial cells in the normal mucosa. (PAP method, magnification X125)

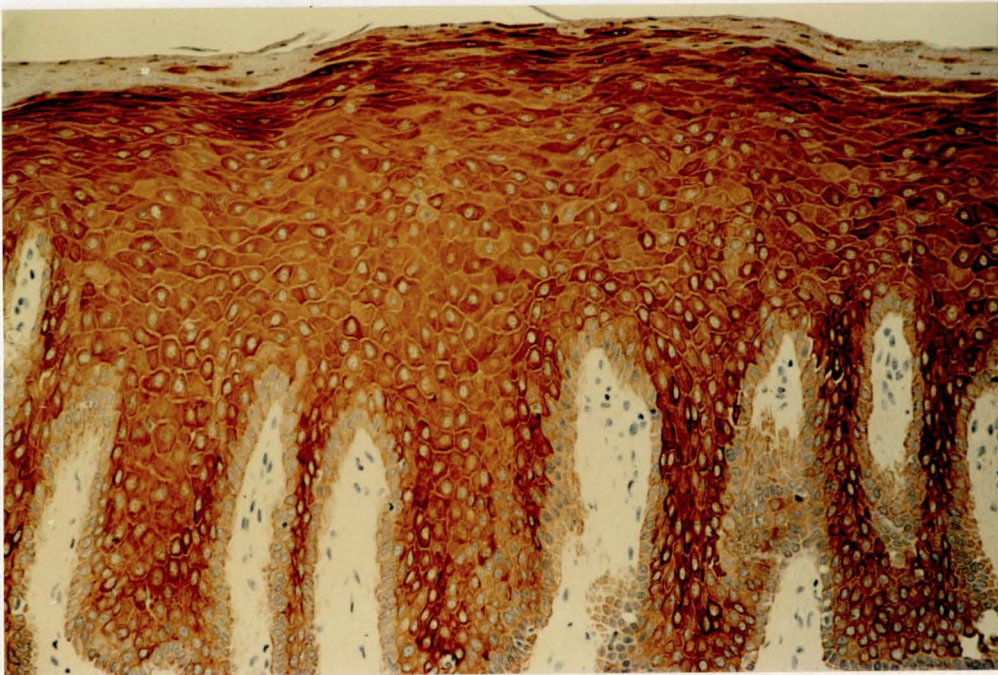


Fig. 2

The pattern of keratin staining similar to Fig. 1 is observed in well differentiated squamous cell carcinoma. (PAP method, magnification X125)

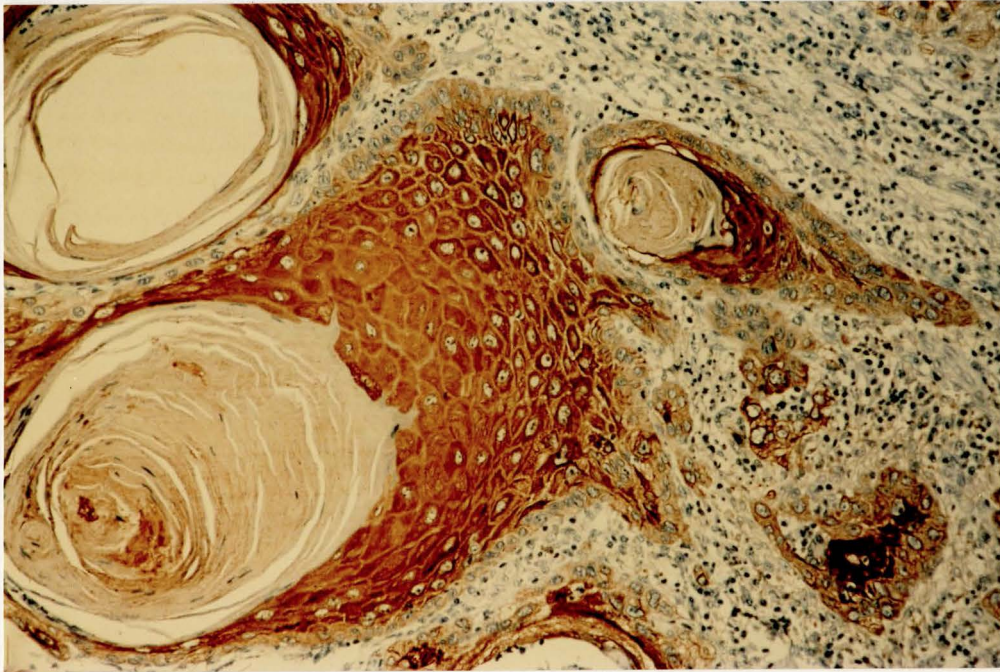


Fig. 3

The invasive clusters of tumor cells show varying degree of positive reaction for keratin in the area of heavy infiltration associated with inflammatory cells. (PAP method, magnification X125)

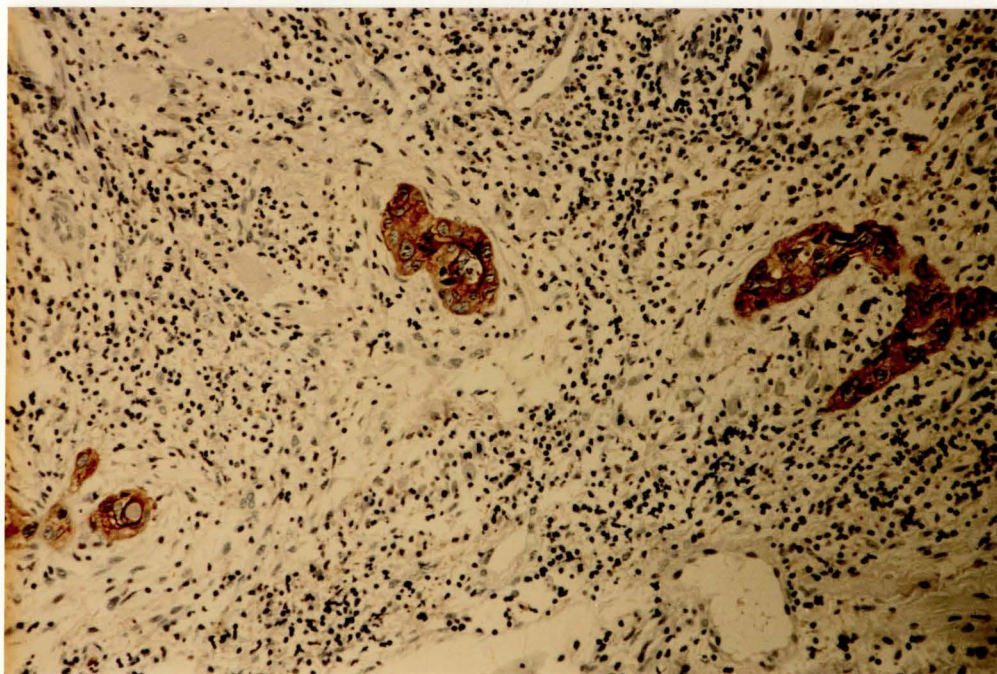


Fig. 4

Keratin positive tumor cells which infiltrate into the supporting connective tissue can be observed with the immunohistochemical method.

(PAP method, magnification X31)

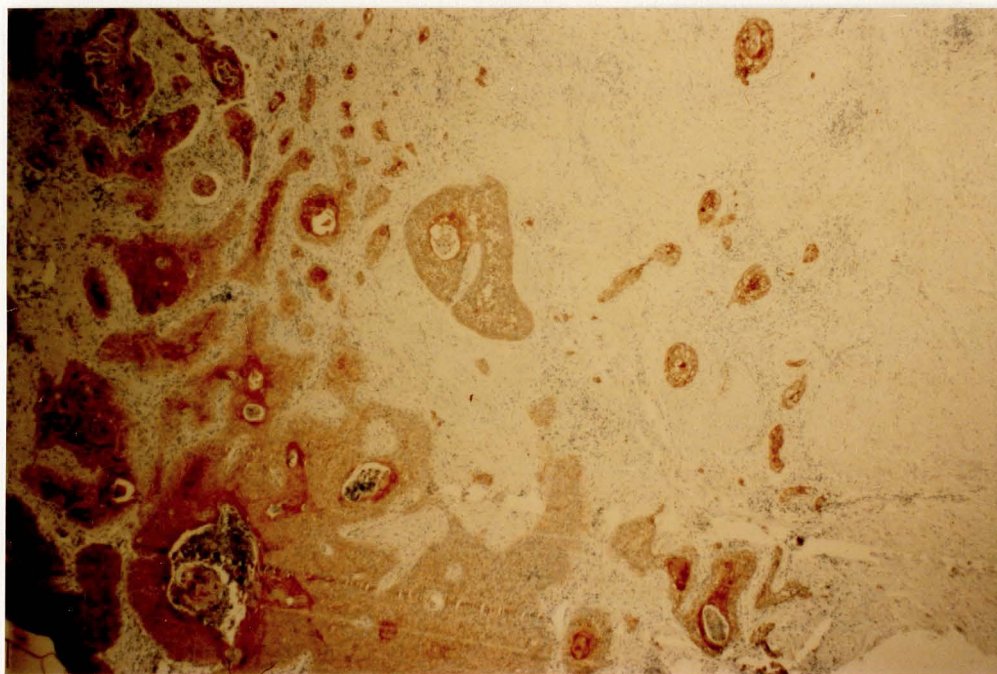




Fig. 5

CEA is shown on the cell surface in the upper stratum spinosum, stratum granulosum, and stratum corneum of the normal mucosa. (PAP method, magnification X125)

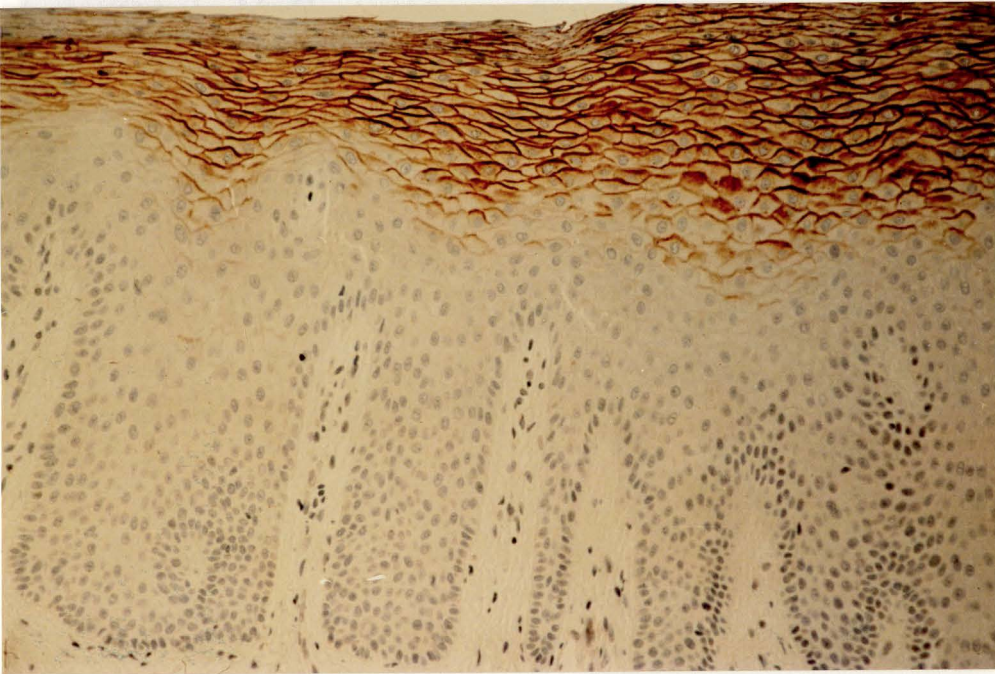


Fig. 6

The positive deposition for CEA is shown both in the cytoplasm and on the surface of the cells of squamous cell carcinoma. (PAP method, magnification X125)

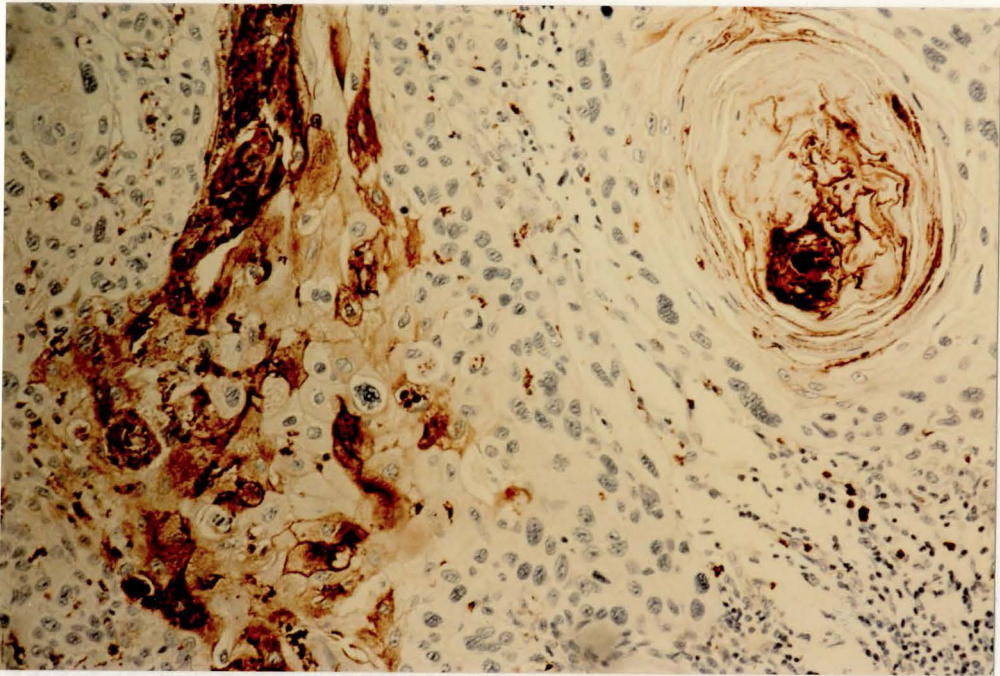


Fig. 7

The continuity of the CEA staining of the epithelium was disrupted at the junctional area where squamous cell become malignant. (PAP method, magnification X125)

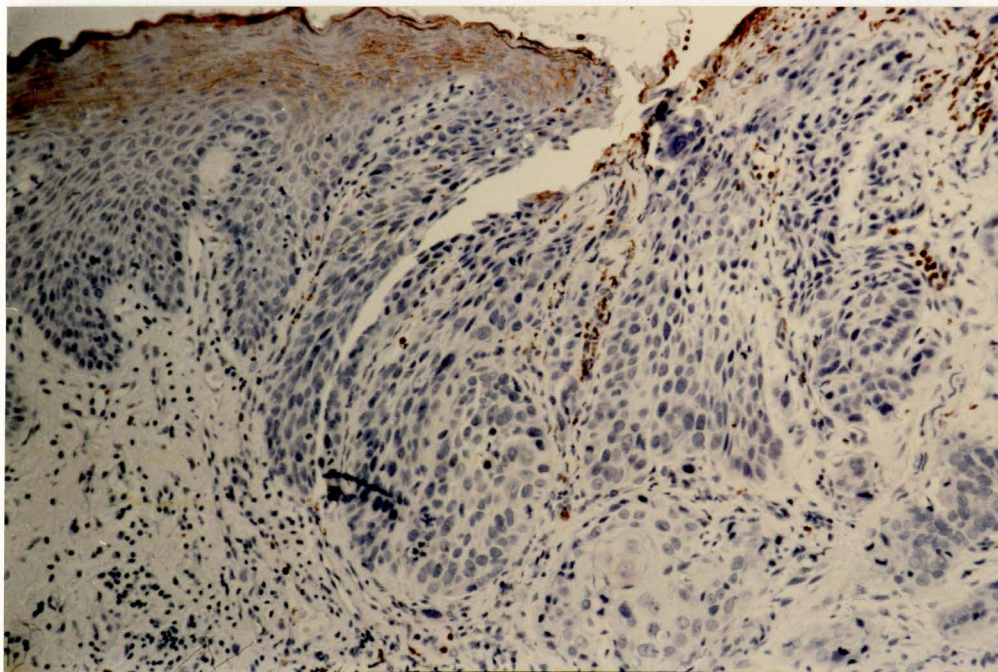


Fig. 8

CEA staining pattern in well differentiated squamous cell carcinoma is similar to that in normal mucosa. (PAP method, magnification X125)

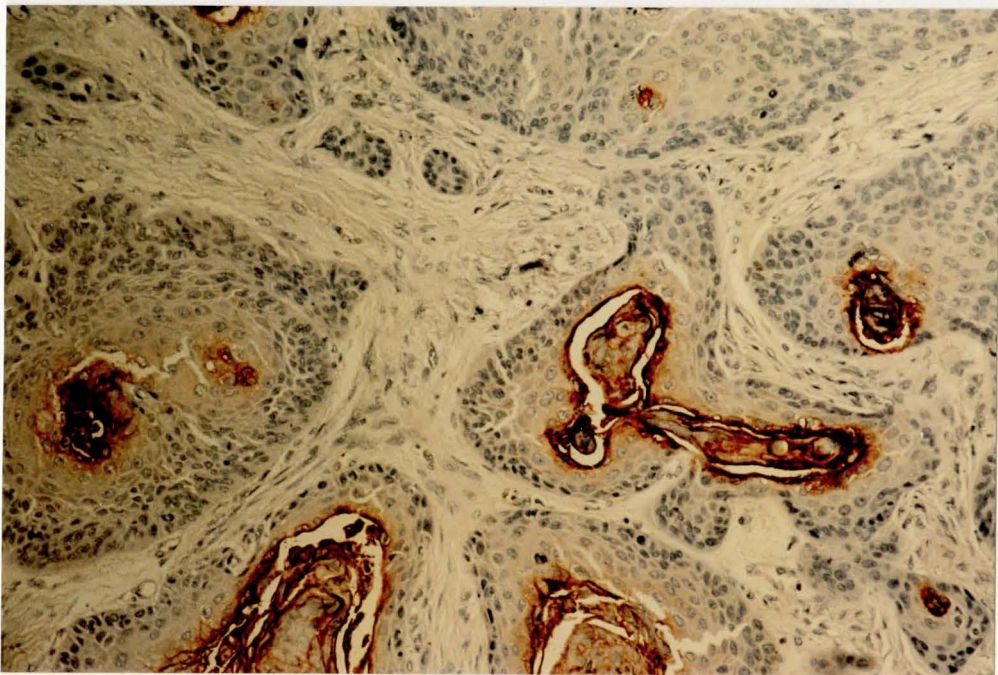


Fig. 9

The uneven distribution of CEA in moderately differentiated squamous cell carcinoma. (PAP method, magnification X125)

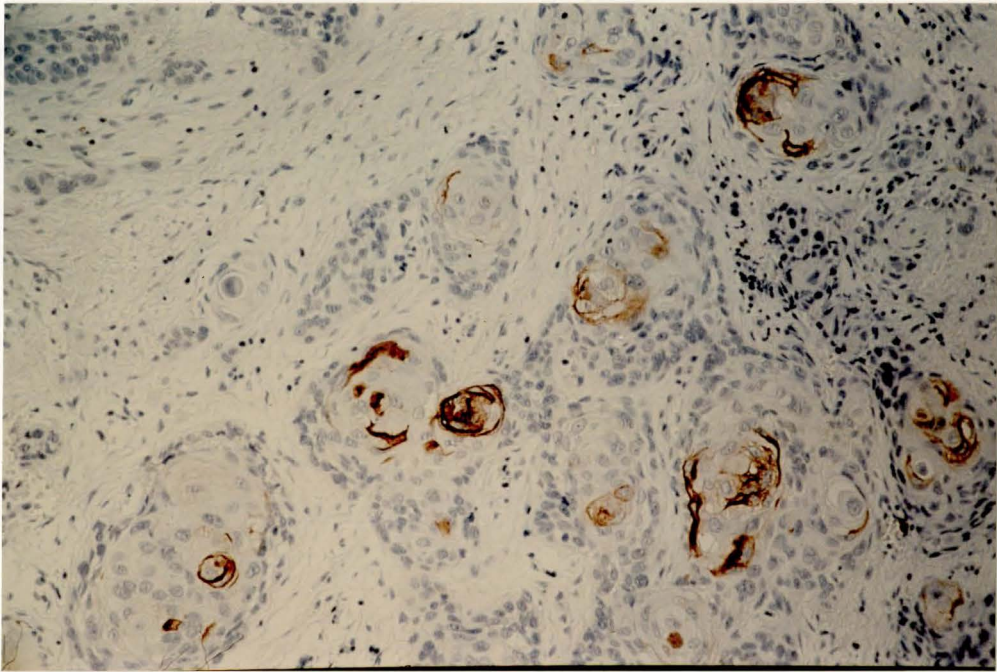


Fig. 10

$\beta$ 2-microglobulin is on the cell surface of stratum basale and stratum spinosum of the normal mucosa. (PAP method, magnification X313)

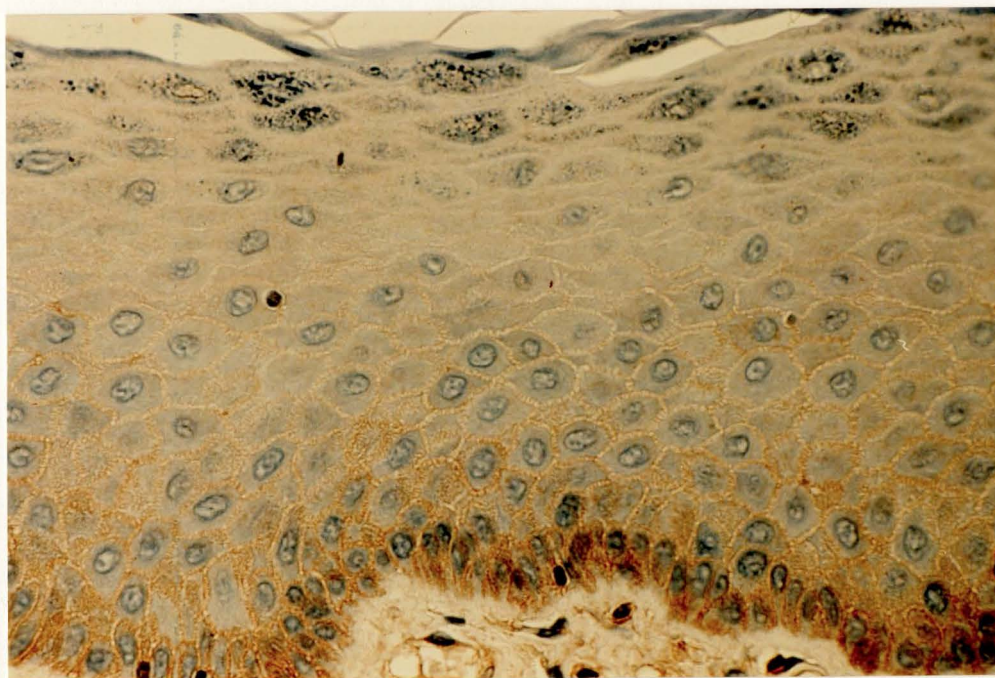


Fig. 11

$\beta$ 2-microglobulin is markedly decreased on the cell surface compared to normal mucosal epithelium at the junctional area where squamous cells become malignant. (PAP method, magnification X125)

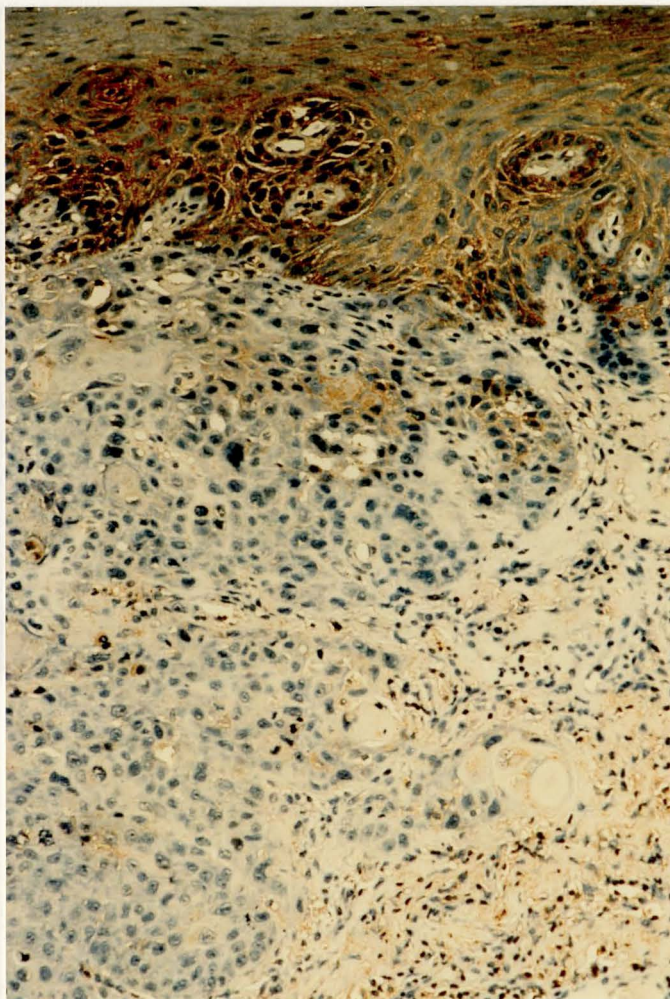


Fig. 12

$\beta$ 2-microglobulin shows both cell surface and cytoplasmic positive reaction in well differentiated squamous cell carcinoma. (PAP method, magnification X33)

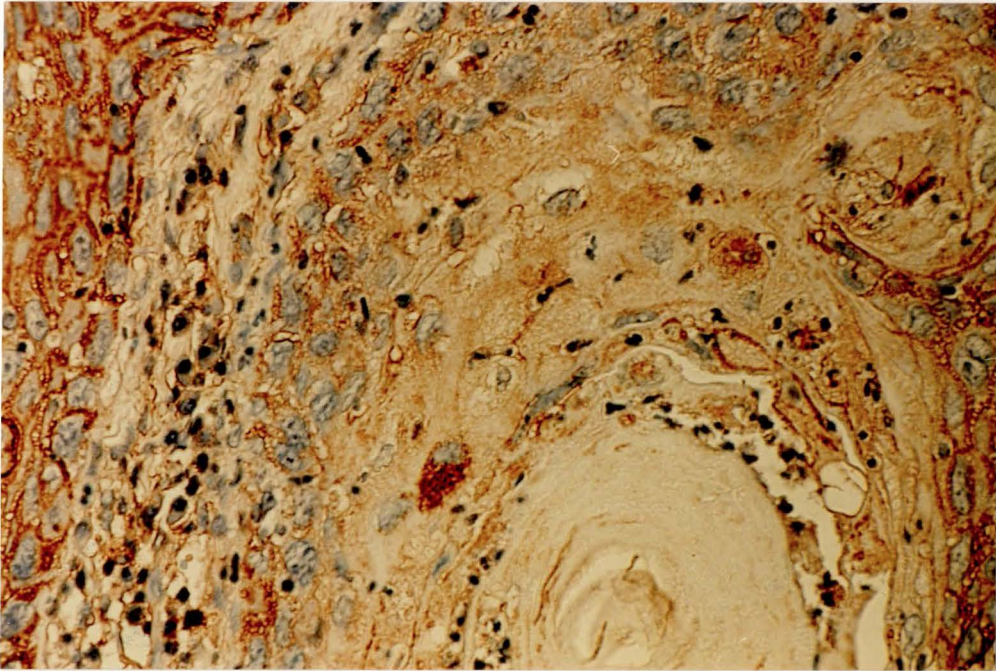




Fig. 13

$\beta$ 2-microglobulin is observed more cytoplasmic positive than cell surface in moderately differentiated squamous cell carcinoma. (PAP method, magnification X125)

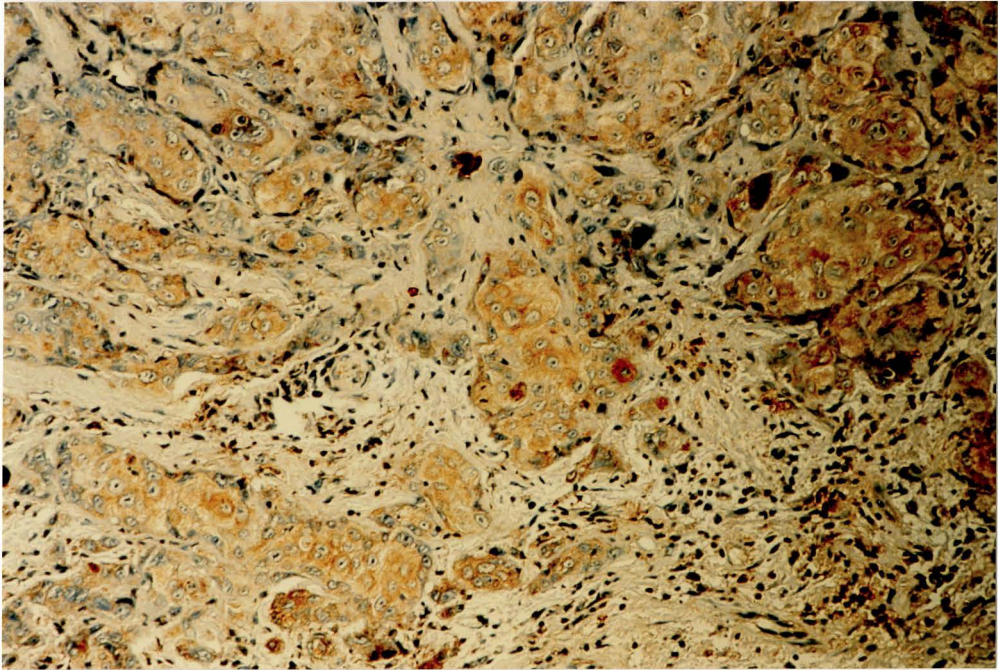


Fig. 14

$\beta$ 2-microglobulin is predominantly shown in the cytoplasm of the tumor cells in the poorly differentiated squamous cell carcinoma. (PAP method, magnification X125)

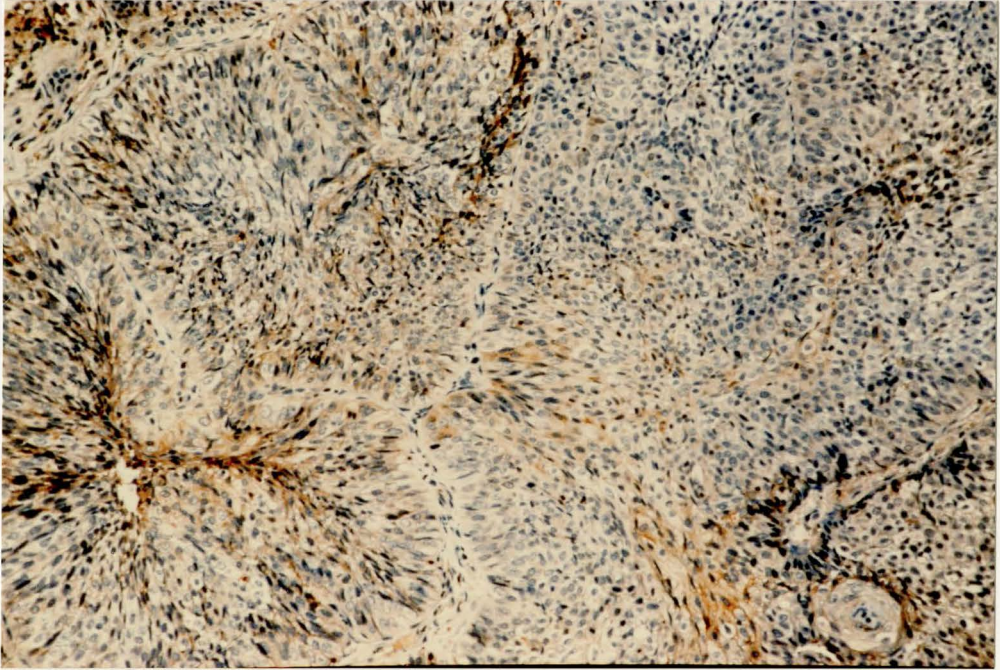


Fig. 15

The positive reaction of laminin on the basement membrane of the normal mucosa epithelium. (PAP method, magnification X125)

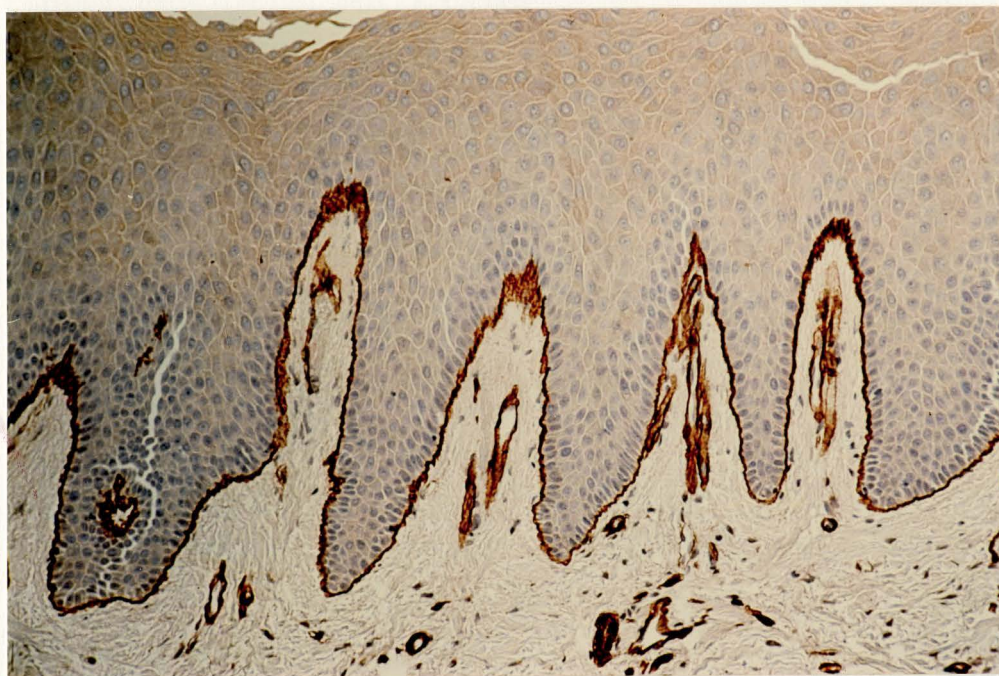


Fig. 16

Laminin delineating the basement membrane in well differentiated squamous cell carcinoma. (PAP method, magnification X125)

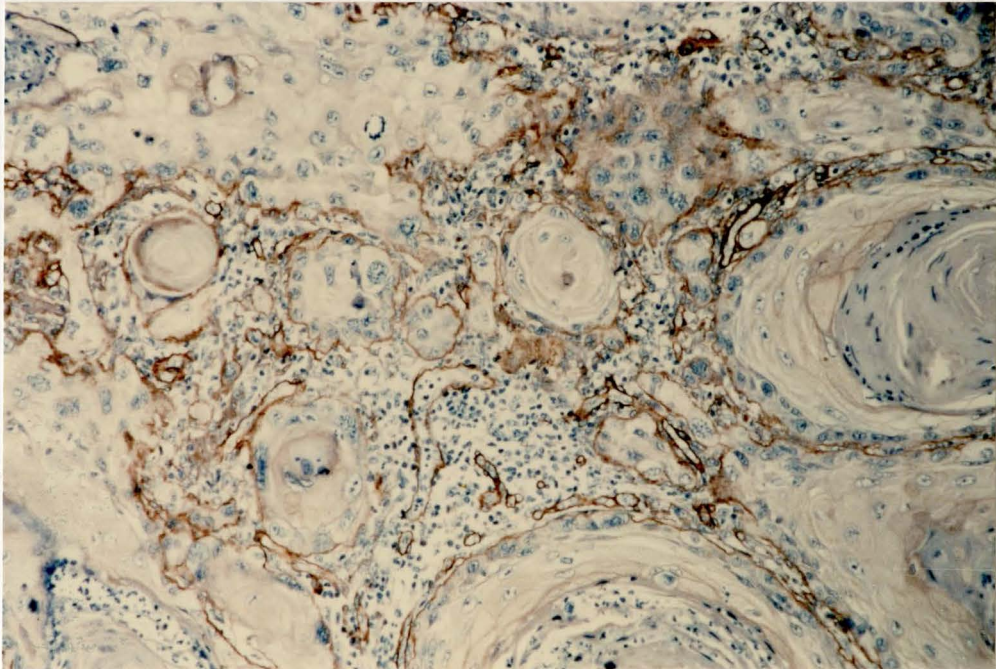


Fig. 17

Laminin delineating the basement membrane in moderately differentiated squamous cell carcinoma. (PAP method, magnification X125)

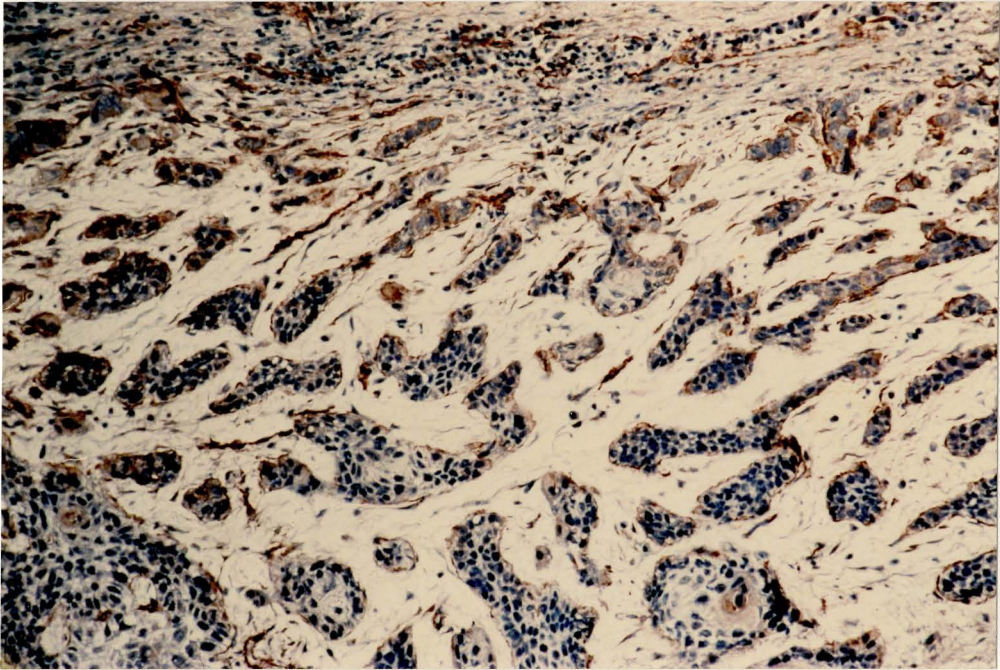


Fig. 18

Laminin delineating the basement membrane in poorly differentiated squamous cell carcinoma. (PAP method, magnification X125)

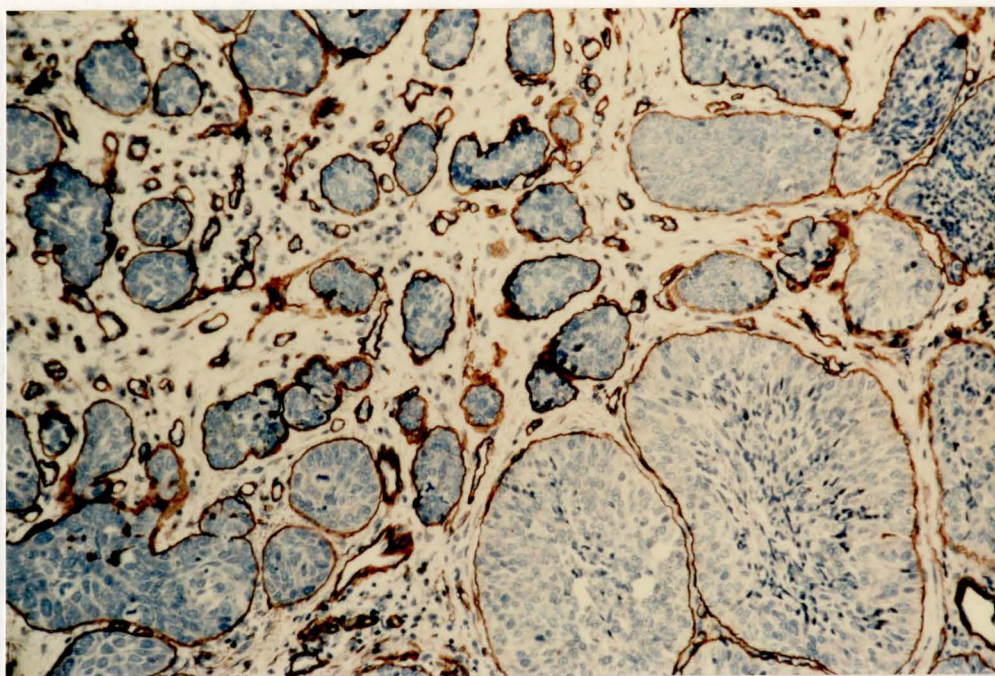


Fig. 19

The positive reaction of fibronectin on the basement of normal mucosal epithelium. (PAP method, magnification X125)

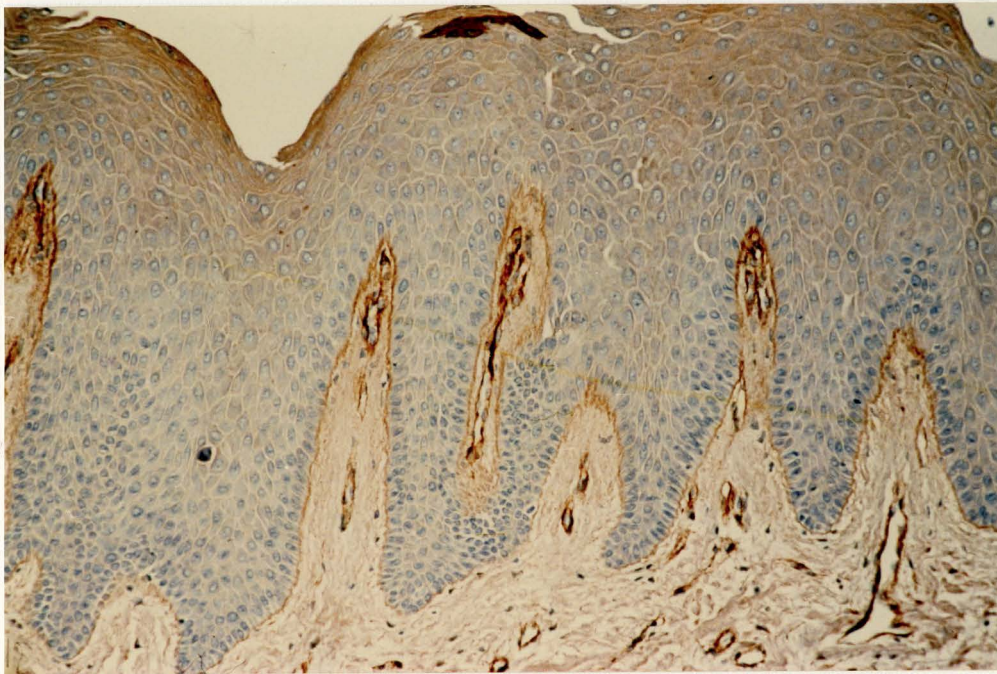


Fig. 20

Fibronectin delineating the basement membrane in well differentiated squamous cell carcinoma. (PAP method, magnification X125)

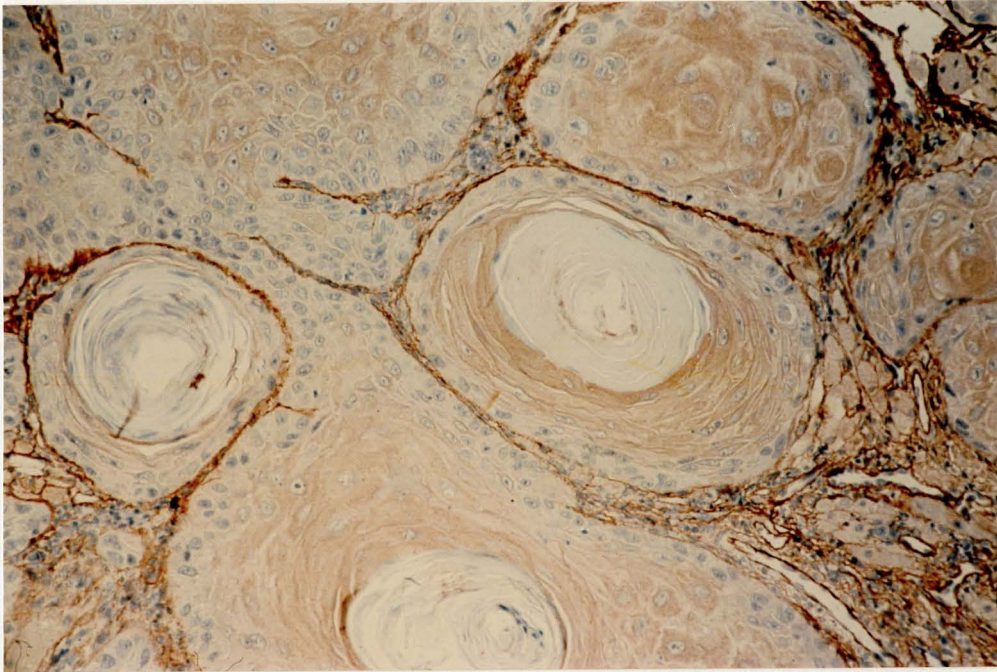




Fig. 21

Fibronectin delineating the basement membrane in moderately differentiated squamous cell carcinoma. (PAP method, magnification X125)

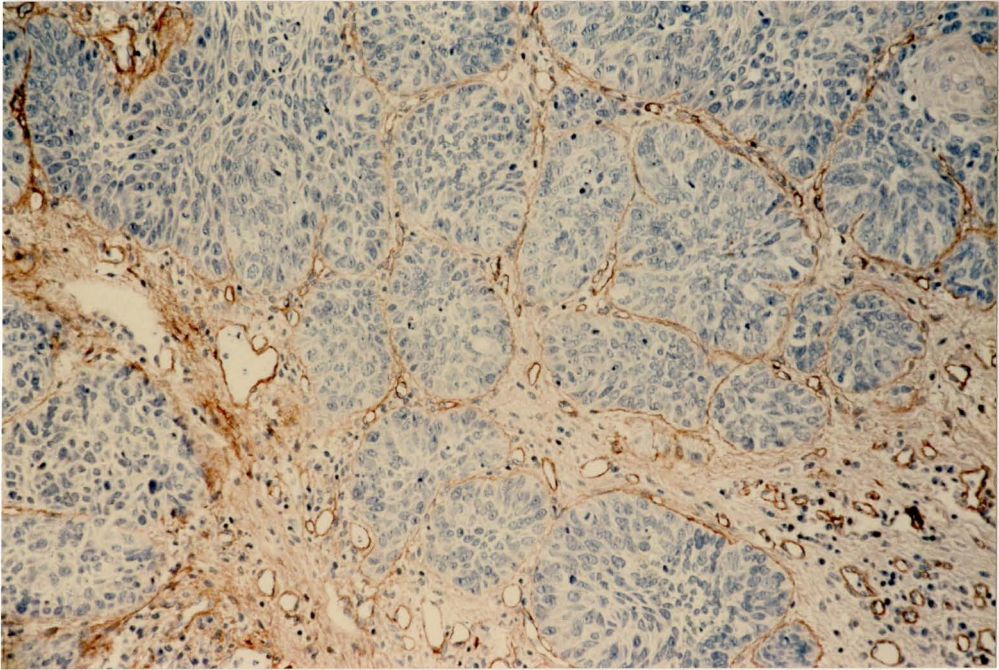


Fig. 22

Fibronectin delineating the basement membrane in poorly differentiated squamous cell carcinoma. (PAP method, magnification X125)

stained by anti-fibronectin antibody (ABC method, magnification X313)

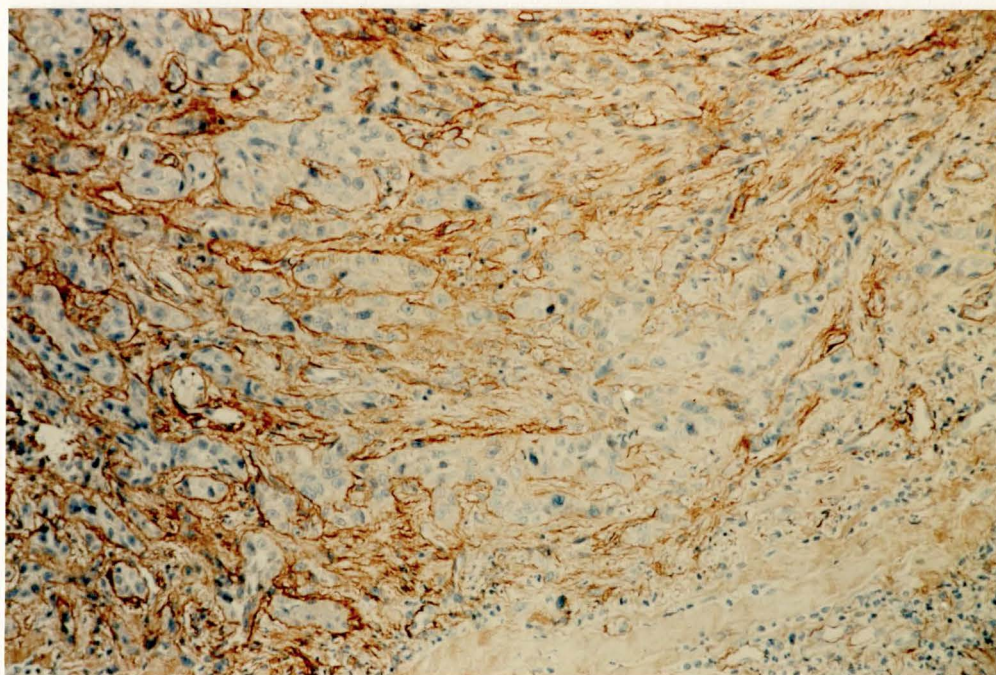


Fig. 23

Positive staining for glucosyl, mannosyl residues in the cytoplasm; and variably positive or negative staining on the cell surface in the normal mucosa by Con A lectin binding. (ABC method, magnification X313)

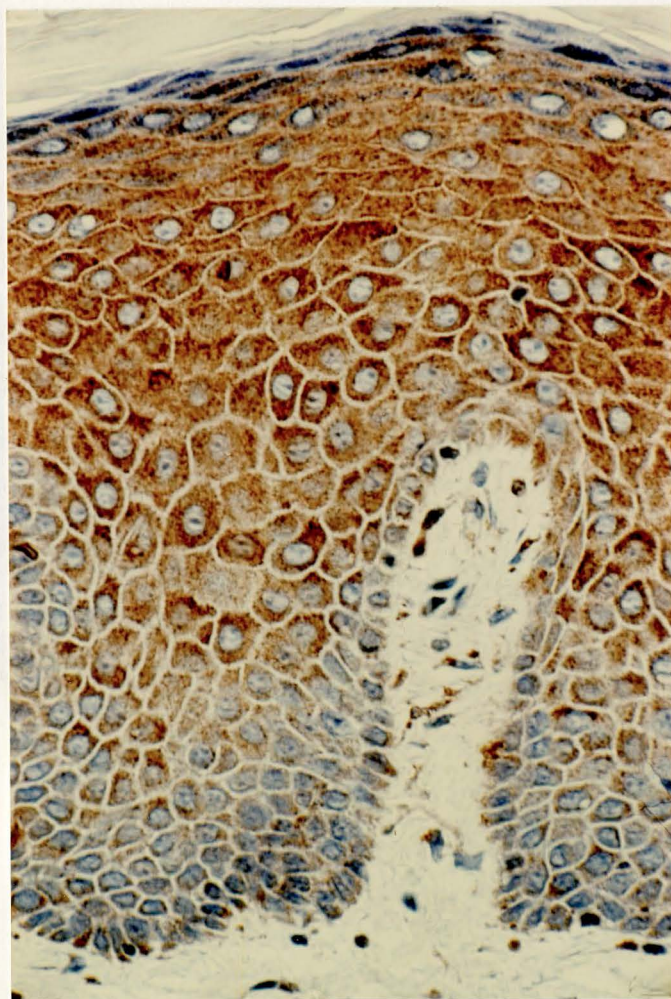


Fig. 24

Similar pattern of staining for glucosyl, mannosyl residues in well differentiated squamous cell carcinoma. (ABC method, magnification X125)

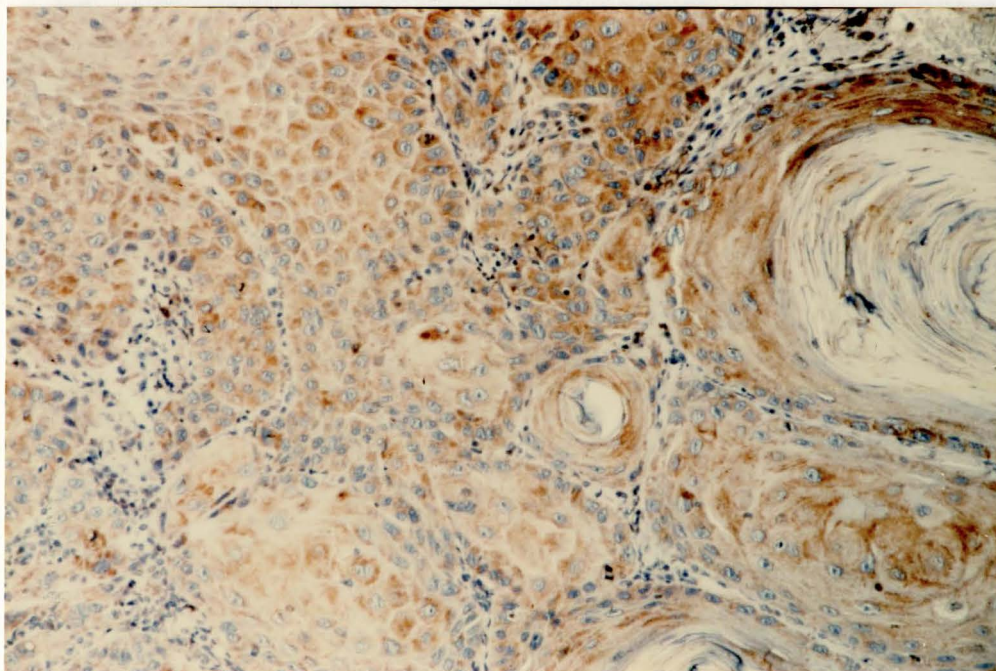


Fig. 25

Less uniform distribution of positive staining for Con A binding shown in less differentiated squamous cell carcinoma. (ABC method, magnification X125)

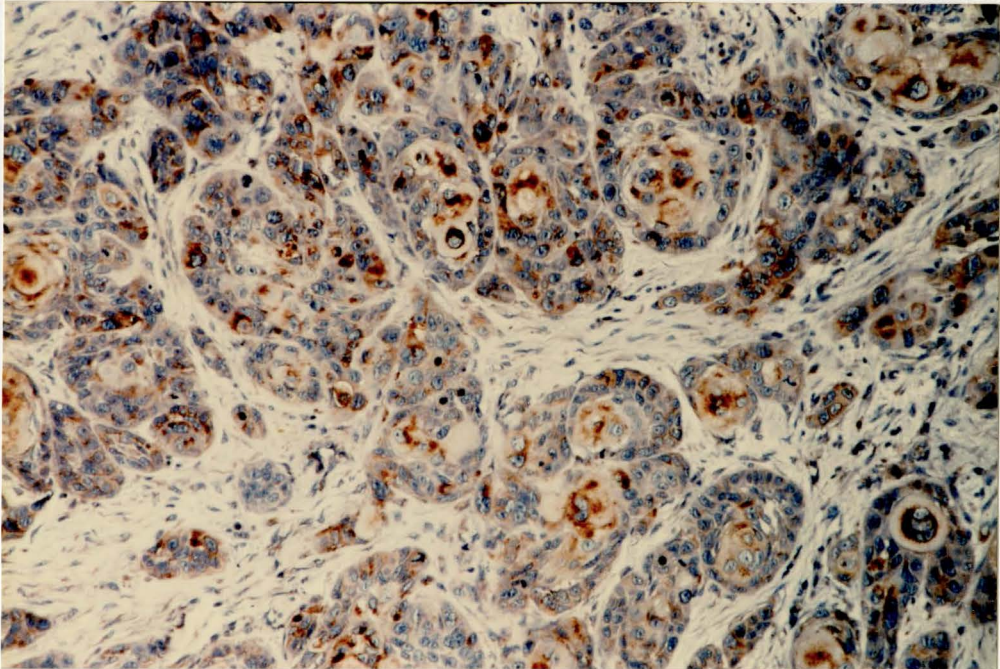


Fig. 26

The uneven staining shows the uneven distribution of glucosyl, mannosyl residues in invasive islets of tumor cells of squamous cell carcinoma.

(ABC method, magnification X313)

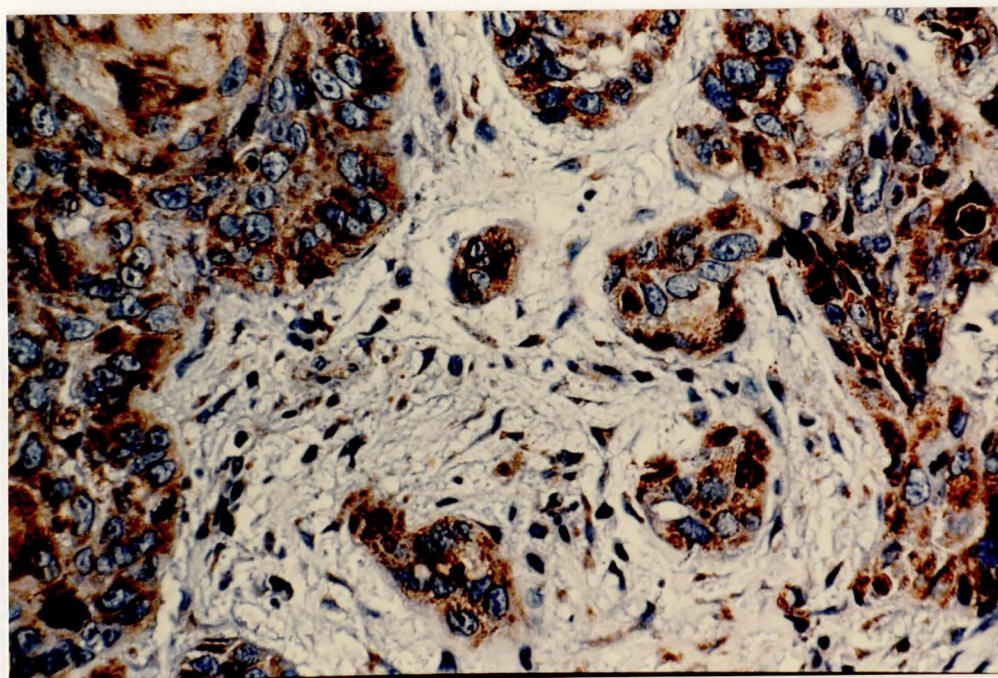


Fig. 27

Galactosyl residues are shown on the cell surface by using RCA I lectin binding ABC method in the epithelium of normal mucosa. (ABC method, magnification X125)



Fig. 28

Similar pattern of positive staining for RCA I receptors shown in well differentiated squamous cell carcinoma. (ABC method, magnification X125)

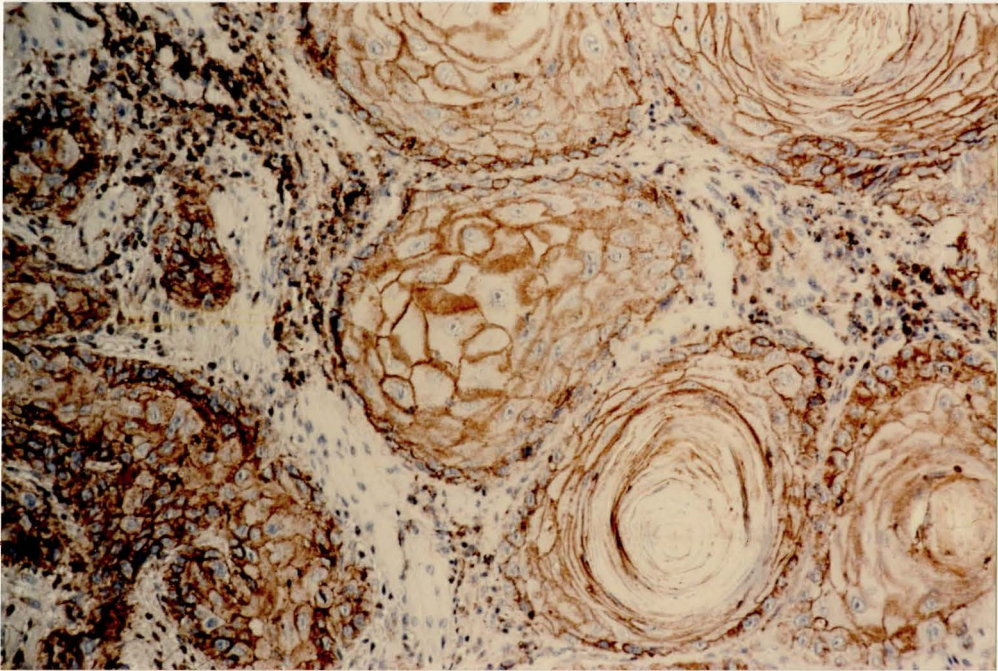




Fig. 29

Positive reaction for galactosyl residues are shown both in the cytoplasm and on the surface of the cells in the less differentiated squamous cell carcinoma. (ABC method, magnification X125)

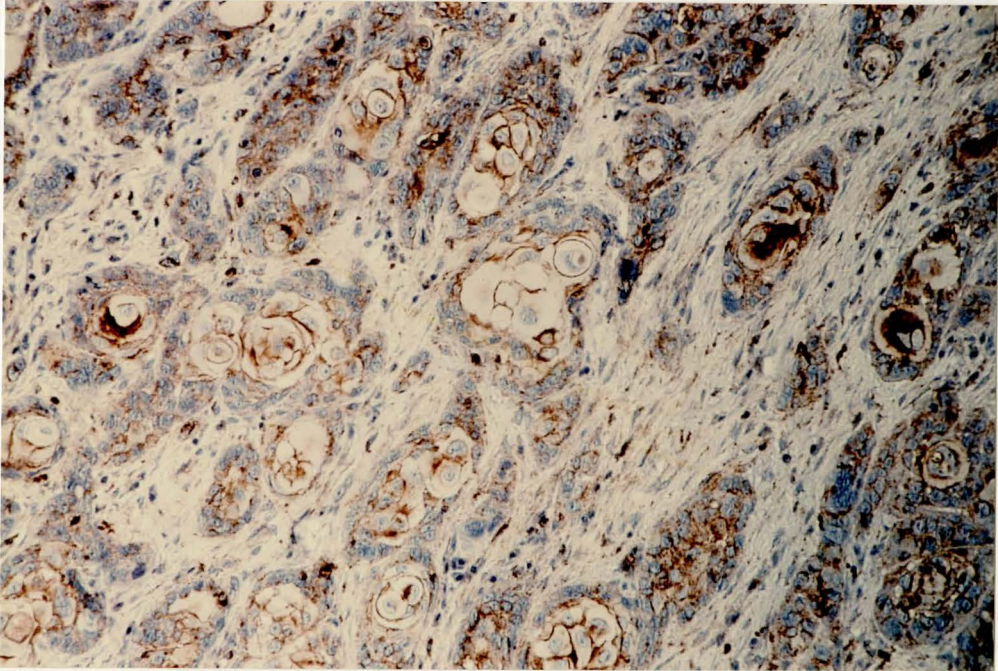


Fig. 30

Strongly positive staining of RCA I receptors in the perinuclear area is observed in the less differentiated squamous cell carcinoma. (ABC method, magnification X313)

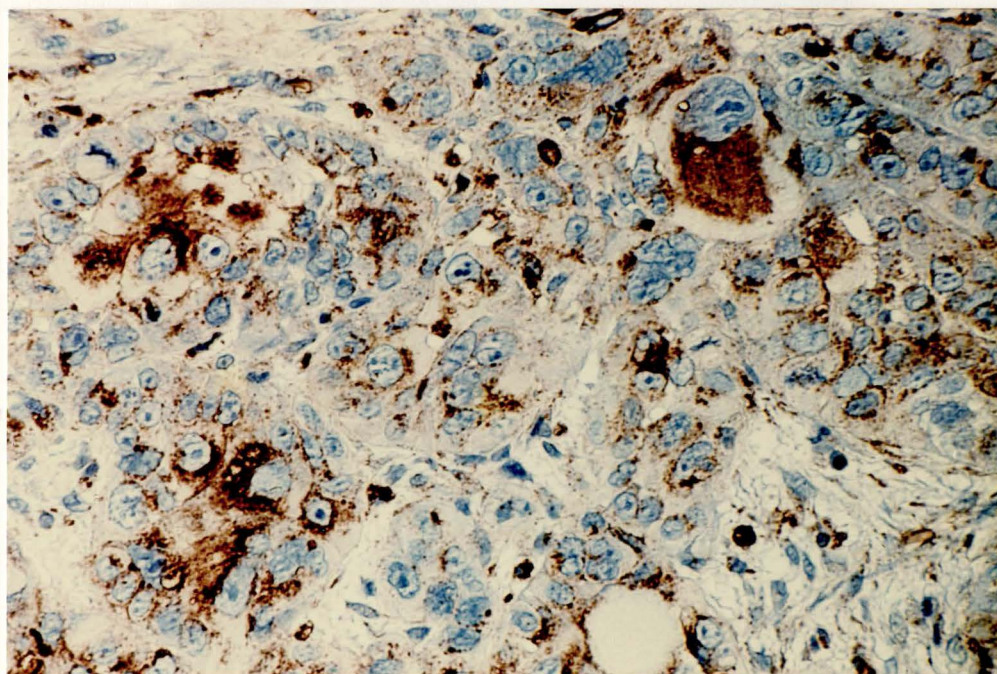


Fig. 31

UEA I, defining fucosyl residues, give a positive reaction on the cell surface of the normal mucosal epithelium. (ABC method, magnification X313)

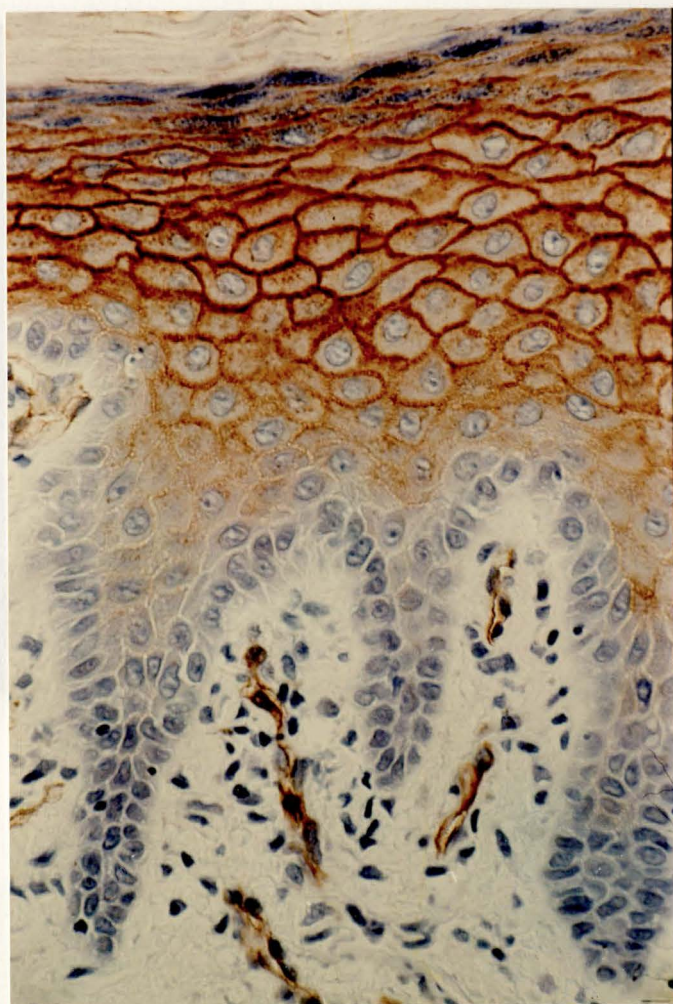


Fig. 32

Similar pattern of positive deposition for fucosyl residues by UEA I lectin binding are identified in well differentiated squamous cell carcinoma. (ABC method, magnification X125)

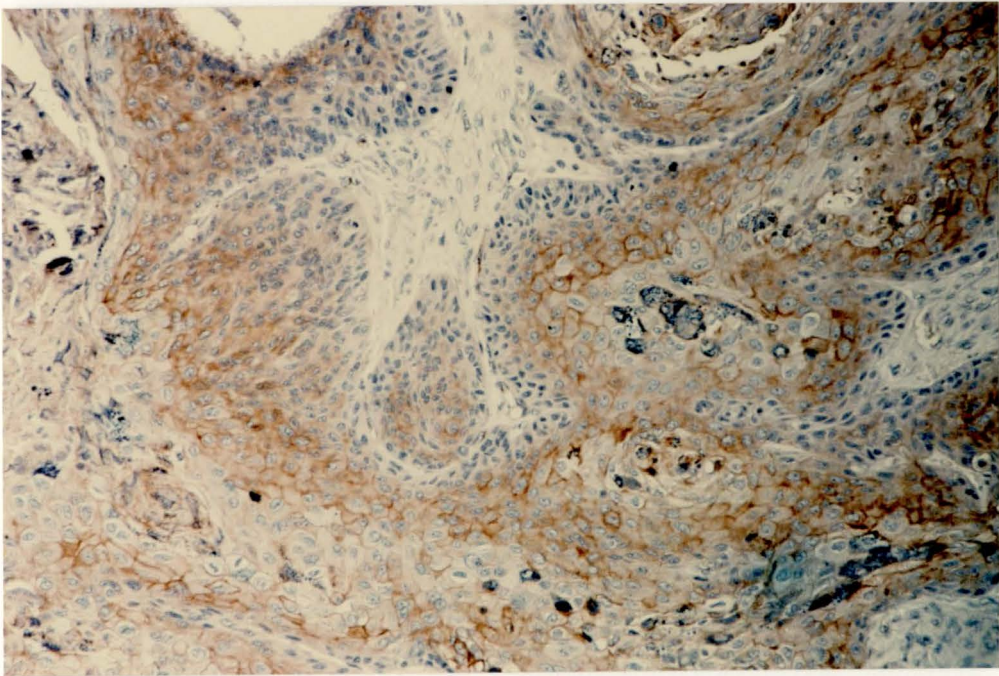


Fig. 33

Positive staining predominantly on cell surface with few cytoplasmic deposition for fucosyl residues are observed in the moderately differentiated squamous cell carcinoma. (ABC method, magnification X125)

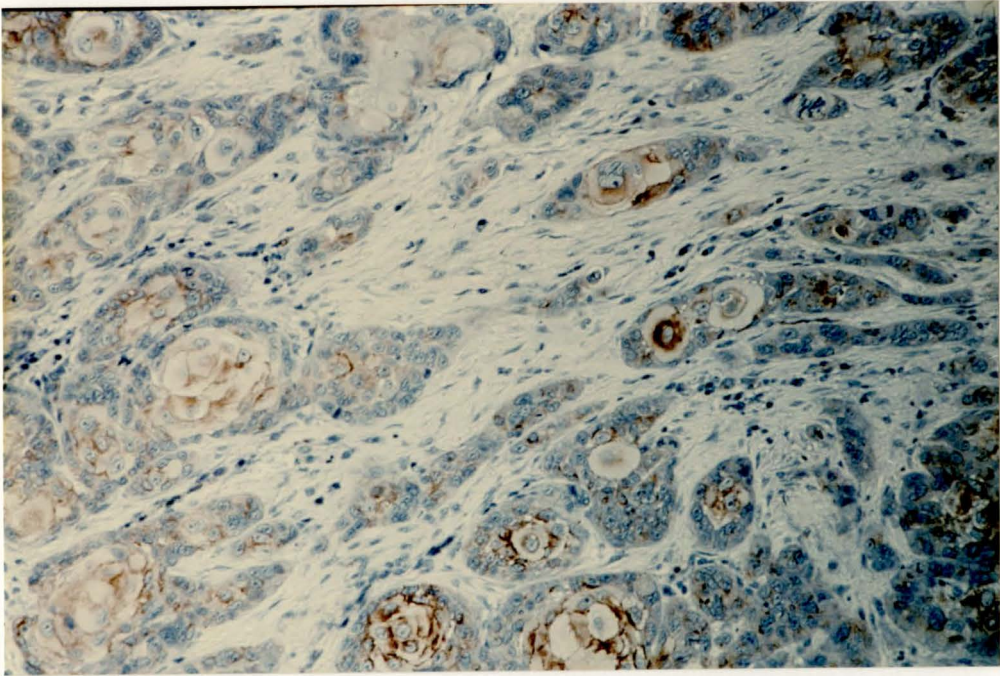


Fig. 34

UEA I receptors for fucosyl residues are shown in the plasma of the tumor cells predominantly in the case of poorly differentiated squamous cell carcinoma. (ABC method, magnification X125)

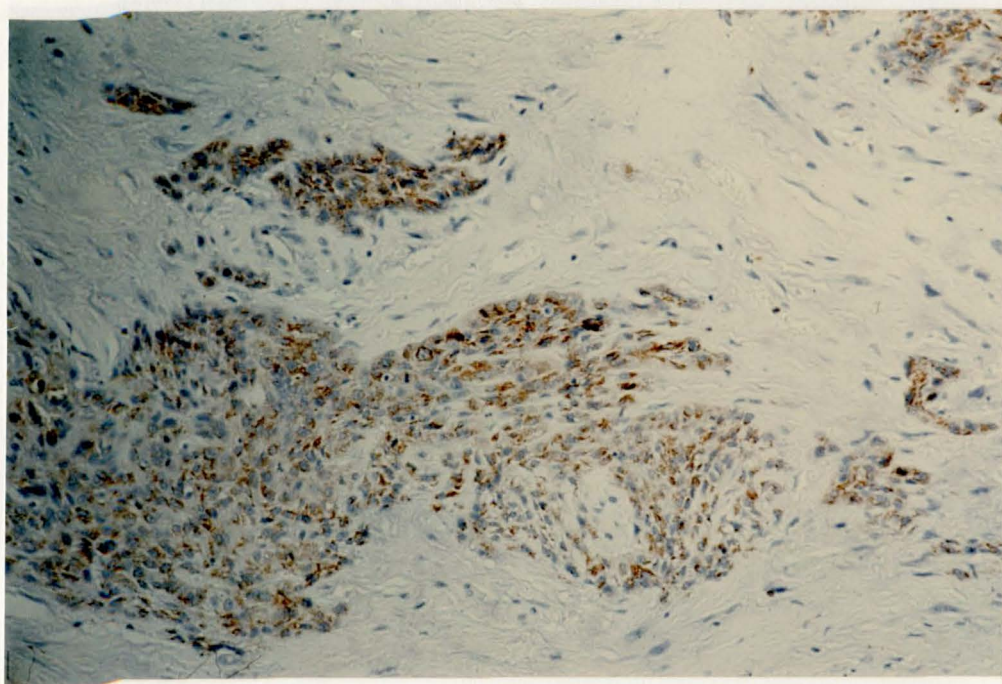


Fig. 35

LFA, defining sialyl residues, is identified on the cell surface of the normal mucosal epithelium. (ABC method, magnification X313)

method, magnification X125)

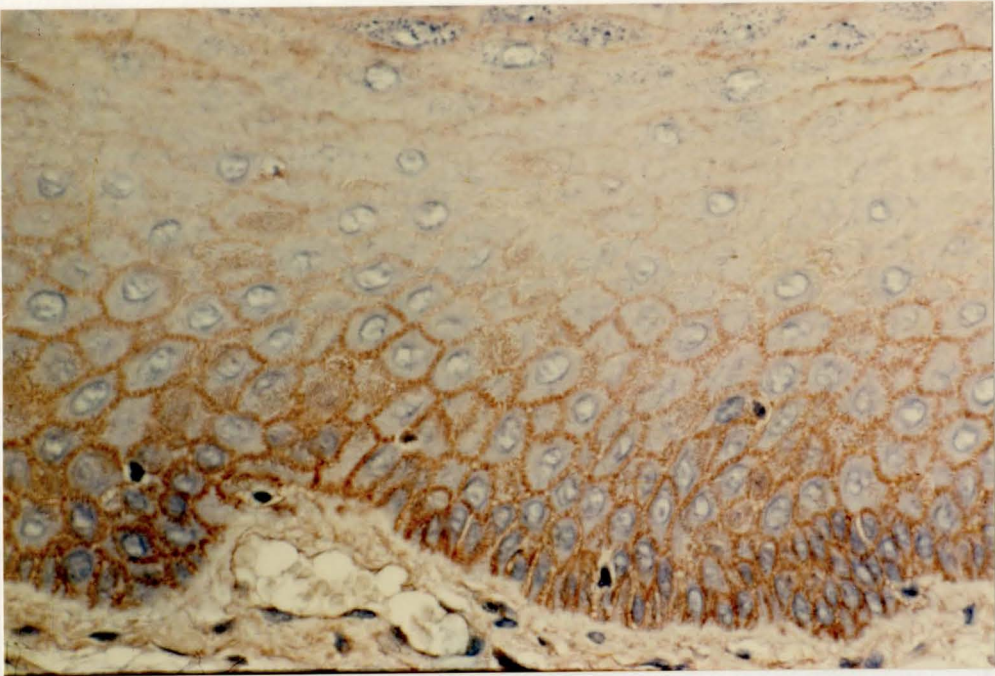


Fig. 36

Similar pattern of positive reaction for sialyl residues in well differentiated squamous cell carcinoma as compared to normal mucosa. (ABC method, magnification X125)

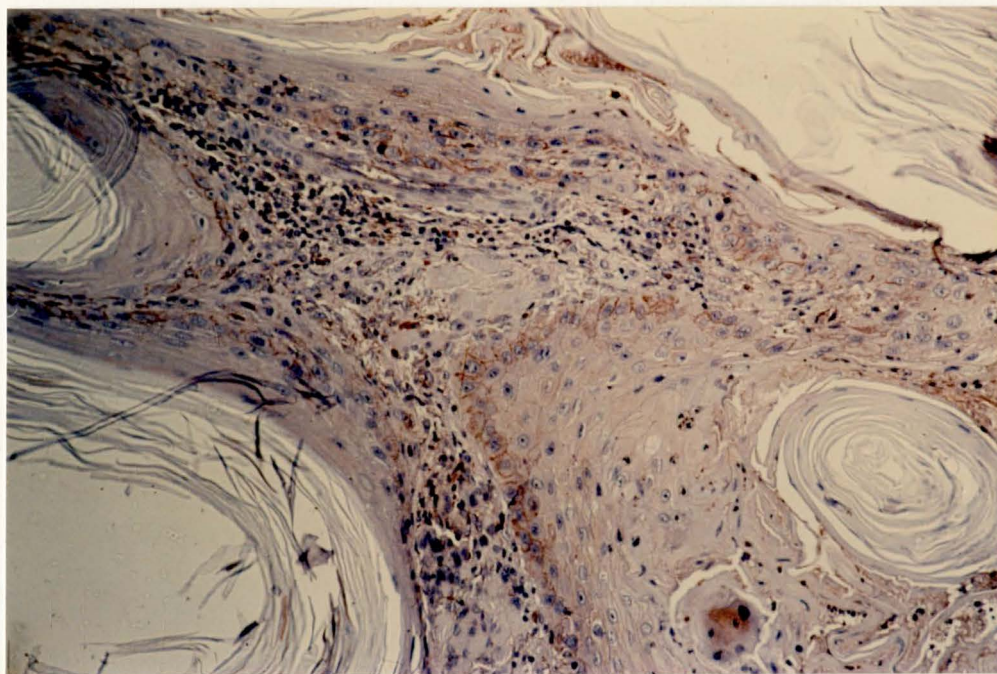
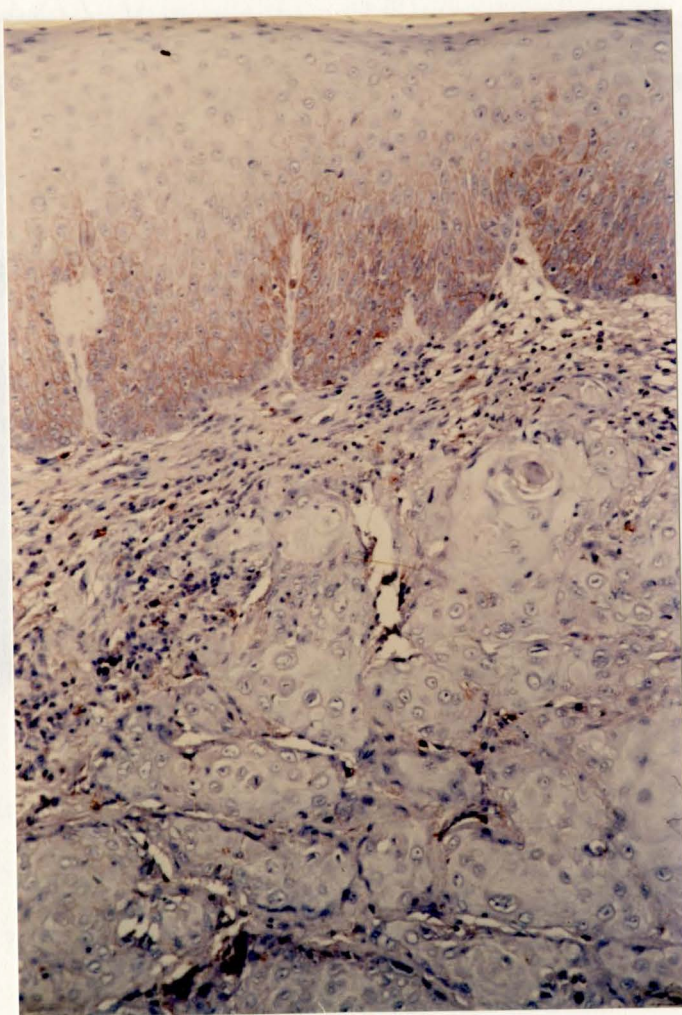




Fig. 37

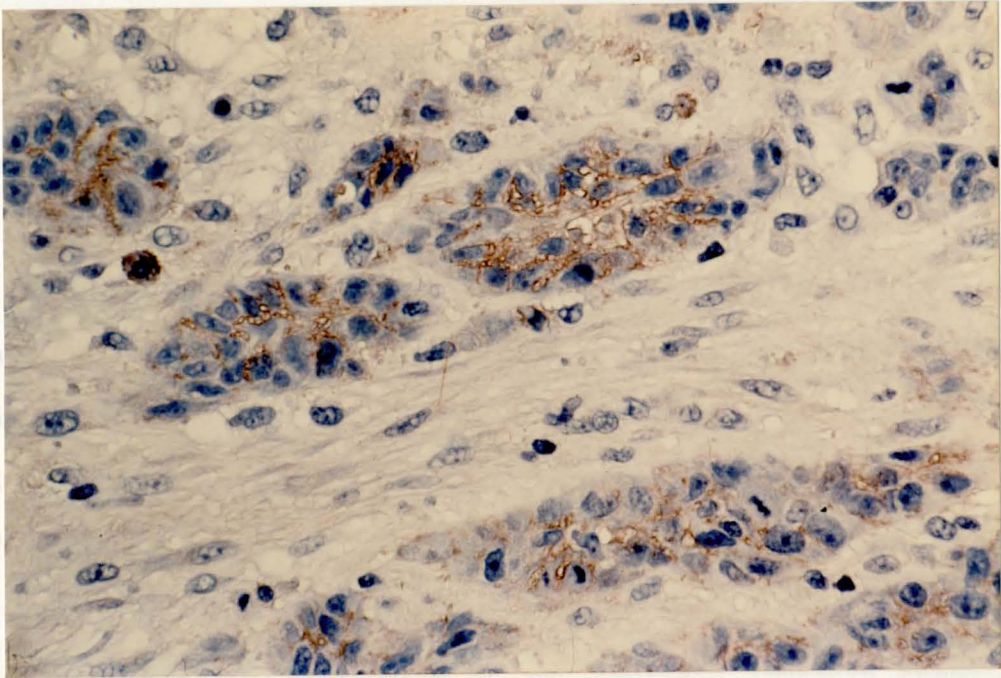
Marked decrease of positive reaction for sialyl residues in squamous cell carcinoma compared to normal mucosa. (ABC method, magnification X125)



## CHAPTER V

Fig. 38

The positive deposition of staining for sialyl residues is observed on the cell to cell contact surface in the invasive islets of tumor cells of squamous cell carcinoma. (ABC method, magnification X313)



## CHAPTER V

### DISCUSSION

#### KERATIN

The pattern of keratin identification as seen in normal squamous epithelium appears similar to that seen in the well differentiated squamous cell carcinoma. The staining reaction for keratin appears with a reduced intensity in the basal cell layer but increases in intensity up to the granular cell layer in well differentiated carcinoma. This observation suggests that an apparent normal differentiation pattern for keratin formation occurs in squamous carcinoma.

The recognition of varying degrees of positive staining for keratin in the clusters of invasive tumor cells, especially in the areas of heavy infiltration with inflammatory cells, is an important feature, since it is useful in localizing such infiltrating malignant squamous cells.

Another important feature of the keratin PAP staining in the study of squamous cell carcinoma is that the deeply invasive, small clusters of tumor cells are detectable by the positive reaction which almost never can be recognized in the H&E stained sections. This raises the question: Can a pathologist who reports that the lesion examined with H&E stained sections conclude that the lesion is well excised, and that the margins are free of tumor? This is doubtful as the keratin stain

can identify some of the few tumor cells separated from the main body of the lesion and in a deep location, whereas H&E stain alone can not. Thus such nests could be easily overlooked.

The positive staining of keratin observed in this study in normal oral mucosa and tumor cells correspond with the results of the study by Schlegel et al. (1980a, b). Moreover, the reduced, variable staining observed in the poorly differentiated tumor cells also correspond with the findings of Viac et al. (1982), who reported the absence or a very low amount of the major protein band of keratin in the squamous cell carcinoma.

#### CEA

The positive staining of CEA in the upper stratum spinosum, stratum granulosum, and stratum corneum indicates that the CEA is expressed in a visible manner immunohistochemically in the more differentiated population of squamous cells as compared to the stratum basale. Interestingly, this is in contrast to those findings in normal cervical epithelium as reported by Bychkow et al. (1983). In his study, the normal epithelium did not show any CEA staining in any of the cell layers.

The similar pattern of staining in the well differentiated squamous cell carcinoma and the normal mucosa suggests that a simulated normal pattern of differentiation occurs in the more differentiated population of squamous cells.

The observation of CEA PAP staining in this study agrees with the studies by O'Brien et al. (1981) in that the poorly differentiated carcinoma usually contains much less demonstrable surface CEA, but may,

occasionally, stain the cytoplasm strongly. Whereas, this observation is different from that reported by Lindgren and Seppala (1979), and Bychkow et al. (1983).

### β2-MICROGLOBULIN

The greater intensity of the positive staining reaction of β2-microglobulin on the surface of the epithelial cells in the stratum basale as compared with the reduced staining in the stratum spinosum suggests that the generating populations of cells contain, and may produce, a greater concentration of β2-microglobulin.

The finding that β2-microglobulin is negative in its reaction with specific antisera in the stratum granulosum and stratum corneum may be due to the reduction of concentration of the β2-microglobulin on the squamous cell surfaces as they become larger in size and fail to be demonstrated by the PAP method as used in this study. The results here are consistent with those studies reported by Forsum and Tjernlund (1977).

The tumor cells markedly show a decrease in the staining on their surfaces at the junctional area where the epithelium apparently begins its malignant change. This suggests that β2-microglobulin in squamous cell carcinoma is lost from the cell surface or fails to form when the cells are less differentiated.

One of the major differences between normal mucosa and squamous carcinoma is that the positive deposition of cell products as manifested by immunohistochemical staining methods can be detected cytoplasmically in the squamous carcinoma.

The observation of β2-microglobulin staining by the PAP method

clearly shows a significant loss of demonstrable surface  $\beta$ 2-microglobulin from the surface of the malignant cells. It suggests that  $\beta$ 2-microglobulin can serve as one of the markers for maturation of the cells using the immunohistochemical method. Furthermore, it appears that the deposits of  $\beta$ 2-microglobulin is related to the morphologic criteria used in spinosum suggests that the generating populations of cells contain, and may produce, a greater concentration of  $\beta$ 2-microglobulin.  $\beta$ 2-microglobulin have a greater tendency to demonstrate the staining for  $\beta$ 2-microglobulin in cytoplasm of the cells. This finding is consistent with those studies reported by Turbitt and Mackie (1981).

Interpretation of the finding of a strongly positive reaction in the cytoplasm of cells in mitotic division may be due to the excess amount of  $\beta$ 2-microglobulin produced during the time of abnormal mitosis.

#### LAMININ AND FIBRONECTIN

Most of the reports of studies on the differences in the localization of laminin or fibronectin between normal cells and tumor spinosum suggests that the generating populations of cells contain, and may produce, a greater concentration of  $\beta$ 2-microglobulin. 1979; Stenman and Vaheri, 1981; Hayman et al., 1981; Albrechtsen et al., 1981). In contrast, the observations in this experiment clearly show that laminin and fibronectin are present on the basement membrane of normal mucosa and also in the well differentiated squamous cell carcinoma. Moreover, they also are present in moderately and poorly differentiated squamous cell carcinomas.

However, unlike biochemical methods, the immunohistochemical

staining method can not show the quantitative differences either of laminin or fibronectin between different tissue sections. Therefore, the positive staining reactions for laminin and fibronectin in tissue sections do not rule out a quantitative decrease of laminin or fibronectin in the basement membrane of squamous cell carcinoma as may be determined by spinosum suggests that the generating populations of cells contain, and may produce, a greater concentration of  $\beta$ 2-microglobulin.

The positive staining of laminin and fibronectin does delineate the basement membrane of squamous cell carcinoma, and proves that such glycoprotein components do exist in the basement membrane. Even in the small clusters of poorly differentiated tumor cells, laminin and fibronectin are evident in a well delineated basement membrane. This suggests that there is no correlation between the presence or absence of laminin or fibronectin in basement membrane of either the squamous cell carcinoma or the normal mucosa. Furthermore, there is no qualitative difference in laminin and fibronectin in the basement membrane between the morphologically determined states of differentiation in the squamous cell carcinoma.

One of the reasons why the results of this study on laminin and fibronectin is different from the other reports is related to the methods employed in the detection. The sensitivity of the PAP method is higher than the immunofluorescence methods used in the other studies. The small amount of antigen, which can not be detected by the immunofluorescence techniques, can be detected by the more sensitive PAP technique as used in this study; which shows beautifully positive staining

of laminin and fibronectin in the basement membrane. Moreover, the positive reaction for laminin and fibronectin in the basement membrane of the blood vessels can serve as an internal control for the PAP technique.

#### CON A

Because of the strong staining of Con A demonstrating the presence of glucosyl-mannosyl sugar residues in the cytoplasm of squamous cells, it spinosum suggests that the generating populations of cells contain, and may produce, a greater concentration of  $\beta$ 2-microglobulin. lens (X400), Con A deposits can be appreciated; positively indicating a reaction in the intercellular spaces and cell surfaces.

One possible explanation for the strong perinuclear deposits of staining with Con A, especially those staining only at one pole of the nucleus, may be due to the glycosyl and mannosyl accumulation or glycosylation in the Golgi apparatus. In squamous carcinoma, it may be that the protein in the Golgi apparatus can not be delivered after glycosylation, and thus becomes accumulated in the Golgi apparatus. Another possible explanation is that the genetic control for glycosylation or mannosylation have been disturbed in squamous cell carcinoma. This may explain why some cells show negative staining while the others are strongly positive appearing phenotypically different. Furthermore, this may explain why, even in the same area of squamous cell carcinoma, the cells show individual differences in product identification.

This study, is the first to report the observation of a predominantly positive staining of Con A and other lectins in the cytoplasm of



squamous cell carcinoma by the ABC method used herein. The positive reactions for Con A both in normal and squamous carcinoma epithelia is contrary to those reported previously (Louis et al., 1981), which showed a more grossly diminished level of staining in normal epithelium than in squamous carcinoma.

#### RCA I

The reduced staining reaction for galactosyl residue receptors with RCA I in the less differentiated squamous cell carcinoma as compared to well differentiated squamous cell carcinoma and normal mucosa suggests that the more differentiated cells may contain more galactose on their surfaces than less well differentiated cells. A possible explanation for the reduction of cell surface staining with RCA I is that the transportation of galactosyl sugar residues is incomplete in less differentiated squamous cell carcinoma so that they can not reach the cell surfaces and accumulate in the cytoplasm.

The finding that the generative population of cells in the less differentiated squamous cell carcinoma showed a decrease in the RCA I surface binding as compared to the well differentiated squamous cell carcinoma; or, the stratum basale of the normal mucosa indicates that the decrease in RCA I binding sites on cell surfaces is associated with the malignant changes in the cell. However, the cells of squamous cell carcinoma retain the ability to produce galactosyl sugars.

The observation of RCA I localization in cells in this experiment is consistent with those reported by Kim et al. (1974, 1975) and contrasts with the findings reported by Dabelsteem and Mackenzie (1978),

which states that the RCA I receptors could not be demonstrated in invading islets of the neoplastic cells.

### UEA I

The generative population of cells in the stratum basale of normal mucosa and those of the squamous cell carcinoma both show negative staining for UEA I receptor. This indicates that either the fucosyl sugar residues are not produced until cells become more differentiated, or that the concentration is too low to show a positive reaction with UEA I by the immunohistochemical method used in this study.

However, like RCA I , the major difference between the squamous epithelium in the normal mucosa and that of squamous cell carcinoma is that there is variably strong staining of the cytoplasm in squamous cell carcinoma. Also, the positive reaction with UEA I on the cell surfaces of the normal mucosa is similar to that observed in well differentiated squamous cell carcinoma. This evidence of similar patterns in the localization of UEA I staining relative to the level of differentiation of squamous cells also occurs in the center of invasive islets of tumor cells of moderately squamous cell carcinoma. Very interestingly, even in the poorly differentiated squamous cell carcinoma, like well differentiated carcinoma, there is no less staining reaction with UEA I associated with the morphologic differentiation of the squamous cell carcinoma. Also, the poorly differentiated carcinoma shows an evenly distributed cytoplasmic positive reaction with UEA I.

As Holthofer et al. (1982) reported, the endothelial lining of the vessels show positive reaction with UEA I by immunohistochemical meth-

ods. This is an excellent internal control for UEA I staining, especially when in a few sections a totally negative reactions is observed both in squamous cells and endothelial cells. In such instances, this would indicate that either the fucosyl sugar residues are lost during the processing of the tissues or the concentration in the tissue is too low to be detected.

### LFA

LFA defines the sialic acid on the epithelial cell surfaces both in the normal mucosa and in the squamous cell carcinoma. However, in contrast to those studies reported by van Beek et al., 1973, 1975; Merritt et al., 1978; Mabry and Carubelli, 1972; Yogeewaran and Salk, 1981, the observation in this study shows less LFA positive reaction in squamous cell carcinoma than in normal mucosa. This indicates that the sialic acid on the malignant cell surface is either reduced or is lost.

Although the staining pattern in well differentiated squamous cell carcinoma is similar to that observed in the normal mucosa, it is unlike that seen in other oligosaccharides in this study. The sialic acid still remains localized on the cell surface, in those cases of moderately differentiated and poorly differentiated squamous cell carcinoma. Whereas galactose, mannose, and fucose localizes in the cytoplasm of the cell as defined with Con A, RCA I, and UEA staining. This consistent cell surface localization, detected with the LFA reaction, may be the unique character of sialic acid in squamous cell carcinoma.

Sialic acid can be detected by immunohistochemical method in generative population of cells in normal mucosa and in well differentiated

squamous cell carcinoma. In the invasive islets of tumor cells, the surface positive reaction for sialic acid only exists on the cell contact surface; but is negative in the basement membrane cell surface area. This observation may suggest that the loss of surface sialic acid, signifies malignant changes of the squamous cells. This is especially true for those invasive clusters of cells in squamous cell carcinoma which are in contact with the connective tissue stroma. The negative reaction for sialic acid on the stromal surface of the cells may indicate that biologic activity associated with the ability of such cells to migrate, infiltrating the stroma.

#### GENERAL PATTERN

The similar staining pattern which is found both in the squamous epithelium of well differentiated squamous cell carcinoma and in the normal mucosa, shows a normal differentiation pattern and the production of glycoproteins and carbohydrates. However, when observed in moderately or poorly differentiated squamous cell carcinoma, the products of the tumor cells show variable changes. They become unevenly distributed and exhibit less staining than that seen in normal mucosa.

Even in those clusters of invasive cells, evidence of normal differentiation usually can be observed in the center portion of the islets, while differentiation is not seen in the peripheral cells. This suggests that when the subpopulation of invasive cells form clusters in the squamous cell carcinoma, even in the poorly differentiated type, such cells still have the tendency to differentiate. This also suggests that the tumor cells can recognize each other, and try to regain the

ability to form normal products which was lost in the beginning of the invasion process. The uniformity difference of staining pattern between normal mucosa and squamous cell carcinoma suggest the possibility of quantitative differences do exist.

The qualitative difference in staining reaction observed between normal mucosa and the squamous cell carcinoma is seen in CEA,  $\beta$ 2-microglobulin, RCA I, and UEA I

in which the products are localized in the cytoplasm of the cells in squamous cell carcinoma but not in the cytoplasm of the normal mucosa. This may serve as a tumor marker for the squamous cell carcinoma. Further, similar studies on the squamous cell carcinoma arising from the skin, esophagus, and lung, is suggested

Although keratin, Con A, and LFA do not show any qualitative differences between normal mucosa and squamous cell carcinoma; the uneven, variable staining seen in the squamous cell carcinoma still may serve as a reference, suggesting the occurrence of malignant changes in the cells. Keratin definition is especially useful when a pathologist is trying to determined how deeply the squamous cell carcinoma has invaded the supporting stroma or if the margin of a specimen is free from the tumor cells.

The findings in this study clearly suggests that further immuno-histochemical studies should include the different tumors of squamous epithelial origin such as basal cell carcinoma, verruca carcinoma, kera-toacanthoma, verruca vulgaris, and condyloma accuminatum. This may provide a method to assess the different products in different tumors of

the same cell of origin.

## CHAPTER VI

### SUMMARY AND CONCLUSION

Sixty cases of oral squamous cell carcinoma (36 cases of well differentiated type, 16 cases of moderately differentiated type, and 8 cases of poorly differentiated type) and 5 cases of normal oral mucosa were studied by immunohistochemical technique for product identification. Using specific antibodies by PAP technique for identifying glycoproteins and using high affinity lectins by ABC technique for identifying cell surface oligosaccharides.

In normal oral mucosa, CEA,  $\beta$ 2-microglobulin, galactose, fucose, and sialic acid were detected on the cell surface; keratin, mannose, and glucose are detected in cytoplasm of the cells and variably positive or negative reaction is observed on the cell surface.

In oral squamous cell carcinoma, CEA,  $\beta$ 2-microglobulin, galactose, and fucose were detected both in the cytoplasm and on the surface of the cells; keratin and Con A have positive reactions in the cytoplasm of the cells, but variably positive or negative reactions occur on the cell surfaces; LFA is detected on the surface of the cells but is negative in the cytoplasm of such cells.

Laminin and fibronectin are observed positively both in the basement membrane of the normal mucosa and in the squamous cell carcinoma.

Normal differentiation does occur in squamous cell carcinoma,

especially the well differentiated type. In the less differentiated squamous cell carcinoma, there is apparently some disturbance in the production of glycoproteins and carbohydrates. It is evident that the normal processing of products of the cells can be disturbed during their malignant transformation. However, after such tumor cells aggregate to form a subpopulation of cell clusters, the normal differentiative products distribution usually can be found in cell to cell contact surface and in the cytoplasm of those cells located at the central portion of that subpopulation.

There is a difference in product identification as noted between the normal oral mucosa and oral squamous cell carcinoma. There is an even pattern of product distribution in normal mucosa, whereas there is an uneven, variable reaction in staining, contrasting with a focal strong product deposition pattern in the squamous cell carcinoma.

Qualitatively speaking, CEA,  $\beta$ 2-microglobulin, galactose, and fucose were observed by positive immunohistochemical reaction in the cytoplasm of the cells in squamous cell carcinoma whereas those products were not detected in the squamous epithelium of the normal mucosa. This provides for a qualitative difference between the normal oral mucosa and the oral squamous cell carcinoma.

The basement membrane, however, does not serve as a factor of malignant transformation or invasion of the squamous cell carcinoma. Laminin and fibronectin always can be observed in the basement membrane of the squamous cell carcinoma and can not serve as a differentiative factor when employing immunohistochemical methods for evaluation.



This study shows that different patterns of product identification exists between normal oral mucosa and oral squamous cell carcinoma. This may serve better to define normal tissues and to differentiate malignant transformation. Also, such a method can improve the utility of current histomorphologic standards employed for criteria in establishing the diagnosis of oral squamous cell carcinoma.

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APPROVAL SHEET

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the Committee with reference to content and form.

The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Master of Science in Oral Biology.

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