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A 22-kd Surface Antigen Detected by Monoclonal Antibody E 48 Is Exclusively Expressed in Stratified Squamous and Transitional Epithelia

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After immunization of mice with viable cells of a metastasis of a laryngeal squamous cell carcinoma, a monoclonal antibody E 48 was obtained that detects an epitope present exclusively in squamous and transitional epithelium and their neoplastic counterparts. Immunoblotting revealed that E 48 recognizes a 22 kd molecule. Seventy-five of 76 squamous cell carcinomas from the head and neck, lung, cervix, and skin stained positively, whereas various adenocarcinomas from the colon, lung, and breast, and small cell lung carcinomas consistently stained negatively. The E 48 antigen, which is formaldehyde resistant, appears to be a reliable marker for differentiation of squamous cell carcinomas from adenocarcinoma. and small cell carcinomas. (Am J Pathol 1990, 136:191–197)

It has been recognized that within a certain epithelial tumor several directions of differentiation can be found.[1] For instance, in cytologic specimens, approximately 50% of all lung tumors can be classified as admixtures of epidermoid and adenocarcinomatous subtypes.[2] Also at the electromicroscopic level, up to 50% of lung tumors classified as squamous cell carcinomas on the base of routine histology show characteristics of adenocarcinomatous differentiation.[3] The monitoring of multidirectional differentiation is thought to be of clinical importance.[4] It is therefore useful to have markers for the different pathways of epithelial differentiation. Squamous cell carcinoma is the most common neoplasm among carcinomas from the head and neck region, lung, cervix, and epidermis. Specific markers for squamous differentiation are involucrin[5] and keratin 10.[6] These antigens, however, are present only in differentiated cells and consequently cannot be used to differentiate poorly differentiated squamous cell carcinomas from morphologically undifferentiated tumors. Therefore, we attempted to produce a monoclonal antibody (MAb) that recognizes poorly differentiated squamous epithelial cells. This study reports such an antibody—E 48—and describes some of the biochemical characteristics and light microscopic and electromicroscopic distribution in cells of tissues with reactive and neoplastic changes.

Materials and Methods

Immunogen

A surgically removed metastasis of a moderately differentiated squamous cell carcinoma from the larynx (T3N1M+) was cut into pieces and treated with a mixture of 0.1% collagenase and 0.03% DNAse in Hank's balanced salt solution (HBSS).[7] The single cells were used for immunization or stored in liquid nitrogen. Screening and immunization were essentially the same as described previously.[8]

Immunization and Hybridoma Production

Balb/c mice were injected intraperitoneally with 10 million viable cells. Four weeks later, an intrasplenic booster was given under general anesthesia.[9] Three days later, the spleen was removed and the dissociated spleen cells were fused with the nonproducing cell line SP-2/0. Growing hybridomas were screened on enzyme-linked immunosorbent assay (ELISA) for immunoglobulin production

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and for reactivity with red blood cells. Selected antibodies were screened further if they reacted with frozen sections from oral mucosa, submandibular gland, and the tumor for immunization. Hybridomas showing selective binding were stabilized by limiting dilution. Large amounts of antibody were obtained by ascites-producing mice primed by incomplete Freund adjuvant.

Isotypes of the MAb

Isotype determination was performed with the use of an 96-well ELISA plate coated with affinity-purified rabbit anti-mouse subclass specific antibodies (IgG1, IgG2a, IgG2b IgG3, and IgM; Nordic, Tilburg, The Netherlands)

Tissues

Neoplastic and non-neoplastic tissues were obtained from surgical procedures. Tissues also were derived from autopsies performed within eight hours after death. Tissues were stored in liquid nitrogen.

Cell Lines

Human squamous carcinoma cell lines of the head and neck were provided by Dr. T. Carey, University of Michigan, Ann Arbor, MI. UMSCC 14-A was derived from a carcinoma of the floor of the mouth, 11-B from a laryngeal carcinoma, 22-A and 22-B originated from a primary and a metastatic carcinoma of the larynx, respectively. A 431, derived from a vulva squamous cell carcinoma, was a gift of Dr. B. Defize, Hubrecht Laboratory, Utrecht, The Netherlands. All cell lines were cultured in RPMI Hepes containing 10% fetal calf serum (FCS).

Cell ELISA

Squamous cell carcinoma cell lines growing as monolayers in 96-well ELISA plates were used for determining binding of the antibody to the outer surface of viable tumor cells. When a confluent culture was seen, plates were washed with phosphate-buffered saline (PBS) and incubated with supernatant of MAb E 48 for one hour at room temperature. Thus, membrane reactivity only was detected and not cytoplasmic or nuclear reactivity; MAb K 112, which reacts with a 43-kd nuclear antigen present in the nuclei of the tested cell lines, served as a negative control.[10] Subsequently the plates were washed, incubated with peroxidase-labeled goat anti-mouse immunoglobulin, washed, and o-phenylendiamine dehydrochloride (Sigma Chemical Co., St. Louis, MO) in citrate buffer (pH 5.0) together with 10 μl of 33% H_2O_2 was added as chromogen. Color development was stopped by adding 2 N H_2SO4. Absorbance was red at 492 nm.

Immunoperoxidase Staining

Four to six micrometer thick sections of frozen tissue blocks were prepared with a cryostat (Jung-Reichert, Nussloch, FRG), mounted on poly-L-lysine coated glass slides, air dried, and acetone-fixed during ten minutes at room temperature. Sections also were cut from neutral buffered, formalin-fixed, paraffin-embedded material. Subsequently these sections were deparaffinized, rehydrated, and treated with 0.5% H_2O_2 in methanol to block endogenous peroxidase activity. Immunoperoxidase staining was performed as described previously.[11]

Enzymatic Sensitivity

The following purified preparations were used: trypsin (227 U/mg, Sigma Chemical Co, St. Louis, MO) pronase (70,000 PUK, Calbiochem, San Diego, CA), and neuraminidase (0.5 U/ml, Vibrio Cholerae, Behring, Amsterdam, The Netherlands). Periodate treatment was essentially the same as described by Woodward, Young, and Bloodgood.[12]

Radioiodination

Iodination of E 48 was performed according to the one-vial method described by Haisma, Hilgers, and Zurawski.[13] In short, 200 μg of antibody in 0.1 M borate buffer (pH 8.2) was mixed with 1 mCi ^{125}I in a vial previously coated with 1 ml Iodogen (50 μg/ml). After ten minutes at room temperature, a sample was taken to determine the amount of incorporated iodine. One milliliter of AG1-X8 resin (Bio Rad, Richmond, CA) previously mixed with PBS containing 1% bovine serum albumin (BSA) was added to absorb unbound iodine.

SDS-PAGE and Immunoblotting

SDS-PAGE was carried out as described by Laemmli,[14] using 5% to 15% linear acrylamide gradient slab gels. Molecular weight markers (Rainbow, Amersham, UK) measured 200 kd (myosin), 92.5 kd (phosphorylase A), 69 kd (BSA), 46 kd (ovalbumin), 30 kd (carbonic anhydrase), 21.5 kd (trypsin inhibitor), and 14.3 kd (lysozyme). Proteins were transferred from the gel to the nitrocellulose with a Multiphor II Nova Blot System (LKB, Bromma, Swe-

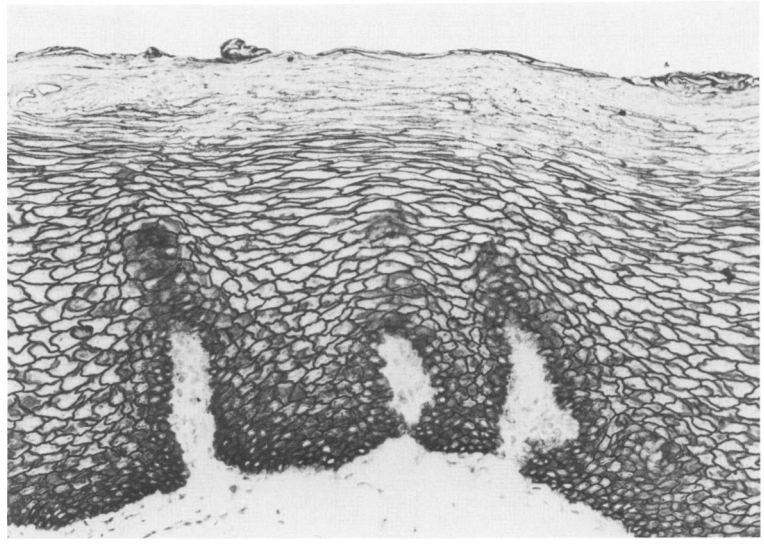

Figure 1. *Indirect immunoperoxidase staining of oral mucosa by MAb E 48 ×240. Note the distinct surface staining in all layers.*

den) for semi-dry electrophoretic transfer using a discontinuous buffer system. After pre-incubation with PBS containing 10% FCS for 30 minutes at room temperature, the nitrocellulose sheets were overlayed with ^{125}I-labeled E 48 (1×10^5 cpm/ml) for two hours. The sheets were then autoradiographed by exposure to x-ray film with a Kodak X-Omatic intensifying screen for 18 to 24 hours at −80 C.

Results

Selection and Isotype

Of the antibody-producing hybridomas obtained by fusion, one was selected on the basis of its specific reactivity with squamous cells, as shown on frozen sections of human oral mucosa and the metastasis of the squamous cell carcinoma used for immunization. Cells obtained after the hybridoma was recloned twice produced an IgG1 antibody called MAb E 48.

Reactivity of MAb with Normal Human Tissues

On frozen sections of nonkeratinizing epithelial cells of oral mucosa, MAb E 48 labeled the surfaces of the keratinocytes exclusively (Figure 1). In this study, all immunohistochemical stainings were performed on frozen sections to exclude the possibility that lack of reactivity was due to masking of the epitope by the fixatives. Experiments then were performed with B5- and formalin-fixed, paraffin-embedded sections and similar results were obtained (Figure 2 is an example of reactivity in formalin-fixed material). All squamous epithelial cells of oral mucosa exhibited reactivity with the E 48 antibody, regardless of their differentiation stage. Basement membranes did not react. Similar staining was observed in stratified epithelia of the esophagus, vagina, and human epidermis. In epidermis, no staining was seen in the stratum cor-

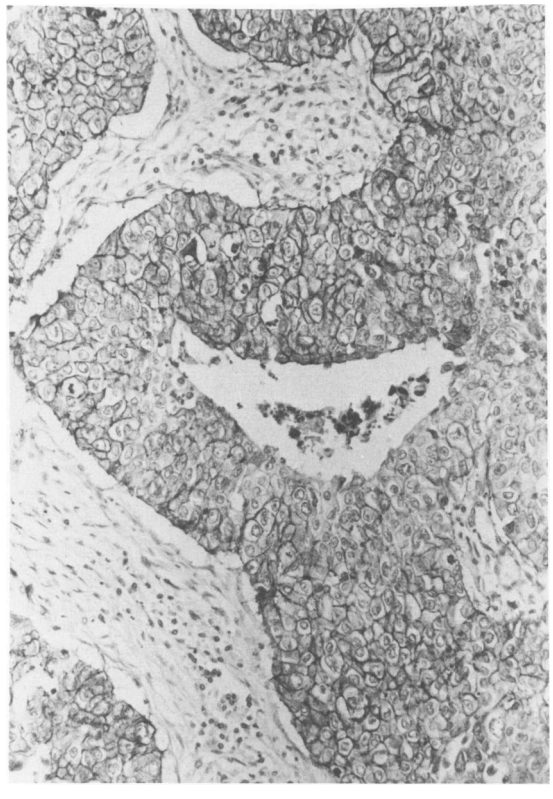

Figure 2. *Large-Cell undifferentiated carcinoma of the lung. Formalin fixation. Indirect immunoperoxidase staining MAb E 48 ×270.*

Table 1. *Reactivity of MAB E 48 with Normal Human Tissue Specimen by Immunoperoxidase Staining*

Organ	Positive/tested
Epidermis corneum	6/6 all layers except stratum
Sebaceous gland	0/6
Sweat gland	0/6
Hair follicle	4/4 outer root sheath
Oral mucosa	10/10
Esophagus	2/2
Cervix	2/2
Vagina	2/2
Bladder epithelium	3/3 all layers
Parotid gland	0/4
Submandibular gland	0/4
Mamma	0/2
Lung including trachea, bronchus pneumocytes	0/6
Stomach	0/2
Jejunum	0/2
Colon	0/9
Pancreas	0/2
Liver	0/3
Kidney	0/2
Adrenal gland	0/2
Testes	0/2
Prostate	0/4
Ovary	0/2
Uterus myometrium	0/2
endometrium	0/2
Spleen	0/3
Tonsil	0/4 except for epithelium
Lymph node	0/4
Thymus	0/2 except for Hassall bodies
Thyroid	0/2
Parathyroid gland	0/1
Muscle	0/4
Vascular endothelial cells	0/10
Brain (cerebellum)	0/2
Pituitary gland	0/2

In negative scored tissue sections not a single cell was labeled by E 48.

neum. Sweat and sebaceous glands were negative, whereas the outer root sheath of a hair follicle did react with the E 48 antibody. Among the non-neoplastic tissues tested further, only transitional epithelial cells of the urinary bladder also reacted with the antibody (Table 1). Simple epithelia or nonepithelial tissues did not bind the E 48 antibody. Exceptions were seen occasionally in stratified cuboidal excretory ducts of glands adjacent to squamous cell carcinomas, which reacted focally with MAb E 48. Stratified squamous epithelia of guinea pig, rat, or of bovine origin did not stain with E 48, indicating lack of interspecies specificity.

Binding of MAb E 48 to Intact Viable Tumor Cells

Membrane binding of MAb E 48 to viable nonfixed cells was assessed by means of a cell ELISA technique. Four different stratified squamous cell carcinoma cell lines, UMSCC 14-A, 22-A and B, and A 431, bound the antibody in this assay. No reactivity was seen with UMSCC 11-B, however, a cell line derived from a laryngeal squamous cell carcinoma (Table 2).

Table 2. *Reactivity of MAb E 48 with Viable Tumor Cells in ELISA*

	SCC 14 A	SCC 22 A	SCC 22 B	A 431	SCC 11 B
E 48	0.43	0.65	0.75	0.69	0.11
K 112	0.09	0.08	0.10	0.12	0.11

Optical readings were taken at 492 nm. Standard deviations were less than 5%. Mab K 112, which detects a nuclear antigen, served as a negative control in order to determine surface binding only.

Identification of the E 48 Antigen in Cultured Cell Lines by Immunoblotting

Immunoblot analysis of three different cell lines of squamous cell carcinomas that reacted with the antibody in the cell ELISA identified a single band with the identical molecular weight value of 22,000 (Figure 3). After reduction of the samples by the addition of β-mercaptoethanol, recognition of the 22 kd band was abolished. When protease inhibitors were omitted, no reactivity was seen. No reactivity was seen with UMSCC 11-B under the appropri-

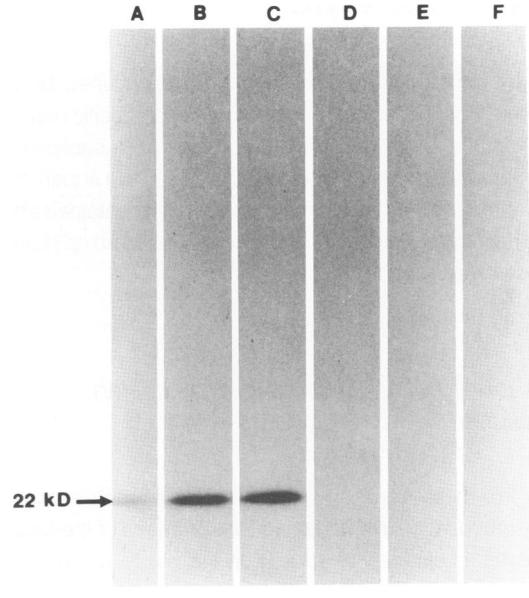

Figure 3. *Western blot analysis of cell lysates of squamous-cell carcinoma cell lines with Mab E 48. Lane A: UM SCC 14-A, lane B: UM SCC 22-B, lane C: A 431, lane D: A 431 when lysed without protease inhibitors, lane E: UM SCC 11-B, and lane F: HL 60 a leukemia cell line, both UMSCC 11-B and HL-60 are unreactive with E 48 in immunoperoxidase staining.*

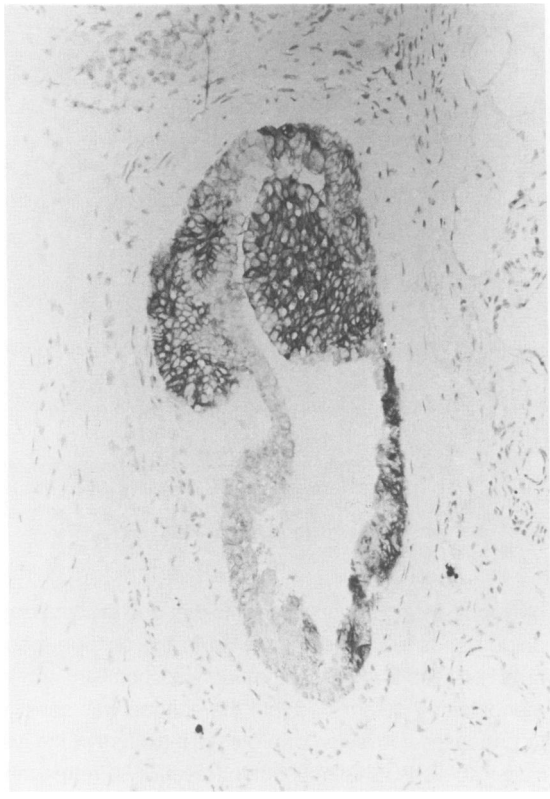

Figure 4. *Squamous-cell metaplasia in a excretory duct of a submandibular gland. Immunoperoxidase staining MAb E 48 ×180. Note the lack of reactivity of MAb E 48 with adjacent simple epithelia and the stratified cuboidal epithelium of the excretory duct.*

ate conditions, nor were cells of the leukemia tumor cell line HL 60 reactive.

Enzymatic Sensitivity

The relative sensitivity of the structure recognized by MAb E 48 to various enzymes was assayed by an immunoperoxidase technique. Pretreatment of tissue sections containing the antigen with neuraminidase or periodic acid did not influence antibody binding. In contrast, after trypsin or pronase treatment, staining disappeared. Similar observations were made for cultured cells of squamous cell carcinoma cell lines. It thus appears that the epitope is protease sensitive, but not associated with a carbohydrate chain or a sialic acid.

Reactivity with Human Reactive and Neoplastic Tissues

Squamous metaplasia, shown in a large excretory duct of the submandibular gland in Figure 4, reacted positively with the antibody. Table 3 lists the reactivity of E 48 with fresh frozen sections of neoplastic tissues as determined by indirect immunoperoxidase staining. With the exception of one solid duct carcinoma of the mamma, only squamous cell and bladder carcinomas stained positive for MAb E 48. Staining was seen on the surface and within the cytoplasm (Figure 5). Whereas in the majority of squamous cell carcinoma all cells were labeled by the antibody, some tumors expressed the antigen only in the more differentiated cells (Figure 6). In general, however, there was no correlation between the intensity of E 48 staining or the number of cells reacting positively with MAb E 48 and the morphologic degree of differentiation. Special attention was given to lung carcinomas, because in these tumors it can be difficult to discriminate between the areas of the various subtypes of differentiation (adenocarcinoma, squamous cell carcinoma, and undifferentiated large and small cell carcinoma). In some lung tumors containing both areas with squamous cell differentiation and large cell undifferentiated areas, positive staining for MAb E 48 occurred in both areas, suggesting that in these cases, early commitment to squamous cell differentiation in light microscopic undifferentiated areas already can be seen with MAb E 48. MAb E 48 also reacted with poorly differentiated areas in two adenocarcinomas and two large cell undifferentiated carcinomas, which all contained squamous cell carcinoma features at the electron microscopic level (ie, the presence of tonofilaments).

Table 3. *Reactivity of E 48 with Human Neoplastic Tissues on Frozen Sections by Immunoperoxidase Staining*

Carcinomas	Positive/tested
Squamous Cell	
Head and neck	58/58
Lung	14/15
Cervix	2/2
Skin	1/1
Nonsquamous Cell	
Urinary bladder carcinomas	3/3
Lung	
large-cell undifferentiated carcinomas*	2/3
Small-cell carcinomas	0/4
Adenocarcinomas*	2/17
Adenocarcinomas of the cervix	0/2
Adenocarcinomas of the breast	1/7
Adenocarcinomas of the colon	0/9
Ovary	
Serous carcinoma	0/5
Mucinus carcinoma	0/3
Sarcomas	0/4
Melanomas	0/2
Non-Hodgkin lymphoma	0/1

* Positive staining focally in poorly differentiated areas, electron microscopic examination revealed the presence of tonofilaments in areas positive for E 48.

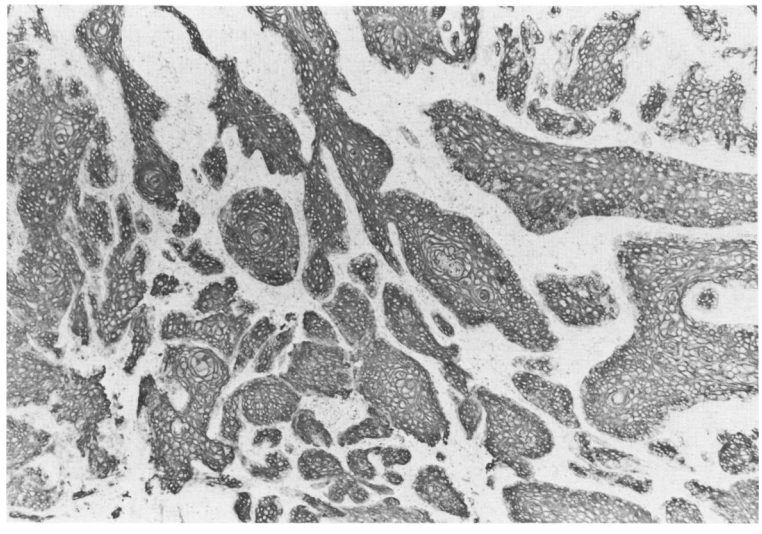

Figure 5. *Indirect immunoperoxidase staining of a moderately differentiated squamous-cell carcinoma of the floor of the mouth by MAb E 48 ×90.*

Discussion

This study demonstrates that MAb E 48 recognizes an epitope that, among normal tissues, is expressed exclusively in stratified squamous epithelia and transitional epithelium. Staining often was seen on the cell membrane in a punctated fashion, with no cytoplasmic reactivity. Stratified cuboidal epithelia present in large excretory ducts generally did not react, nor did any other tissue containing simple epithelium. Exceptions were occasionally seen among apparently normal excretory ducts of a gland adjacent to a squamous cell carcinoma. The antigen detected by MAb E 48 appears to be specific for humans because stratified squamous epithelia of guinea pig, rat, and bovine origin (muzzle) failed to exhibit any reactivity.

Immunoblot analysis revealed that the antigen is a 22 kd molecule under nonreducing conditions. The susceptibility to reducing agents suggests that one or more disulphide bands are directly involved in the epitope recognized by MAb E 48. Both immunohistochemistry and immunoblotting determined that the epitope was sensitive to proteases. Omission of protease inhibitors in the lysis buffer abolished the immunoblot reactivity. Pretreatment of frozen sections with pronase also resulted in loss of reactivity. In contrast, pretreatment with periodic acid or neuraminidase did not alter the labeling of the, antibody suggesting that E 48 recognizes a peptide epitope rather than a carbohydrate or sialic acid structure.

The specificity of MAb E 48 for the recognition of squamous epithelial differentiation was underlined further by the finding that 75 of 76 squamous cell carcinomas reacted with MAb E 48, whereas no adenocarcinomas or small cell carcinomas were reactive. Only one poorly differentiated solid duct carcinoma of the mamma

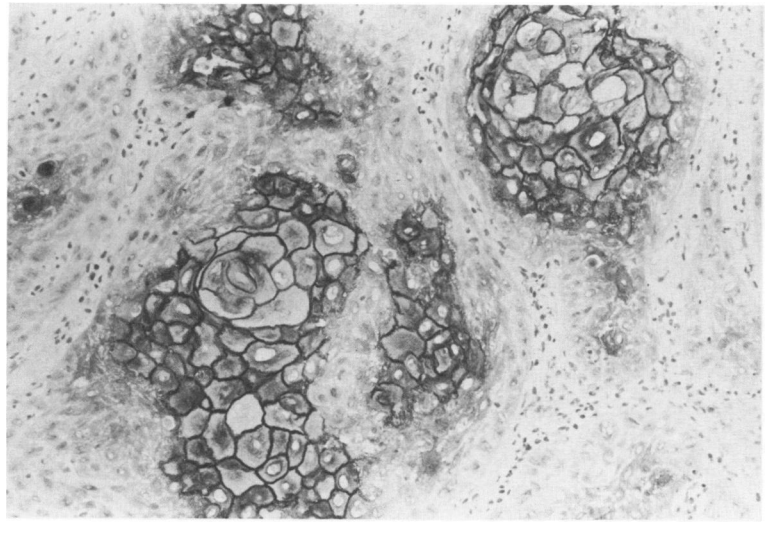

Figure 6. *Moderately differentiated squamous-cell carcinoma of the tongue showing loss of antigen expression in the more peripheral cells of the tumor nests. Indirect immunoperoxidase staining MAb E 48 ×240.*

showed focal staining. Differentiation into squamous cell direction could not be demonstrated at the electromicroscopic level due to lack of tissue. All squamous cell carcinomas reacted with Mab E 48 regardless their site of origin and their morphologic degree of differentiation. Heterogeneity of E 48 expression was not observed in the majority of squamous cell carcinomas. In 6 of 76 squamous cell carcinomas, a restricted number of cells reacted with the antibody. Two morphologically diagnosed adenocarcinomas and two large cell undifferentiated carcinomas that reacted focally with E 48 also showed characteristics of squamous cell differentiation at the subcellular level. In squamous cell carcinomas the antigen was detected both on the membrane and within the cytoplasm. Experiments with viable tumor cells showed clearly that the antigen was located on the outer surface. Compared with normal squamous epithelium, squamous cell carcinomas showed enhanced cytoplasmic staining, which might be an indication of internalization of the antigen.

The antigenic structure of the E 48 antigen is unknown presently. On the basis of its molecular weight and tissue distribution, it represents an antigen not previously described in human tissues. The high specificity of MAb E 48 for squamous cell differentiation and the related transitional epithelial differentiation, as well its reactivity with formalin-fixed tissue, make the antibody valuable for diagnostic purposes. Moreover, the accessibility of the epitope on the outer cell surface in the majority of squamous cell carcinomas and the restricted expression in normal tissues suggest that the antibody is a suitable candidate for immunotargeting. In fact, squamous cell carcinoma xenografts in nude mice could be localized successfully with radiolabeled MAb E 48.[15] Systemic studies on the nature of the epitope must determine its precise role as a tissue-specific antigen.

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