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MONOCLONAL ANTIBODIES TO SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK

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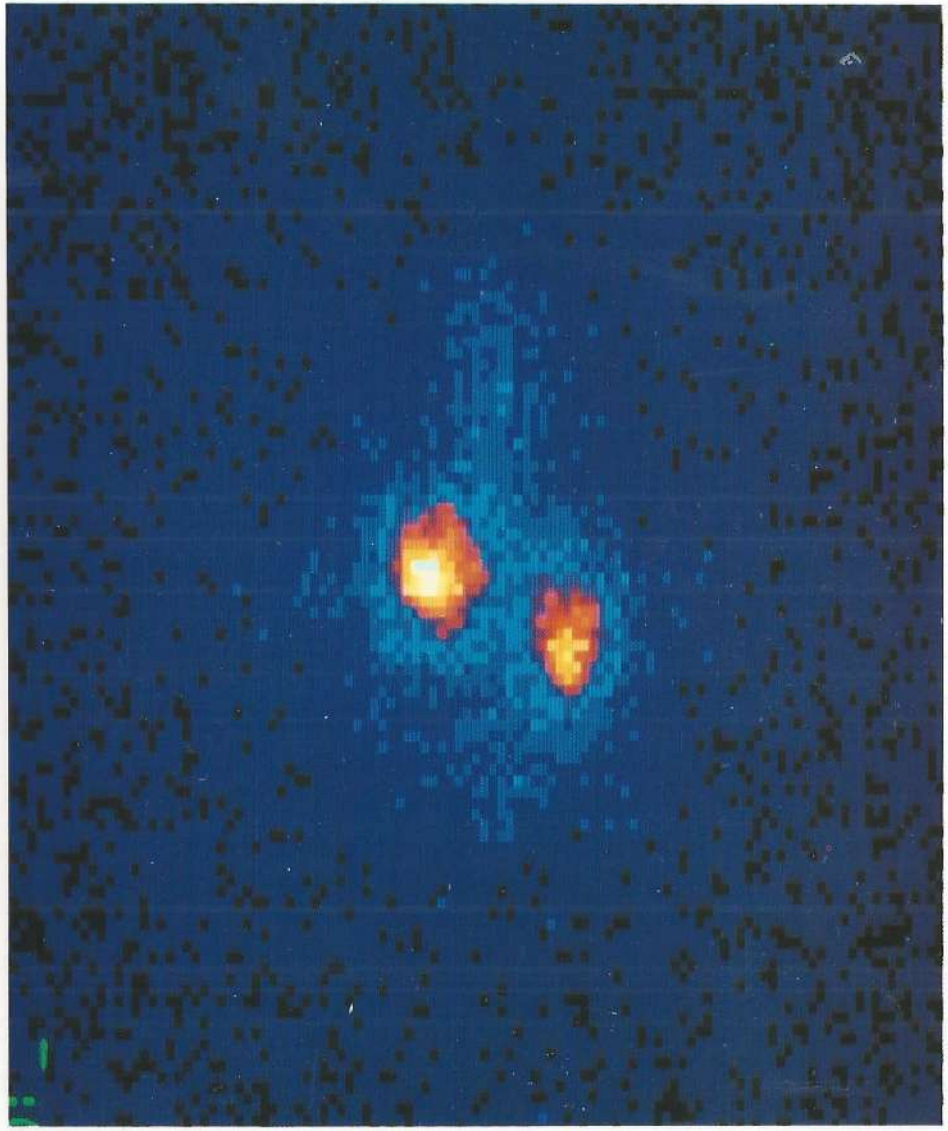

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VRIJE UNIVERSITEIT TE AMSTERDAM

MONOCLONAL ANTIBODIES TO SQUAMOUS CELL CARCINOMAS OF THE HEAD AND NECK

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Voor Esther

Voor Esther

CHAPTER 1

INTRODUCTION TO THE THESIS

1.1.

Squamous cell carcinomas of the head and neck

Incidence,diagnosis and therapy. Tumours of the head and neck include all neoplasms originating from the upper aerodigestive tract. Squamous-cell carcinoma (SCC) is the predominant histological type among these malignancies (1). They are composed by atypical squamous cells. SCC is classified histologically into well, moderately and poorly differentiated types. Well differentiated SCC show few mitoses, little anaplasia and abundant evidence of keratinization such as formation of horn pearls and numerous intercellular bridges. Poorly differentiated SCC exhibit many mitoses, anaplasia and minimal or absent keratinization. In cases where light microscopy fails to demonstrate any evidence of keratinization, electronmicroscopy may show tonofilaments and desmosomes. This is often helpful to discriminate poorly differentiated SCC from other poorly differentiated neoplasms (2). Moderately differentiated SCC show features intermediate between well and poorly differentiated types.

The incidence of SCC of the head and neck accounts for 5-6% of all newly diagnosed malignancies in North-Western Europe and the United States of America (3). A markedly higher incidence can be found in Mediterranean and South-American countries (4). This is partly related to the higher alcohol and tobacco consumption in these countries, which are major risk factors for head and neck SCC (5). Dietary factors may also play a role as risk factor in that high doses of vitamins A and C have been suggested to prevent oral cavity and laryngeal cancers, but this still has to be proven (6). Human Papilloma Virus is also linked to SCC (7), but it is yet not clear whether it serves as an innocent bystander or as a causative agent.

Patients with cancer of the head and neck are classified according the TNM classification of the Union Internationale contre le Cancer (UICC) or that of the American Joint Comittee on Cancer (AJC) (8,9). These systems are based on the assessment of three components: T -extent of primary tumour; N -status of regional lymph nodes in the neck; M -presence or absence of distant metastases. The addition of numbers to these three components (eg T1, T3 etc.) indicates the extent of the malignant disease. After assigning T, N, and M categories these may be grouped into stages. Small tumours (T1 and T2) without lymph node involvement (N0) and distant metastases (M0) are classified as stage I and II, the advanced cases as stage III or IV. Staging is important for treatment planning (10). Also survival rates are directly linked to the tumour stage. Patients with a stage I or II have a relative good prognosis, while patients in stage III or IV are much more difficult to treat curatively.

It is generally recognized that the status of the cervical lymph nodes is the single most important prognostic factor in SCC of the head and neck (11,12). Both AJC and UICC attach importance to size and number of involved nodes. Clinical assessment of the status of neck nodes is still mainly based on palpation (13), although this is not a very reliable method. The overall error in assessing the presence or absence of neck node metastases from palpation alone is reported to be in the range of (20-30 %) (12-14). Also the accuracy of palpation leaves much to be desired regarding the determination of number and size of involved nodes. Modern imaging techniques like ultrasonography (US), computed tomography (CT) and magnetic resonance imaging

(MRI) have the advantage over palpation that they are objective and provide a permanent record. However, their capability of detecting small nodes is limited, whereas very often they can not discriminate between normal nodes, reactively enlarged nodes and tumour infiltrated nodes. Because of these problems with the pretreatment assessment of the status of neck nodes, it is often very difficult to decide whether the neck nodes should be treated or not and this leads to both over- and undertreatment of the neck. There is thus a great need for specific methods of detection of lymph node metastases in the neck.

Failure to eradicate the disease above the clavicles is traditionally regarded as the major cause of death in patients who have local or regional advanced squamous-cell carcinoma of the head and neck. But with a treatment consisting of surgery followed by radiation therapy a changing pattern of failure is occurring (15). Many studies report a decrease of local and regional failure rates from this type of combined therapy (12,16,17), but observe that this is not reflected in a proportionate increase of 5 year survival rates. As fewer patients die from uncontrolled disease above the clavicles, more are exposed to the risk of disseminated disease below the clavicles (and to the appearance of second malignant neoplasms). The lungs are the most frequent site of metastases, followed by the skeletal system (12).

The diagnostic methods available today do not allow for the detection of small deposits of tumour at distant sites. More importantly, there is at present no adequate therapy available for distant metastases. The high expectations as to chemotherapy have not been become true (18,19). There is thus a great demand for more sensitive diagnostic methods and more selective therapies for distant metastases.

1.2.

Perspectives of monoclonal antibodies in head and neck oncology

Based on the assumption that tumours possess specific antigens, Ehrlich proposed the concept of the magic bullet (20). He envisioned antibodies delivering toxic reagents directly to tumour cells to eradicate these cells. With the ability to couple radio-isotopes to an antibody, it should be possible to use the bullet both for diagnosis and therapy of cancer. The introduction of monoclonal antibody technology (21) created an opportunity to test the theory *in vitro* and *in vivo*. In contrast to polyclonal antisera, monoclonal antibodies are specific for single antigenic determinants and can be produced in large quantities with high degrees of purity. If Ehrlichs hypothesis would be correct, the magic bullet could be applied in 2 areas of head and neck oncology, where there is a great need for such innovative approaches as outlined above: 1. the assessment of the status of neck nodes. 2. diagnosis and therapy of distant metastases.

1.3.

The concept of the magic bullet

The immune system can differentiate between 'self' and 'non-self' and protects the integrity of the organism. Neoplastic transformation of cells may be associated with phenotypic changes. The loss of 'self' antigens and the induction of 'non-self' antigens on the surface of the neoplastic cell especially by differentiation can induce a

cellular or humoral immune response. The concept of the magic bullet is based upon the assumption that tumours express specific antigens, which can be recognized by selective antibodies and thus can be used for targeting of a neoplasm. These two aspects will be discussed in detail here.

1.3.1.

Tumour associated antigens

Tumour specific antigens (TSA), antigens exclusively found in tumour tissue but not in any normal tissue, are readily identified on experimental tumours induced by chemical carcinogens, radiation or oncogenic viruses (22), but evidence is still lacking that they are present on so-called spontaneous tumours. Instead, most identified tumour antigens of human carcinomas represent tumour associated antigens (TAA), present on tumour tissue but also detectable among normal tissues although in some cases in lower quantities (23). The relationship of tumour associated antigens with (proto) oncogen and growth factor receptors is beyond the scope of this introduction. For further reading one is referred to the review of Bishop (24).

As will become clear from the following sections this does not necessarily indicate that the magic bullet theory is not valid, since normal tissues expressing the TAA might be protected from the antibody by factors influencing antibody penetration into tissue. For therapeutic purposes this is a critical point. Therefore, we will give a detailed description of the accessibility factors influencing the targeting of antibodies into normal and malignant tissues.

1.3.2.

Antibody extravasation

The barrier offered by normal capillary endothelial cells varies greatly between tissues (25). In liver, spleen and bone there is virtually no barrier because the endothelium is fenestrated and a basement membrane is lacking. At the other end of the scale the endothelium of lung and skin is particularly poorly permeable to molecules with a mass of 100-200 kD like immunoglobulins. The way of passaging across the endothelial cells is thought to be by way of intracytoplasmic vesicles and transendothelial channels in these organs (26). Intermediate permeability is seen in organs as intestine, endocrine and exocrine glands and kidney where the endothelial cells have fenestrations but also a basement membrane (27).

In tumours, the endothelium is usually fenestrated, even in tumours arising from those tissues that normally have no fenestrated capillaries (27). In addition, the basement membrane of the tumour endothelium is frequently defective and this is likely to give rise to an increased permeability (28). It is obvious that an extensive vascularization in tumours is a prerequisite to reach the majority of the tumour cells. Neoplasms, especially the better differentiated types often do have a well developed blood supply, since their unrestricted growth is dependent upon angiogenesis, induced by angiogenetic factors produced by neoplastics cells (29). The frequent histological observation of necrosis especially in poorly differentiated and/or rapidly growing tumour is

often due to insufficient vascularization of the neoplastic cells. Consequently this may also limit the accessibility of the tumour cells by the antibody.

1.3.3.

Transport throughout the extracellular space of tissues

After extravasation immunoglobulins are found in the extracellular space. Antibodies move through this space by diffusion and convection (bulk fluid movement) (26,30). In normal tissues antibodies are removed by the lymphatics or by re-entry into small low pressure blood vessels (26). Lymphatics are not formed by tumours and this results into pooling of extravasated antibodies (31). The constituents of the extracellular space vary greatly between tumours. The most common structural proteins are collagen and elastin, which will interfere little with the free movement of immunoglobulins in the tumour. Components as proteoglycans, however, will offer resistance to Ig diffusion. As a consequence in tumours in which the extracellular space is composed by a high amount of proteoglycans eg. some scirrhous breast and lung carcinomas, fibrosarcomas and osteosarcomas (32,33) diffusion and convection will severely be hampered.

Another factor influencing the diffusion through the extracellular space is the intratumoural pressure. As the tumour starts to grow, there is also an increase in interstitial pressure (34). This may lead to a reduced blood flow and ultimately central necrosis.Consequently this will also reduce extravasation of antibody. Thus, smaller tumours are more favourable for antibody penetration than the larger tumours.

1.3.4.

Access to the tumour cell

When the antibody finally has reached the peripheral cell layer of a tumour nest, the intercellular junctions are the last barriers that can withhold the antibody from binding to its counterpart, being the tumour associated surface antigen. In normal squamous cell epithelium the intercellular junctions are formed by desmosomes. Squamous cell carcinomas are assumed to have less desmosomes, particularly the poorly differentiated types, but this has never been convincingly demonstrated (35).

1.3.5.

Intratumour antigen-antibody binding

The actual accessibility of antigenic sites *in vivo* has been studied in xenograft bearing nude mice injected with radiolabelled antibodies. Autoradiographic studies indicate that antibody localization is confined to the periphery of tumour cell nests adjacent to the vascularized stroma (36,37). Apart from other factors hampering the success of radio-immunotargeting such as the proportion of antigen-positive cells, tumour vascularization, the presence of circulating antigens and the accessibility of antigenic sites, it has been suggested that the size of the antibody is related to penetration. When $F(ab')_2$ fragments are used instead of whole IgG the central tumour layers are also reached (37). Small tumour nests are usually completely penetrated by antibody irrespective whether $F(ab')_2$ fragments or intact IgG molecules are used (37).

1.3.6.

Antigen heterogeneity

Heterogeneity in the expression of tumour associated antigens, as defined by the binding of monoclonal antibodies, is a characteristic common to most, if not all human carcinoma cell populations (38,39). Antigen-negative cells within the population can escape detection and therapy by their failure to bind the appropriate antibody. Therefore, the extent of antigenic heterogeneity is an important consideration when designing protocols for the management of cancer by administration of monoclonal antibodies. One of the approaches to counteract the effect of heterogeneity is the use of mixtures of MAbs, recognizing different TAA's (40). Another possibility has been reported by the administration of recombinant interferon. This induced increased expression of TAA and consequently augmented the localization of a radiolabeled monoclonal antibody to the tumour site in an experimental model (41).

1.3.7

Antigen shedding

The shedding of antigen by the tumour into blood is the basis of serum cancer diagnosis (42). Theoretically, circulating antigen can react with the antibody and thus impair the effectiveness of radio-immunoscintigraphy. Indications were found that this, indeed, occurred when the OC 125 antibody, recognizing an antigen present on ovarian carcinomas and in serum of ovarian bearing patients, was injected intravenously in patients (43). Squamous cells are not actively involved in the secretion of substances as is the case for glandular epithelia and therefore it is less probable to expect active shedding of tumour antigens into serum by SCC.

1.3.8.

Internalization

After binding of MAb to a surface molecule, the antigen-antibody interaction can result in internalization of the antibody. As a consequence the antibody can be found within the cytoplasm. This creates opportunities to deliver toxic reagents close to the tumour nucleus, thereby irreversibly damaging the DNA double helix (44). The variables which can predict internalization of an antibody are unfortunately unknown and therefore, spontaneous internalization upon binding to the tumour cell surface has to be evaluated for every antibody.

1.4.

Selection criteria for antibodies

Taking into account the above mentioned parameters influencing the success of the magic bullet theory, certain criteria can be set, which antibodies have to meet. Firstly, antibodies should be preferentially of IgG subclass rather than IgM because extravasation and penetration into tumour will be better for the smaller IgG molecules. For the same reason, it is important that the IgG antibody should remain immunoreactive after its digestion to a $F(ab)_2$ fragment. Secondly, the antibody should not be reactive at all with circulating blood cells and blood vessels. To what extent the antibody can

react with other normal tissues is a matter of controversy. We consider reactivity with organs which have fenestrated endothelial cells and no basement membranes, such as liver, spleen and bone marrow, as a conclusive disability for usage of an antibody *in vivo*. Binding of the MAbs to tissues as skin, lung and brains is not likely to cause problems, because of the supposed decreased permeability of these tissues for macromolecules (26). A third requirement is that the antibody should at least react with as many as possible tumour cells. It is not realistic to expect reactivity with all cells because of antigen heterogeneity. Within tissue sections of tumours, the peripheral layers of tumour nests should preferentially react, since these are the best accessible for the antibody. The recognized epitope should have a surface domain rather than a cytoplasmic localization. The following criteria are less absolute, but they may contribute to the success of the magic bullet: the antibody should not react with an antigen shed into the blood; the antibody should be internalized upon binding to the tumour cell. Finally, intrinsic biological activity of the MAb can also augment the effect of the antibody. With regard to this we think of antibodies capable to disrupt cellular interactions (45,46) or which can inhibit proliferation of tumour cells, usually by binding to a receptor for a growth factor (47).

1.5

Experiences of monoclonal antibody administration in other solid tumours than SCC

Numerous studies have reported the use of radiolabelled antibody for detection of human tumour xenografts in animal models, usually the nude mice. Successful localization with radiolabelled MAbs under these experimental conditions have been described for colon, ovarian, breast and renal carcinomas and also for melanomas and neuroblastomas (48-53). The average percentage of radioactivity which is found in the xenograft upon intravenous injection of a radiolabelled antibody varies between 1-20%. Tumour to blood ratios range between 0.5 and 8, although higher rates have been observed in renal tumours, probably caused by extraordinary vascular permeability of the evaluated xenograft (54).

Considerable less experiments have been undertaken to demonstrate the feasibility of radiolabelled MAbs to achieve therapeutic effects in an experimental setting. These studies did however show the ability of radiolabelled MAbs to eradicate selectively xenograft tumour cells with limited toxicity to other tissues (55,56). Only a few antibodies have reached the stage of clinical application. The great majority of the performed studies sofar are phase I/II studies with the aim to test the antibody for radioimmunolocalization. The general conclusions from these studies are that intravenously injected MAbs provoke little or no adverse reactions and that larger lesions (> 1 cm) are better depicted than smaller ones. A considerable amount of radioactivity (in case of radiolabelled antibody) can be found in liver and spleen, thereby masking potential metastases. Despite the disadvantages most authors claim that radio-immuno scintigraphy yielded useful information not obtained by other techniques (43,57-62). Pectasides *et al.* for instance, claimed superior results with ^{131}I-labelled MAbs in detection of residual disease in ovarian cancer patients as compared to CT scan (58). Immunotherapeutic studies have been performed in ovarian cancer patients and in

patients with lymphomas (63-67). While in some cases partial remissions and sometimes even complete remissions were observed, other patients failed to respond. Since for therapeutic purposes large doses of antibody have to be administrated, most patients develop an anti-mouse immunoglobulin response (42,68). This will ultimately limit the possibilities of intact mice antibodies. One way to circumvent this problem is the use of $F(ab)_2$ fragments. The most encouraging results, sofar, were reported by Hale *et al.* (68), who used an anti-leukocyte antibody (Campath-1H) in the treatment of 2 non-Hodgkin lymphoma patients. In order to avoid an antiglobulin response, an immunoglobulin was constructed by genetic engineering, containing the hypervariable region of the original rat immunoglobulin and the constant region of the human immu-noglobulin. Intravenous administration of the reshaped antibody resulted in complete remissions in the 2 patients without any significant side effects. One of the patients was refractory to any conventional therapy. It is likely to expect that further therapeutic studies will be performed with genetically reconstructed antibodies (69,70).

1.6.

MAbs and head and neck squamous cell carcinomas

At present there is only a small number of monoclonal antibodies which have been produced after immunization with head and neck cancer material. Zenner and Hermann were the first who described a MAb to a squamous-cell carcinoma cell surface antigen (71). However, their reports are limited to the documentation of selective killing of laryngeal carcinoma cells with this antibody in an *in vitro* assay without further identification of the antigen(72). In 1983 Carey *et al.* described the A9 and G10 antibodies directed against surface molecules of SCC (73). Later studies revealed that G10 reacts with a precursor blood group antigen (74) and that MAb A9 recognizes a basement membrane component (75,76). Detailed studies on the reactivity of MAb A9 in normal human tissues were not presented, although they noted reactivity with normal blood vessels and epithelial basement membranes (75). Boeheim *et al.* produced the SQM-1 antibody, a murine IgM antibody preferentially reactive with a SCC surface antigen of 48 kD (77). A cytoplasmic antigen, recognized by MAb 17.13.C1.10 was found to be selectively expressed in squamous-cell epithelia and myoepithelial cells (78,79). An identical tissue distribution was found for the recently described MAb 174H64 (80). Biochemical characterization made clear that this antigen has a relative molecular weight of 57 kD and is probably a cytokeratin protein. Myoken *et al.* described 2 MAbs to oral SCC which were also found to be reactive with keratin proteins. These antibodies lacked further specificity for SCC (81).

Concerning the antibodies mentioned above we may consider that these MAbs possess characteristics likely to hamper successful use for immunodiagnosis and therapy. MAb A9 shows reactivity towards normal blood vessels and we also observed this for the SQM-1 antibody. 17.13.C1.10 and 174H64 are highly selective reagents for SCC, but the location of the antigen is predominantly intracellular, which is thought to be less accessible for an antibody than a surface domain.

Our group also considered MAbs, originally developed to non-SCC tumours but known to be reactive with SCC of the head and neck, as potential tools for detection and therapy of SCC. Among these antibodies we evaluated anti-CEA, anti-MAM-6 and anti-epidermal growth factor receptor (EGF) antibodies on frozen sections in an immunoperoxidase assay. Anti-CEA antibodies were reactive in a minority of SCC as has also been documented by others (82). MAbs to the MAM-6 antigen have been used to identify the epithelial nature of head and neck tumours (83). A decrease in the reactive cells was seen from well differentiated SCC to the poorly differentiated types. In addition, we noted that the MAM-6 antigen is predominantly expressed in the more differentiated cells localized in the central layers of tumours. Anti-EGF receptor antibodies could be useful to fulfil our study purposes, since a considerable number of investigations has reported that overexpression of the EGF receptor occurs frequently in SCC (84,85). In a pilot study with a radiolabelled MAb (EGFR1) only relative large tumours (> 3 cm) could be visualized in patients with SCC tumours (86).

It thus appeared at the beginning of our study that the few antibodies available did not seem suitable for *in vivo* diagnosis and therapy of SCC. The reason for the low number of existing MAbs is obscure. It may be that TAA of SCC are less immunogenic in evoking a murine response since even groups which have been working for prolonged periods of time on the production of MAbs to SCC only succeeded in the establishment of small numbers of antibodies. The production of immunosuppressive factors by the tumour itself may also contribute to this phenomemon (87).

The large number of SCC, one of the major carcinoma types, in the head and neck region, lung, esophagus, cervix, vagina, vulva and epidermis justifies the production of MAbs to this tumour type on a large scale.

1.7.

Aim of the study

The ultimate goal of our long term research is localization and therapy of SCC of the head and neck with radiolabelled or toxin conjugated (in case of therapy only) monoclonal antibodies. To achieve this, monoclonal antibodies with specificity to SCC cells had to be produced, the antigens to be characterized while potential applications had to be tested in an *in vitro* and in an animal model. This preclinical study is the subject of this thesis. Chapter 2 describes the establishment of MAbs to head and neck squamous-cell carcinomas. In chapter 3 through 6 those antibodies showing selective binding (see selection criteria for MAb production) were further investigated for their reactivity with normal and transformed tissues and the recognized antigens were characterized. One antibody was further investigated for its capacity to inhibit the growth of *in vitro* cultured SCC cell lines. Finally, chapter 7 describes the first attempts to evaluate the potential applications of the produced antibodies for *in vivo* use. The most promising antibody was radiolabelled and tested for its ability to localize human SCC tumours growing in the nude mouse model.

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CHAPTER II

PRODUCTION OF MONOCLONAL ANTIBODIES TO SQUAMOUS CELL CARCINOMA ANTIGENS.

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ABSTRACT

For therapeutic or diagnostic use of monoclonal antibodies in clinical oncology, high affinity IgG antibodies to Tumour Associated Antigens (TAA) have to be generated. In order to find out by what immunization schedule the chance to generate such antibodies is increased, we evaluated 3 different immunization protocols with and without attempts to induce tolerance to common tissue antigens. Mice were immunized either by repeated intraperitoneal injections (I), by a single intrasplenic injection (II), or by an intraperitoneal injection followed by an intrasplenic booster (III). Whereas a single intrasplenic immunization resulted in low affinity antibodies to TAA, high affinity antibodies were generated with the other two protocols although at a lower frequency. No benefit was seen from tolerance induction. The intraperitoneal/intrasplenic protocol was found to be superior over the other protocols because of minimal antigen dose and immunization time as well as a higher frequency of hybridoma formation.

INTRODUCTION

Squamous Cell Carcinomas (SCC) of the head and neck are aggressive tumours which must be treated by radical surgery and radiotherapy. The extent of surgical treatment largely depends on the presence of metastatic lymph nodes in the neck. Despite increased sensitivity of Magnetic Resonance Imaging and Computed Tomography, there is still no reliable method to establish the lymph node status in head and neck tumour bearing patients preoperatively (1). Since radiolabeled monoclonal antibodies to SCC could have a potential role in detection of lymph node and distant metastases, we have been active in the production of monoclonal antibodies (MAbs) to SCC.

For the use of MAbs *in vivo*, the antibody should be preferentially a high affinity IgG immunoglobulin rather than an IgM, since the the smaller the antibody the better it will penetrate into tissues (2). Second, the antigen recognized by the antibody should be a tumour associated antigen (TAA): strongly expressed by tumours and limited expression in normal tissue (3,4). The production of monoclonal antibodies to Tumour Associated Antigens is hampered by 2 major problems. Firstly, purification of TAA of SCC is impossible because they have not been characterized and so for immunization mixtures of antigens have to be injected. As a consequence antibodies recognizing different epitopes are produced. To select the appropriate antibody, a careful and extensive screening has to be performed. The second problem is caused by the murine immune response which shows preferential reactivity to certain antigens, a phenomenon which is called immunodominance (5). The fact that antibodies to the human major histocompatibility antigens HLA-A,B,C and Dr antigens are frequently produced after immunization of mice with human tumour cells suggests that these molecules, if present on tumour cells, are higly immunogenic (6-8). In order to counteract the bias of the murine immune response to the immunodominant antigens, we concentrated upon 2 stategies. The first is based upon the assumption that prolonged feeding of a specific antigen results in a state of immunological tolerance to these antigens, which can persist during subsequent immunizations (9-11). So mice were fed with a mucosal homogenate prior to immunization to suppress the antibody response to common tissue antigens. Another approach has been suggested by Spitz *et al.*(12), who reported the production of MAbs to weakly immunogenic antigens by a single intrasplenic injection. Since this schedule yielded especially IgM class antibodies, we modified it by adding an intraperitoneal priming to increase the percentage of IgG antibodies production.

In this report we describe the results of the production of high affinity MAbs preferentially reactive with SCC obtained with 3 different immunization protocols. Since we also noted strong differences between the protocols for several important parameters as antigen dose, immunization time and fusion efficiency, these data are also presented here.

MATERIALS AND METHODS

Cell lines and tissues

Tumour and normal tissues were obtained from surgical procedures or from autopsies within 8 hrs. after death. Tissues were stored in liquid nitrogen. Tissue homogenates were prepared essentially as has been described by Oosterwijk *et al.* (13). Human squamous carcinoma cell lines were kindly provided by Dr. T. Carey, University of Michigan, Ann Arbor, MI.

Immunizations

Protocol 1

- Repeated intraperitoneal injections (14).

Female BALB/c mice (12 weeks of age) were immunized by intraperitoneal (i.p.) injection of 10^7 whole cells. The cells were combined with complete Freund's adjuvant at the first injection and with incomplete Freund's adjuvant at the second and third injections given at 2-weeks interval. After one month an i.p. booster injection was given without adjuvant. The final injection (1×10^6 cells) was given i.v. one week later. Three days following this last immunization, the mice were killed and the spleen cells were harvested for fusion.

Protocol 2

- Intrasplenic immunization.

This was basically performed as described by Spitz *et al.*(12). In short, mice were anesthesized by an i.p. injection of 6-7 µgram of Nembutal per gram of body weight. The animal was placed on its right side and the fur of the left side was shaved. After the abdomen was swabbed with 70% ethanol, a skin incision was made. Fifty µ l PBS containing antigen was injected deeply into the spleen. After injection peritoneum and skin were sutured.

Protocol 3
-Intraperitoneal/intrasplenic immunization:
10^7 viable cells without adjuvans were injected i.p.. An intra- plenic booster of 10^6 cells was given 2-4 weeks later as has been described above.
-Tolerance induction by oral feeding.
From three weeks after they had been born, mice were fed with a daily consumption of 1 mg mucosa homogenate in drinking water. The period of daily oral feeding of mucosa homogenate varied from 10 weeks to 30 weeks before immunization. Afterwards mice were immunized according to protocol one or three.

Fusions

Three days after each final immunization, the mouse was killed and the spleen was immediately removed and mechanically dissociated in RPMI-1640 medium. The spleen cells were subsequently transferred to two 75 cm^2 tissue flasks in order to allow the macrophages to adhere. After 4 hours of incubation the spleen cells were collected. Myeloma cells (Sp-2/0-Ag 14) and spleen cells were fused at a ratio of 1 : 6 in 1 ml of 50% polyethylene glycol-1450 and 10% DMSO, for 1 min. The fusion was stopped by adding slowly RPMI to the cells. The suspension was centrifuged at 500 g for 5 minutes. The fused cells were then gently resuspended in fusion medium containing RPMI, 15% Fetal Calf Serum (FCS), 10^{-4} M Hypoxanthine, 1.6 x 10^{-7} Thymidine, 4 x 10^{-7} M Aminopterine and 1% hybridoma growth factor (HGF, Janssen, Beerse, Belgium). In general, the fused cells were plated in a concentration of 10^5 cells/well. Clones became visible as a clump of cells 4-6 days after fusion and were suitable for screening at 10-12 days.

Screening

For objective evaluation of the different immunizations, all fusions were screened in an identical manner. Ten to 12 days after the fusion, hybridomas were screened in an ELISA system

In this way the number of producing clones could be determined, reactivity to surface determinants of SCC cell lines, as well binding to red blood cells. One day after this screening, hybridomas reactive with SCC cell lines but unreactive with red blood cells, were judged upon their reactivity with frozen sections of metastatic SCC, normal squamous epithelium and normal colon since the latter contains a variety of different tissues as muscular, neural and epithelial tissue. Suitable hybridomas were then stabilized by limiting dilution.

Elisa

In the screening protocol the following ELISA systems were used: 1. Cell ELISA: SCC tumour cell lines growing as monolayers were brought into suspension by trypsinization (0.25% trypsin, 0.2% EDTA) washed and resuspended in RPMI medium containing 10% FCS. Aliquots of 4 x 10^4 cells/ml. were transferred to 96 wells plate. After 2 days a monolayer of cells was seen.
2. ABO erythrocyte ELISA: freshly prepared red blood cells with blood group A,B and O were added to a 96 well plate in a concentration of 5 x 10^7 cells/ml. Membranes were disrupted by distilled water to avoid endogenous peroxidase activity.
3. Immunoglobulin production ELISA: 96 microtest well plates were coated with affinity purified rabbit anti-mouse immunoglobulins (10µ g/ml, Dakopatts Copenhagen, Denmark) with 0.1 M Na_2CO_3 pH 9.4.

For determination of binding reactivity the following steps were performed: After coating, ELISA plates were washed with PBS Tween (0.05% v/v) and incubated with 3% BSA in PBS(w/v) to prevent non-specific binding of immunoglobulins to plastic. This was followed by a incubation of supernatant for one hour at room temperature. After washing and incubation with rabbit anti-mouse horse radish peroxidase conjugated immunoglobulins (Dakopatts) the plates were washed again and developed with 100 µl ortho phenyl diamine. Colour development was stopped after 10 minutes by adding 100 µl 1 N H_2SO_4 to the wells and optical readings were taken at 492 nm. As controls non-relevant antibodies were used.

Immunoperoxidase staining

Four to 6 µm thick sections of frozen tissue blocks were cut on a cryostat microtome mounted on poly-L-lysine coated glass slides,dried, acetone fixed and tested in the indirect immunoperoxidase assay as described elsewhere (15).

Affinity determination

A simple and rapid method was used to estimate the affinity of the antibodies. Frozen sections, which under standard conditions stained positive, were incubated with hybridoma supernatant in the presence of 1 M NaCl. Afterwards the slides were washed in the presence of high molar salt. Further testing was performed as described in the immunoperoxidase assay. Antibodies binding in the presence of high molar salt concentration were regarded as high affinity antibodies in contrast to non-binding antibodies (16).

RESULTS

Antigen dose, immunization time and fusion efficiency

- Single intrasplenic immunization:

Initially, low numbers of spleen cells were obtained and consequently less hybridomas were seen after a single intrasplenic immunization as compared to the hyperimmunization protocol. When the antigen dose was raised to 1 x 10^6 cells, an equivalent amount of immune spleen cells was obtained (data not shown). It is clear from table 1 that this required antigen dose is considerably less than for the other two protocols. This is especially important in case of a limited availability of the antigen. A second consequence of this regimen is the minimal time necessary for immunization. As a disadvantage we noted that the hybridomas were found to be rather unstable since less than 25% of the selected hybridomas still produced immunoglobulins after subclonation.

-Intrasplenic immunization preceded by intraperitoneal priming:
In an attempt to improve the results obtained after a single intrasplenic immunization an intraperitoneal priming was given. This resulted in an increased fusion efficiency. The relation between the minimal necessary number of fused spleen cells per well to be plated to obtain more than 90% producing clones is shown in table 1.

TABLE I -RELATION BETWEEN IMMUNIZATION PROTOCOL AND THE REQUIRED ANTIGEN DOSE, IMMUNIZATION TIME AND MINIMAL NUMBER OF FUSED SPLEEN CELLS.

fusion protocol	injected tumour cells x 10^6	immunization time (days)	no of fused spleen cells x 10^4*
protocol I	52	45	15
protocol II	1	3	15
protocol III	11	17	1

*: mean number of fused spleen cells, which had to be plated to obtain in more than 90 % of the wells producing hybridomas.
Protocol I represent the repeated intrasplenic immunizations, II the single intrasplenic immunizations and III intraperitoneal/ intrasplenic immunization.

After a single intrasplenic immunization or after the hyperimunization protocol fused spleen cells had to be plated in a concentration of 1.5 x 10^5 cells per well. Following the protocol of intraperitoneal/intrasplenic immunization, it was possible to plate out in a concentration of 1 x 10^4 fused spleen cells per well. About 80% of the selected hybridomas continued to produce immunoglobulins after prolonged *in vitro* culturing.
- Hyperimmunization protocol:
Stability of the selected clones is similar to results obtained with intraperitoneal/intrasplenic immunzation. Fusion efficiency is however lower, while the necessary antigen dose is larger and the time needed for immunization is longer (table 1).

Isotype and affinity

Both hyperimmunization and intraperitoneal/intrasplenic immunization protocol resulted in a high percentage of high affinity antibodies of the IgG class (table II). Following a single intrasplenic immunization all selected antibodies belonged to the IgM class. These antibodies showed also less binding affinity to the recognized antigens as compared to the antibodies obtained with the other protocols.

TABLE II - RELATION BETWEEN IMMUNIZATION PROTOCOL, ISOTYPE AND AFFINITY OF THE SELECTED HYBRIDOMAS.

Fusion protocol	IgM producing clones affinity	percent high
protocol I	10 %	75 %
protocol II	90 %	25 %
protocol III	0	80 %

Immunodominance
Hyperimmunization protocol:

In our initial experiments we noted strong immunodominance of common tissue antigens as bloodgroup and stromal antigens, when mice were hyperimmunized with tissue homogenates. The number of antibodies to stromal antigens reduced upon immunization with tumour cells only, indicating the importance of the immunogen in this aspect. Independent of this partial purification was the generation of antibodies reactive in the red blood cell ELISA, which consisted of about 30% of the newly produced antibodies. Table III lists the number of antibodies directed against these antigens.
-Single intrasplenic immunization protocol:
When a single intrasplenic immunization injection was given, less than 10% of the producing hybridomas reacted in the red blood cell ELISA. Also, upon screening on frozen sections no or few hybridomas gave staining patterns identical to those from other clones, but a broad band of apparently unique antigens was observed.
-Intraperitoneal/intrasplenic immunization:
The mean number of produced hybridomas reacting in the red blood cell ELISA is comparable to that obtained in the hyperimmunization protocol. Also, on frozen sections apparently identical immunohistochemical patterns were repeatedly identified, indicating the immunogenicity of a restricted group of antigens to which a high number of antibodies was formed.
-Effect of oral tolerance protocol:
No benefit was seen from this protocol with regard to avoiding immunodominance, regardless whether mice were 8 or 25 weeks of a daily fed dose of mucosa homogenate (table II).

TABLE III - RELATION BETWEEN IMMUNIZATION PROTOCOL WITH AND WITHOUT ORAL TOLERANCE INDUCTION AND THE PERCENTAGE OF PRODUCED HYBRIDOMAS REACTING WITH RED BLOOD CELLS.

Protocol red blood cells	percent reacting with	no. of fusions
I	33	2
II	10	3
III	28	3
I*	31	2
II*	ND	ND
IIIa*	33	2
IIIb*	27	2

indicates that tolerance induction was attempted. IIIa mice were fed 8-12 weeks prior to immunization, IIIb* mice 12-25 weeks. ND: not done.

Immunohistochemical reactivity patterns of the selected MabS

Out of 14 different fusions 12 monoclonal antibodies have been selected. None of these antibodies are truly SCC specific, as all show some reactivity with normal tissues. All selected antibodies react with surface antigens present on squamous cell carcinomas. They can be divided in five different groups according to their reactivity in normal stratified squamous epithelia (table IV).

TABLE IV - REACTIVITY PATTERN OF SELECTED MABS ON FROZEN SECTIONS OF NORMAL STRATIFIED SQUAMOUS EPITHELIUM

Group	Structure recognized	MAbs
I	Basement membrane	K 19, K 52, K 58, K 271
II	Basal cells	K 984, K 959, K 938
III	Suprabasal cells	K 928, K 9613, K 9615
IV	None	K 931
V	All keratinocytes	E 48

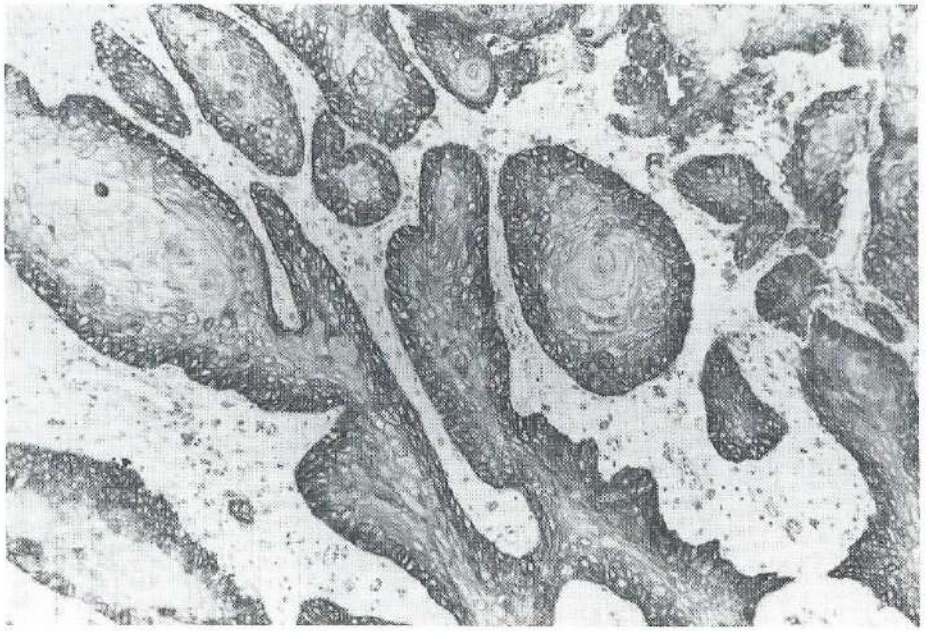

FIGURE 1. Reactivity of MAb K 984 with a SCC of the larynx.

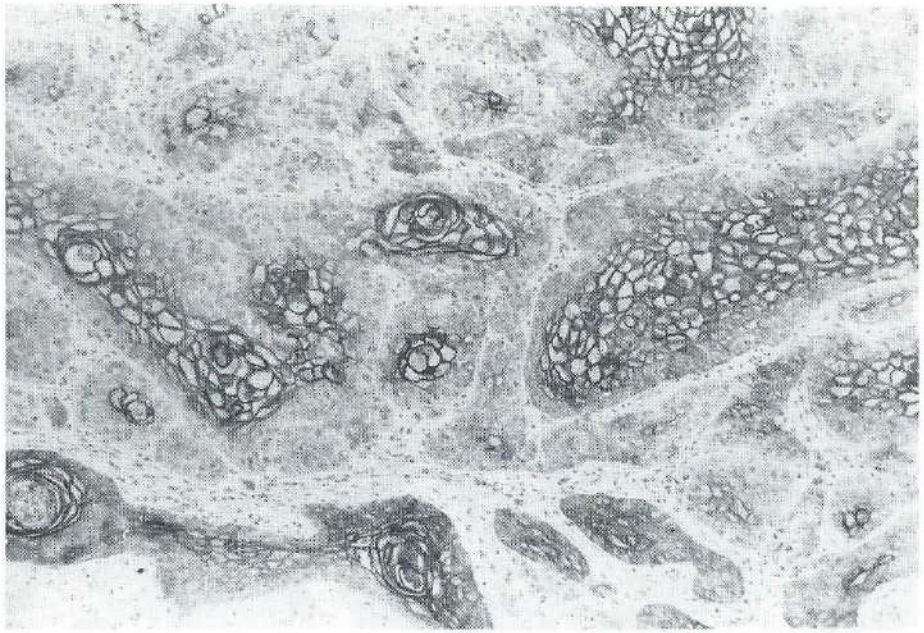

FIGURE 2. Reactivity of MAb K 928 with a SCC of the larynx.

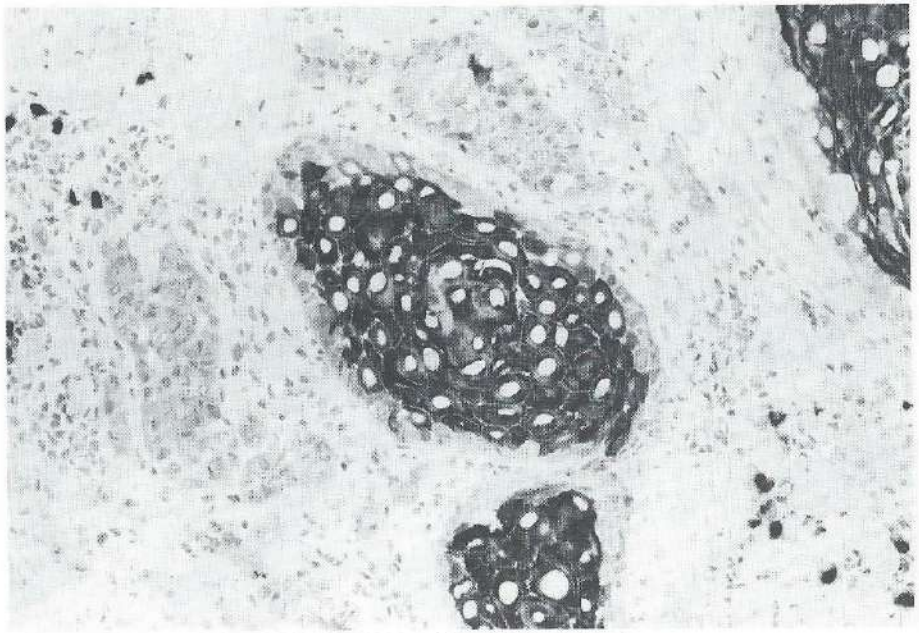

FIGURE 3. Reactivity of K 931 MAb with a SCC of the pharynx.

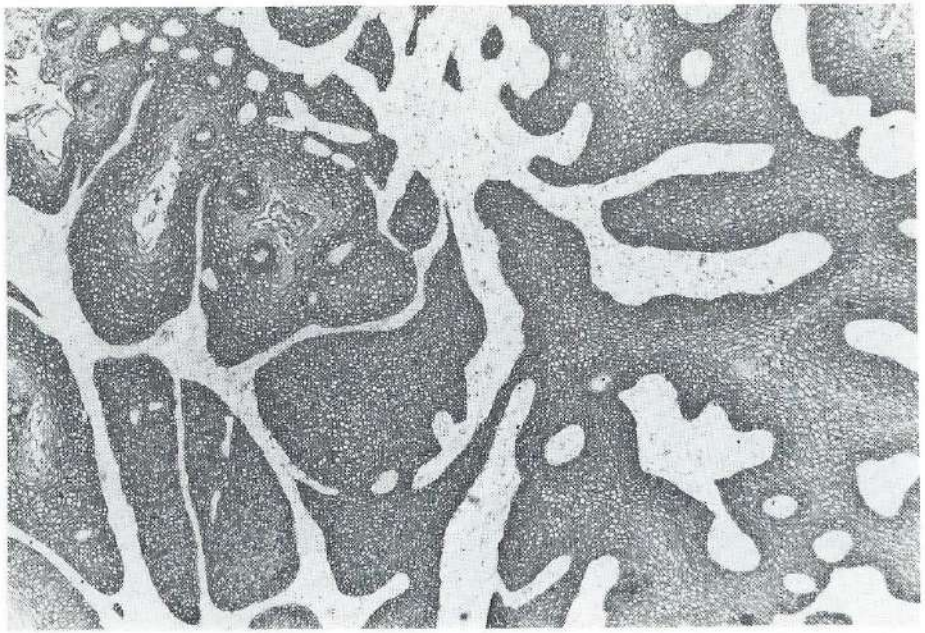

FIGURE 4. Reactivity of MAb E 48 with a verrucous carcinoma of the maxillary sinus.

Group 1 antibodies react with basemement membrane antigens, which are frequently overexpressed in SCC. They also show reactivity with blood vessels. Group 2 and 3 antibodies can discriminate basal (proliferating) and suprabasal cells in normal stratified squamous epithelia and their neoplastic derivatives (figure 1 & 2), respectively. The K 931 antigen (group 4) is absent in normal stratified squamous epithelia, but abundantly expressed in simple epithelia and squamous cell carcinomas (fig. 3). MAb E 48 (group 5) reacts with all layers in normal stratified squamous epithelia and transitional epithelia. The antigen is not found in any other tissue throughout the human body. Strong reactivity is seen with all squamous cell carcinomas so far tested (fig. 4).

DISCUSSION

This study confirms the results of Spitz *et al.*(12) that a single intrasplenic injection is sufficient to produce antibodies even to the more weakly immunogenic determinants although we found it necessary to increase the injected dose as compared to the quantity originally described. A major drawback of this method is the instability of the newly formed hybridomas which results in the loss of antibody production after some weeks of *in vitro* culture. Twenty five percent of the selected hybridomas still produced after 2 subclonations, which is considerably less than 80% we obtained with other immunization routes, or 60% as has been reported by others (17). It was not unexpected that a single intrasplenic immunization preferentially yields IgM antibodies, however this isotype has several disadvantages in tumour imaging or therapy *i n vivo*, as compared to IgG antibodies. We circumvented the mentioned problems by giving the mice an intraperitoneal priming before the intrasplenic injection. Unexpectedly this also enhanced the efficiency of the fusion. Fused splenocytes could be plated in a concentration of 10^4 / well to obtain in 90% of the wells producing clones. With other immunization routes, we and others (17) have found a minimal number of 1.5×10^5 fused spleen cells to obtain this result. Therefore a more efficient way of producing hybridomas is generated. Since it is thought that a recently activated B cell (blast) presents the cell which fuses with myeloma cells to form stable hybrids, this protocol probably maximizes the number of specific B cell blasts in the spleen by direct local antigen stimulation.

MAbs derived from hyperimmunized mice show many similar characteristics (affinity, isotype) of MAbs obtained after the intraperitoneal/intrasplenic immunization protocol. However, the hyperimmunization protocol requires obviously more time and a larger antigen dose.

Immunodominance is reflected in the production of a high number of antibodies to identical antigens. As already mentioned we noticed a strong immunodominance from non-relevant bloodgroup and stromal antigens. In order to reduce this we varied several immunization protocols. Our results, however, show that immunodominance was present in every immunization protocol, with the exception of the single intrasplenic immunizationschedule. Noteworthy is the high immunogenicity of basement membrane antigens, reflected in the production of a high number of MAbs to such struc-

tures by us and others (18) using SCC tumour cell lines as immunogen.

Feeding mice over a period of time with a homogenate of oral mucosal tissue did not result in immunological tolerance to these antigens. The existence of immunological tolerance as a result of oral feeding has been well documented by several basic immunological studies (9-11). All studies which succesfully reported tolerance induction had been limited to the tolerance induction of a single antigen, in contrast to this report in which it was attempted to induce tolerance to a mixture of antigens. A more succesful approach to obtain tolerance in this way may be the limitation of number of antigens to which tolerance has to be induced, and increasing the dose of daily fed antigen.

The usefullness of the MAbs presented here are under current investigation. Others (19) have already shown the importance of monoclonal antibodies to basement membranes (group 1) as biological markers for prognosis. The selective capacity of identifying basal and suprabasal cells (group 2 and group 3 respectively) may prove valid in monitoring the level of differentiation in SCC tumours, since it is thought that the basal cells represent the proliferative fraction and the suprabasal cells the differentiated cells (20,21). For *in vivo* use, MAb E 48 seems to be the most promising since it is strongly expressed in SCC, whereas among normal tissues it is only found in stratified squamous and transitional epithelia. Furthermore the antibody has been succesfully used after radiolabeling in localizing SCC xenografts in the nude mice (22).

We evaluated three immunization protocols with and without attempts to induce tolerance to normal tissues. No benefit was seen from oral tolerance induction in this study. A single intrasplenic injection is sufficient for hybridoma production to less immunogenic antigens, the results however can be dissapointing because of loss of antibody production after some weeks.

We strongly recommend the single intraperitoneal injection followed by an intrasplenic booster as a first choice for the production of MAbs to surface antigens. Minimal time and antigen dose are required, while stable and suitable hybridomas are formed in a highly efficient manner.

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CHAPTER III

A 22-KD SURFACE ANTIGEN DETECTED BY MONOCLONAL ANTIBODY E 48 IS EXCLUSIVELY EXPRESSED IN STRATIFIED SQUAMOUS AND TRANSITIONAL EPITHELIA.

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ABSTRACT

After immunization of mice with viable cells of a metastasis of a laryngeal squamous cell carcinoma, a monoclonal antibody E 48 was obtained which detects an epitope exclusively present in squamous and transitional epithelium and their neoplastic counterparts. Immunoblotting revealed that E 48 recognizes a 22 kDa molecule. Immunoelectronmicroscopy showed that the antigen is expressed along the membrane of squamous cells, and is also associated with desmosomes. Seventy five out of 76 squamous cell carcinomas from head and neck, lung, cervix and skin stained positive, whereas various adenocarcinomas from colon, lung and breast and small cell lung carcinomas consistently stained negative. The E 48 antigen, which is formaldehyde resistant, appears to be a reliable marker for differentiation of squamous cell carcinomas from adeno-, and small cell carcinomas.

INTRODUCTION

It has now been recognized that within a certain epithelial tumour several directions of differentiation can be found (1). For instance, in cytologic specimens about 50% of all lung tumours can be classified as admixtures of epidermoid and adenocarcinomatous subtypes (2). Also at the electronmicroscopic level up to 50% of lung tumours 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 head and neck region, the lung, the cervix and the epidermis. Specific markers for squamous differentiation are involucrin (5) and keratin 10 (6). These antigens are, however, only present in differentiated cells and consequently can not be used to differentiate poorly differentiated squamous cell carcinomas from morphologically undifferentiated tumours.

Therefore, we have tried to produce a monoclonal antibody, which recognizes poorly differentiated squamous epithelial cells. Here we report such an antibody i.e. E 48 and describe some of the biochemical characteristics and the light and electronmicroscopical distribution in cells of tissues with reactive and neoplastic changes.

MATERIALS AND METHODS

Immunogen

In short, 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 Hanks Buffered Salt Solution (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 non-producing cell line SP-2/0. Growing hybridomas were screened on ELISA for immunoglobulin production and for reactivity with red blood cells. Selected antibodies were further screened upon reactivity with frozen sections from oral mucosa, submandibular gland and the tumour which had been used for immunization. Hybridomas showing selective binding were stabilized by limiting dilution. Large amounts of antibody were obtained by ascites producing mice which had been primed by incomplete Freund adjuvant.

Isotypes of the MAb

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

Tissues and cell lines

Neoplastic and non-neoplastic tissues were obtained from surgical procedures. Tissues were also derived from autopsies performed within 8 hrs. after death. Tissues were stored in liquid nitrogen. Human squamous carcinoma cell lines of the head and neck were kindly provided by Dr. T. Carey, University of Michigan, Ann Arbor, MI). UM-SCC-14A was derived from a carcinoma of the floor of the mouth, 11B from a laryngeal carcinoma, 22A and 22B 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% FCS.

Cell Elisa

Squamous cell carcinoma cell lines growing as monolayers in 96-wells ELISA plates, were used for determining binding of the antibody to the outer surface of viable tumour cells. When a confluent culture was seen, plates were washed with PBS and incubated with supernatant of MAb E 48 for one hour at room temperature. In this way only membrane reactivity was detected and not cytoplasmic or nuclear reactivity; MAb K 112, which reacts with a 43 kDa 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 Company, 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 6 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 10 minutes at room temperature. Sections were also 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 in detail elsewhere (11).

Enzymatic sensitivity

The following purified preparations were used: trypsin (227 U/mg Worthington N.J.) pronase (70.000 PUK, Calbiochem, San Diego, Ca.) neuraminidase 0.5 U/ml (Vibrio Cholerae, Behring, Amsterdam, The Netherlands). Periodate treatment was essentially the same as described by Woodward (13).

Radioionation

Iodination of E 48 was performed according to the one vial method described by Haisma *et al* (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 10 minutes at room temperature a sample was taken to determine the amount of incorporated iodine. One ml of AG1-X8 resin (Bio Rad, Richmond, CA) previously mixed with PBS containing 1% 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^{TM}, Amersham UK) ranged from 200 kDa (Myosin), 92.5 kDa (Phosphorylase A), 69 kDa (Bovine Serum Albumin), 46 kDa (Ovalbumin), 30 kDa (Carbonic Anhydrase), 21.5 kDa (Trypsin inhibitor) to 14.3 kDa (Lysozyme). Proteins were transferred from gel to nitrocellulose with a Multiphor II Nova Blot System (LKB, Bromma, Sweden) 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 overlayered with ^{125}I-labeled E 48 (1 x 10^5 cpm/ml) for 2 hours. The sheets were then autoradiographed by exposure to X-ray film with a Kodak X-Omatic intensifying screen for 18-24 hours at -80°C.

Immunoelectronmicroscopy

Oral mucosa tissue was frozen in liquid nitrogen. 10 µm thick cryosections were prepared and mounted on poly-L-lysine coated glass slides. Sections were immediatedly fixed in PBS containing 1% formaldehyde and 0.1 % glutaraldehyd for 20 min. at 4° C. The sections were subsequently washed in PBS and incubated overnight with undiluted supernatant of MAb E 48 and iso-type matched control antibody JSB-1 directed against the P 170-Glycoprotein and not present in oral mucosa (11). Next, the sections were incubated for 3 hours with goat anti-mouse immunoglobulins conjugated with peroxidase (Dakopatts, Copenhagen Denmark) and after washing in PBS, peroxidase reactivity was revealed by incubation of the sections with 1% 3,3'-diaminobenzidine and 0.01% H_2O_2 for 15 min. After extensive washing the sections were postfixed with 1% osmium tetroxid in 1 M cacodylate buffer (pH 8.2) for 45 min. Finally they were dehydrated in ethanol and embedded in epoxy resin (EPON 812, Fluka, Switzerland), ultrathin sections were made and observed by a JEOL-electronmicroscope without counterstaining.

RESULTS

Selection and isotype

Among 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 twice recloning of the hybridoma produced an IgG1 antibody called MAb E 48.

MAB E 48 detects a membrane component associated with desmosomes

On frozen sections of non-keratinizing epithelial cells of oral mucosa, MAb E 48 labeled exclusively the surfaces of the keratinocytes (Fig 1.).

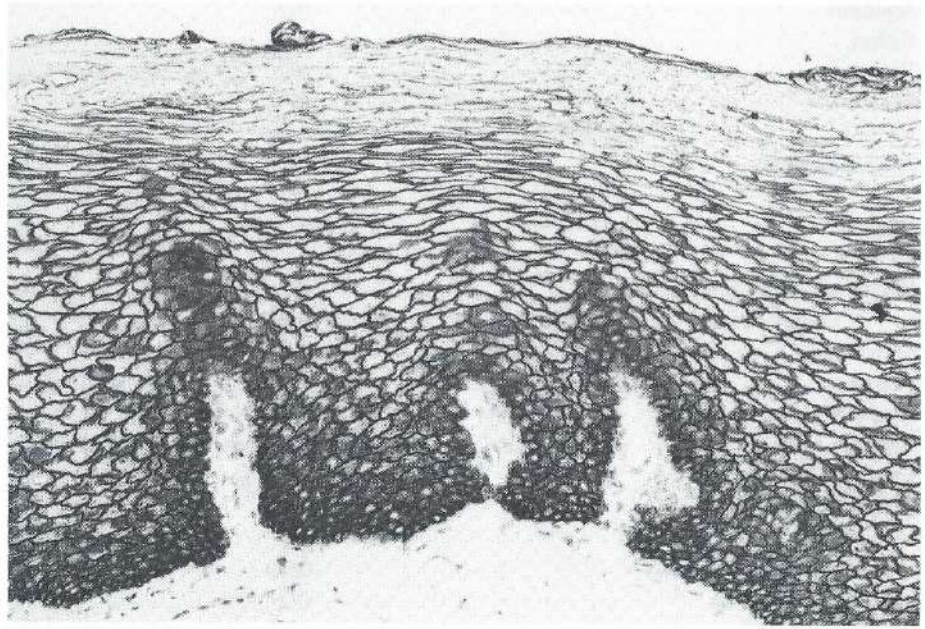

FIGURE 1. Indirect immunoperoxidase staining of oral mucosa by MAb E 48 x 240. Note the distinct surface staining in all layers.

TABLE I-REACTIVITY OF MAB E 48 WITH NORMAL HUMAN TISSUE SPECIMEN BY IMMUNOPEROXIDASE STAINING

ORGAN	Positive/Tested
Epidermis	6/6 all layers except stratum corneum
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
Jejunem	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 labelled by E 48.

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. Afterwards, experiments were performed with B5- and formalin fixed paraffin embedded tissues and we obtained similar results (Figure 7 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 esophagus, vagina and human epidermis. In epidermis no staining was seen in the stratum corneum. Sweat and sebaceous glands were negative, whereas the outer root sheath of a hair follicle did show reactivity with the E 48 antibody. Among all non-neoplastic tissues further tested, only transitional epithelial cells of the urinary bladder also reacted with the antibody (table I.).

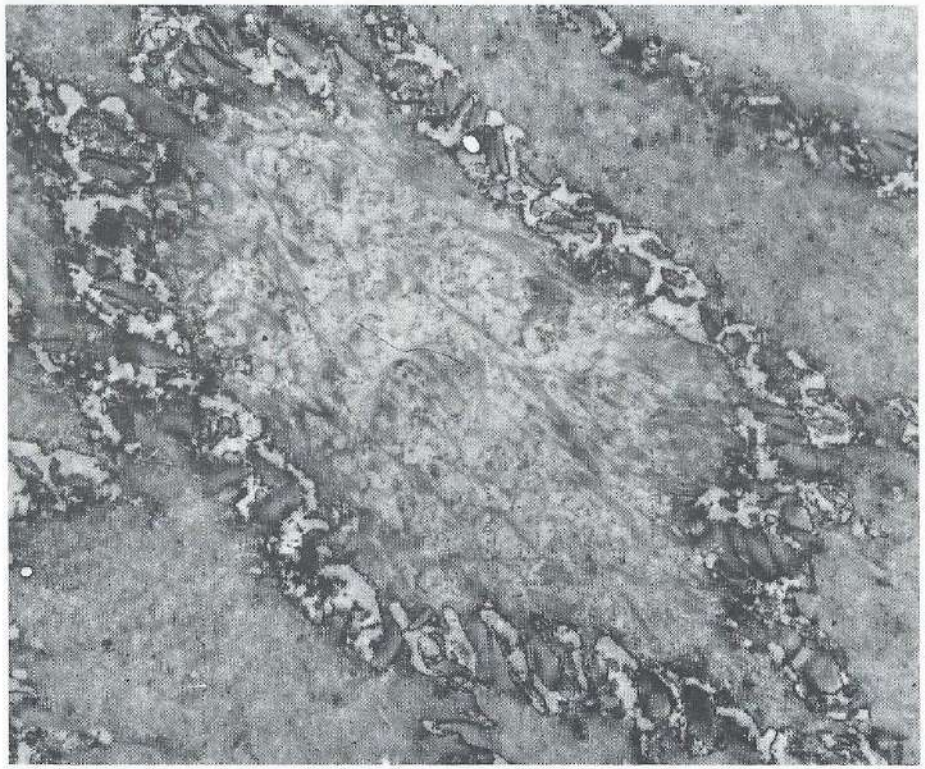

FIGURE 2. Ultrastructural localization of E 48 in cells of oral mucosa. Immunoperoxidase assay x 3762.

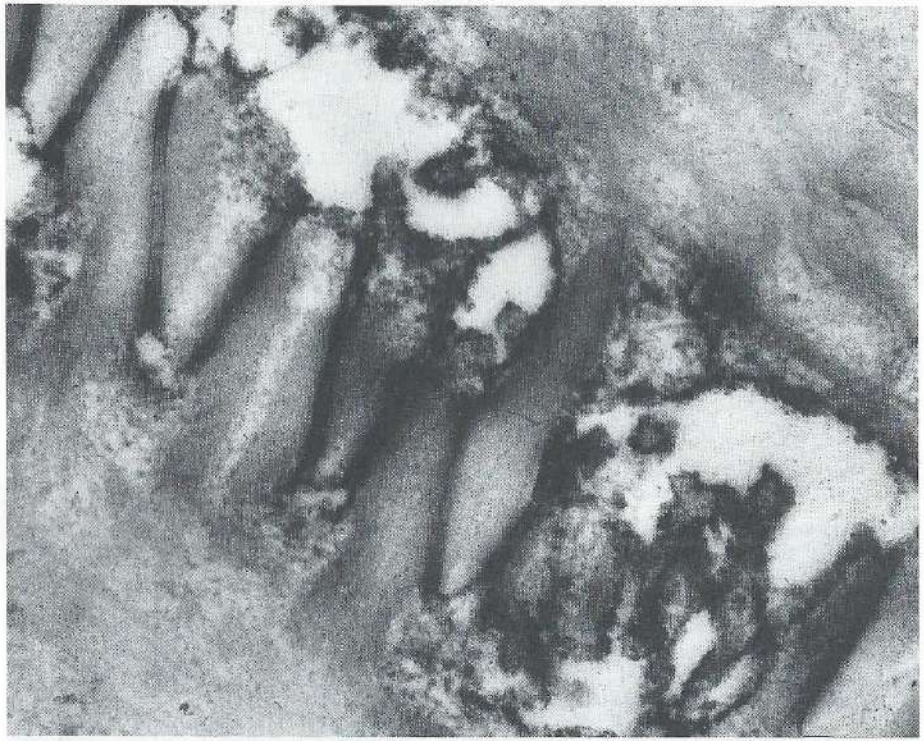

FIGURE 3. High power magnification. Continous labelling along the cell surface. Immunoperoxidase assay.

Adeno-epithelia or non-epithelial tissues did not bind the E 48 antibody. Exceptions were occassionally seen in stratified cuboidal excretory ducts of glands adjacent to squamous cell carcinomas, which focally reacted with MAb E 48.

Stratified squamous epithelia of guinea pig, rat or from bovine origin did not stain with E 48, indicating lack of interspecies specificity.

Immunoperoxidase labelling of oral mucosa at the ultrastuctural level showed that MAb E 48 antigen was present along the cell membrane as well on desmosomes (Fig. 2 and 3). Labelling was not seen on the basement membrace, nuclear or cytoplasmic components.

Binding of MAb E 48 to intact viable tumour cells

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

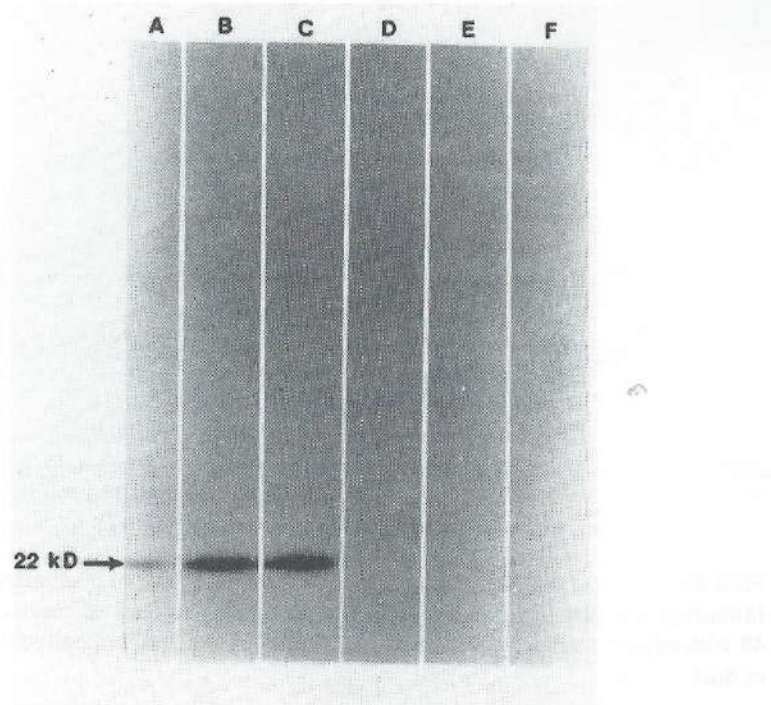

FIGURE 4. Western blot analysis of cell lysates of squamous cell carcinoma cell lines with MAb E 48. Lane A: UM-SCC-14A, lane B: UM-SCC-22B, lane C: A 431, lane D: A 431 when lysed without protease inhibitors, lane E:UM-SCC-11B and lane F: HL 60 a leukemia cell line, both UMS-CC-11B and HL-60 are unreactive with E 48 in immunoperoxidase staining.

TABLE II-REACTIVITY OF MAB E 48 WITH VIABLE TUMOUR 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

Using immunoblot analysis of 3 different cell lines of squamous cell carcinomas, which reacted with the antibody in the Cell ELISA, we have identified a single band

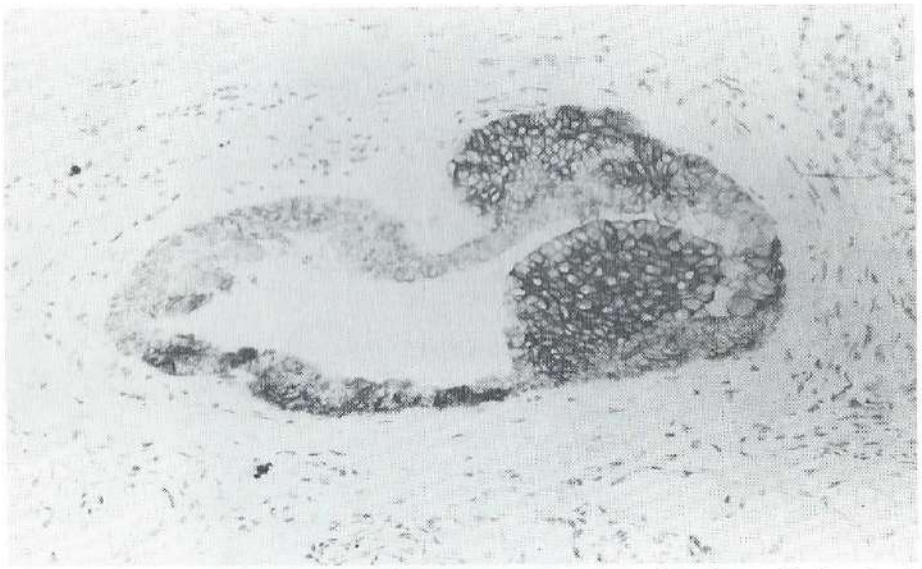

FIGURE 5. Squamous cell metaplasia in an excretory duct of a submandibular gland. Immunoperoxidase staining MAb E 48 x 180. Note the lack of reactivity of MAb E 48 with adjacent simple epithelia and the stratified cuboidal epithelium of the excretory duct.

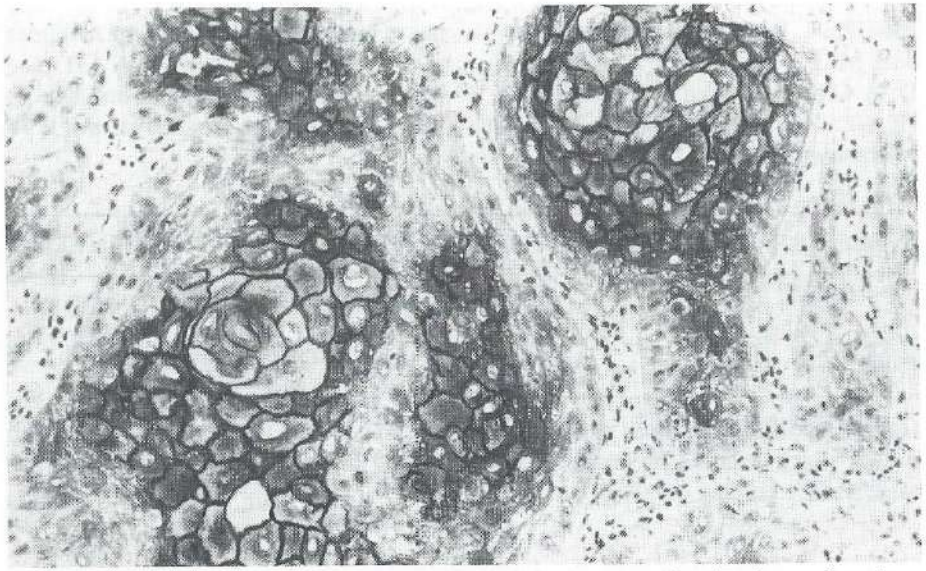

FIGURE 6. Moderately differentiated SCC of the tongue showing loss of antigen expression in the peripheral cells of the tumor nests.Indirect immunoperoxidase staining MAb E 48 x 240.

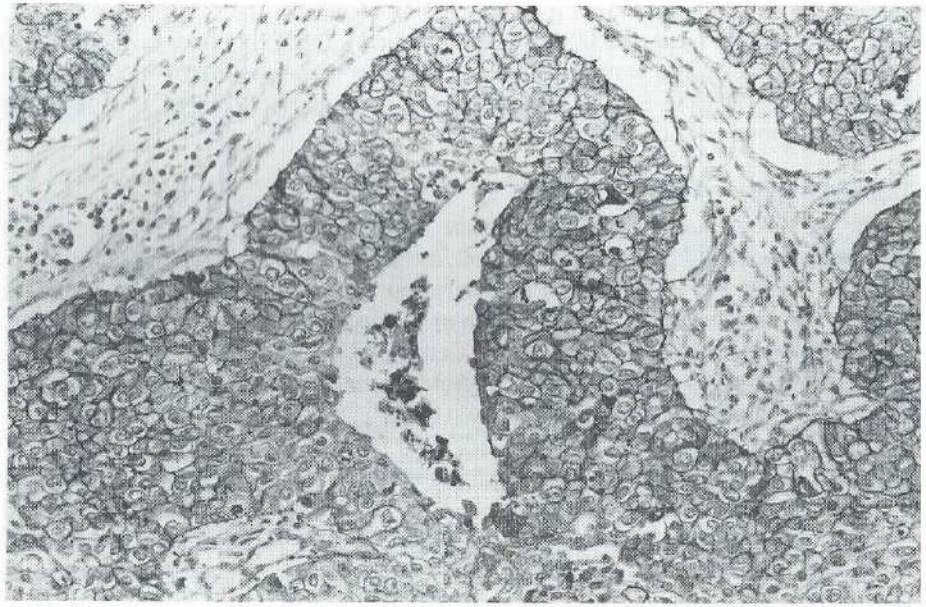

FIGURE 7. Large cell undifferentiated lung carcinoma. Formalin fixation. Indirect immunoperoxidase staining MAb E 48 x 270.

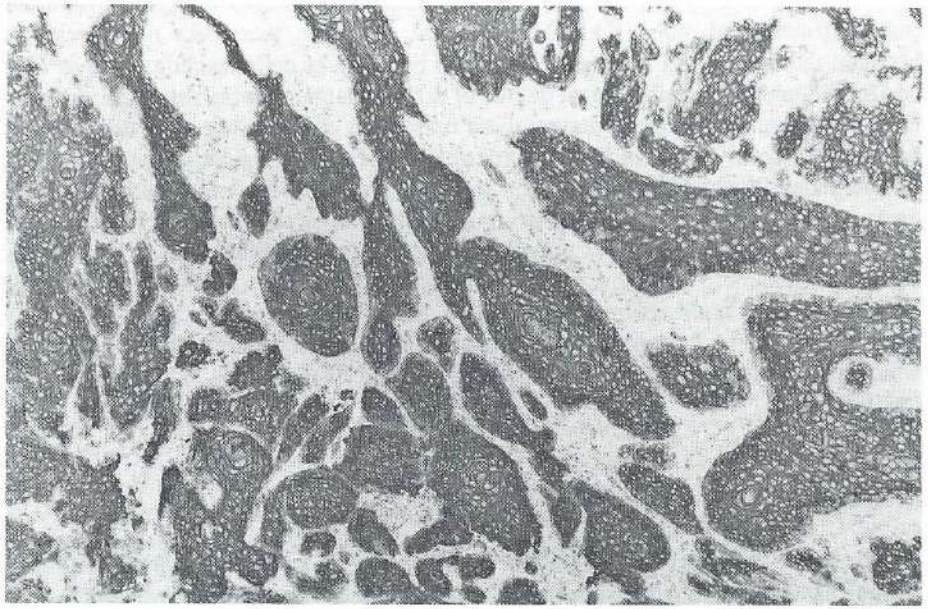

FIGURE 8. Indirect immunoperoxidase staining of a moderately differentiated SCC of the floor of the mouth by MAb E 48 x 90.

of identical Mr value of 22,000 (Fig. 4). Upon reduction of the samples the recognition of the 22 kDa band was abolished. Also when protease inhibitors were omitted, no reactivity was seen. No reactivity was seen under the appropriate conditions mentioned with UMSCC 11-B, which was also negative in the Cell ELISA, or cells of the leukemia tumour cell line HL 60.

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. For cultured cells of squamous cell carcinoma cell lines similar observations were made. 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, as seen in a large excretory duct of the submandibular gland in Fig. 5, reacted positive with the antibody. Table III 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. Whereas in the majority of SCC all cells were labelled by the antibody, some tumours expressed the antigen only in the more differentiated cells (Fig. 6). In general, however, there was no correlation between the intensity of E 48 staining or the numbers of cells reacting positive with MAb E 48 and the morphological degree of differentiation. Special attention was given to lung carcinomas, since among these tumours it can be difficult to discriminate between the areas of the various subtypes of differentiation (adeno-, squamous-, undifferentiated large and small cell ca.). In some lung tumours, which contained both areas with squamous cell differentiation as well as 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 2 adenocarcinomas and 2 large cell undifferentiated carcinomas, which all contained at the electron microscopic level SCC features ie. the presence of tonofilaments.

DISCUSSION

This study demonstrates that MAb E 48 recognizes an epitope which is among normal tissues exclusively expressed in stratified squamous epithelia and transitional epithelium of the urinary bladder. Staining was seen on the cell membrane often in a punctated fashion, with no cytoplasmic reactivity. Stratified cuboidal epithelia, as pre-

TABLE III-REACTIVITY OF E 48 WITH HUMAN NEOPLASTIC TISSUES ON FROZEN SECTIONS BY IMMUNOPEROXIDASE STAINING

SQUAMOUS-CELL CARCINOMAS	positive/tested
Head and Neck	58/58
Lung	14/15
Cervix	2/2
Skin	1/1
NON SQUAMOUS-CELL CARCINOMAS	
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
Mucinous carcinoma	0/3
Sarcomas	0/4
Melanomas	0/2
Non-Hodgkin lymphoma	0/1

*Positive staining: electron microscopic examination revealed the presence of desmosomes and tonofilaments in these 2 tumours.
@Focal reactivity in poorly differentiated areas, electron microscopic examination revealed the presence of tonofilaments in such areas. #Positive reactivity in case of an undifferentiated solid duct mamma carcinoma. Sections were scored positive when at least 30% of all tumour cells were stained by E 48 MAb, in cases of less cells staining, reactivity was considered as focally.

sent inlarge excretory ducts, in general did not react nor did any other tissue containing simple epithelium. Exceptions were occasionally seen among apparent 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 since stratified squamous epithelia from guinea pig, rat and bovine origin (muzzle) failed to exhibit any reactivity.

Immunoblot analysis revealed that the antigen is a 22 kDa molecule under non-reducing 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 by immunohistochemistry and immunoblotting it was found that the epitope was sensitive to proteases. Omission of protease inhibitors in the lysis buffer abolished the immunoblot reactivity. Also pretreatment of frozen sections with pronase resulted in loss of reactivity. In contrast, pretreatment with periodic acid or neuraminidase did not alter the labelling of the antibody suggesting that E 48 recognizes rather a peptide epitope than a carbohydrate or sialic acid structure.

With the use of immunoelectron microscopy the antigen could be traced both on desmosomes and along the cell surface. Desmosomal reactivity was seen in the inter-desmosomal cleft. No labelling was seen with basement membranes (fig 2, 3).

The specificity of MAb E 48 for the recognition of squamous epithelial differentiation was further underlined by the finding that 75 out of 76 squamous cell carcinomas reacted with MAb E 48, whereas no adenocarcinomas or small cell carcinomas did. Only one poorly differentiated solid duct carcinoma of the mamma showed focal staining. Differentiation into squamous cell direction could not be demonstrated at the electronmicroscopic level, due to lack of tissue. All squamous cell carcinomas did react with Mab E 48 regardless their site of origin their and morphological degree of differentiation. Heterogeneity of E 48 expression was not observed in the majority of squamous cell carcinomas. Only in 6 out of 76 squamous cell carcinomas a restricted number of cells reacted with the antibody.

Two morphologically diagnosed adenocarcinomas and two large cell undifferentiated carcinomas which focally reacted with E 48, also showed at the subcellular level characteristics of squamous cell differentiation. In squamous cell carcinomas the antigen was detected both on the membrane and within the cytoplasm. Experiments with viable tumour cells made clear that the antigen was located on the outer surface. Compared to normal squamous epithelium, squamous cell carcinomas showed enhanced cytoplasmic staining, which might be an indication of internalization of the antigen.

In its restricted pattern of occurrence in stratified squamous and transitional epithelia, the MAb E 48 antigen resembles desmoplakin II (215 kDa) and the recently described Band 6 protein (15,16). In contrast to band 6 protein (75 kDa), the E 48 antigen can not be detected in stratified cuboidal epithelia as present in the trachea or large excretory ducts of glands. Moreover, band 6 protein and desmoplakin II are present in the desmosomal plaque and not on the cell surface (15,17). Further biochemical studies are now in progress to identify the E 48 antigen including experiments to find whether the E 48 antigen can be related to a 22 kDa polypeptide, which is together with other glycoproteins selectively enriched in preparations of desmosomal cores (15,18-20).

The high specificity of MAb E 48 for squamous cell and the related transitional epithelial differentiation as well the 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 large majority of squamous cell carcinomas and the restricted expression in normal tissues suggest that the antibody is a suitable candidate for immunotargetting. In fact, squamous cell carcinoma xenografts in nude mice could be successfully localized with radiolabelled MAb E 48 (21). Systemic studies on the nature of the epitope will have to elucidate its precise role as a tissue specific antigen.

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CHAPTER IV

EXPRESSION AND CHARACTERIZATION OF TWO DIFFERENTIATION ANTIGENS IN STRATIFIED SQUAMOUS EPITHELIA AND CARCINOMAS

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ABSTRACT

Using viable cells of a squamous cell carcinoma cell (SCC) line as immunogen, 2 monoclonal antibodies (MAbs K 984 and K 928) to SCC surface antigens were generated. Histopathological studies on frozen tissue sections demonstrated that MAb K 984 preferen- tially reacts with basal keratinocytes in normal epidermis and light microscopically poorly differentiated cells in SCC. In contrast, MAb K 928 binds to suprabasal keratinocytes and in SCC tumour sections with light microscopiccally more differentiated cells, located in the central parts of tumour nests. MAb K 984 did bind to all cells in poorly differentiated SCC, as opposite to K 928 which was totally unreactive in these tumours. Biochemical characterization made clear that MAb K 928 reacted with a 50-55 kDa molecule under non-reducing conditions. MAb K 984 was shown to recognize a carbohydrate chain epitope of a glycoprotein, however, attempts to detect a distinct band in immunoblot analysis with K 984 failed. The antigen detected by K 984 is probably associated with the proliferating fraction of SCC, while the K 928 antigen can be regarded as a marker associated with differentiation of keratinocytes and SCC cells.

INTRODUCTION

Squamous cell carcinomas (SCC) is one of the most common types of carcinomas of the head and neck, esophagus, lung, cervix, vulva and epidermis. Identification and characterization of antigens associated with SCC can be of help in diagnosis of these neoplasms. Monoclonal antibodies (MAbs) are important probes to characterize such antigens and they are also useful tools to study the function of SCC associated antigens. When these antigens are localized on the outer surface of the tumour cell, they may also be used for radio- immunolocalization and therapeutic purposes. Since it became clear that most tumour associated antigens are in fact differentation antigens (1), MAbs to such antigens may be suitable for the above mentioned studies. The number of MAbs to SCC associated antigens is still relatively low as compared to that of MAbs directed to other types of tumours (2-8). In this study we describe two newly developed antibodies which recognize surface antigens associated with differentiation. One antigen is present on on basal squamous cells in epidermis and poorly differentiated cells in SCC while the other antigen is expressed by suprabasal squamous cells in epidermis as well as moderately differentiated neoplastic squamous cells. The antigens are characterized for their expression in normal and neoplastic transformed tissues and some of their biochemical and ultrastructural features.

MATERIALS AND METHODS

Immunization and hybridoma production

Immunization and screening were essentially the same as described previously (8).

In short, Balb/C mice were injected intraperitoneally with 10^7 viable cells of the cell line UM-SCC-22A. Four weeks later, an intrasplenic booster was given under general anesthesia. Three days later the spleen was removed and the dissociated spleen cells were fused with the non-producing cell line SP-2/0. Supernatants of growing hybridomas were screened on ELISA for determining binding to intact viable tumour cells and for lack of reactivity with red blood cells. Selected antibodies were further screened upon reactivity with frozen sections derived from oral mucosa and a squamous cell carcinoma from the head and neck.

Isotypes of the MAbs

Isotype determination was performed on 96-wells ELISA plates which had been coated with affinity purified rabbit anti-mouse subclass specific antibodies (IgG1, IgG2a, IgG2b, IgG3 and IgM, Miles, Elkhard IN).

Tissues and cell lines

Neoplastic and non-neoplastic tissues were obtained from surgical procedures and they were also derived from autopsies. Tissues were stored in liquid nitrogen.

The degree of differentiation of SCC was determined by light microscopy. Well-differentiated SCC showed architecturally keratin and keratin pearl formation and the individual well differentiated cells showed a low nucleus/cytoplasm ratio and individual cell keratinization as judged by their eosinophilic cytoplasm. Poorly differentiated SCC did not show keratin pearl formation and the cells showed a high nucleus/cytoplasm ratio and no or very little individual cell keratinization. Moderately differentiated SCC had characteristics in between. Some tumours which were classified as well differentiated SCC by the presence of keratin pearl formation were composed to a high extent of poorly differentiated tumour cells as determined by a high nucleus/cytoplasm ratio and individual cell keratinization.

Human SCC cell lines of the head and neck were kindly provided by dr. T. Carey, University of Michigan, Ann Arbor, MI. UM-SCC-14A, 14B and 14C were derived from a carcinoma of the floor of the mouth (respectively primary tumour, lymph node and skin metastasis), UM-SCC-11B from a laryngeal carcinoma, UM-SCC-22A and UM-SCC-22B are originating from a primary and a lymph node metastasis of the hypopharynx, respectively. A 431, derived from a vulva squamous cell carcinoma, was a gift of dr. B. Defize, Hubrecht laboratory Utrecht, the Netherlands.

Cell ELISA

SCC cell lines growing as monolayers in 96 wells ELISA plates, were used for determining binding of the antibody to the outer surface of viable tumour cells. Confluent cultures were washed with PBS and incubated with supernatant of MAbs K 984 and K 928 for one hour at room temperature. In this way only membrane reactivity was detected and no cytoplasmic or nuclear reactivity; MAb K 112, which reacts with a 43 kDa antigen present in the nuclei of these cell lines, served as a negative control (9). Subsequently the plates were washed, incubated with peroxidase labeled goat anti-mouse immunoglobulin (Dakopatts, Copenhagen Denmark), washed and ortho phe-

nyl diamine (OPD, Sigma St. Louis MO) was added as chromogen. Color development was stopped by adding 2 N H_2SO_4. Absorbance was red at 492 nm.

Immunoperoxidase staining

Surgically removed tumour specimens were snap frozen in liquid nitrogen and cryostat sections of frozen tissue blocks were mounted on poly-L-lysine coated glass slides, air dried and fixed in acetone for 10 minutes at roomtemperature. Immunoperoxidase staining was performed as previously described (10).

Enzymatic sensitivity

Neuraminidase was obtained from Behring (0.5 U/ml, Vibrio Cholerae, Behring, The Netherlands). Periodate treatment was essentially the same as described by Woodward (11). NP-40 lysates of whole cells were prepared by incubation of whole cells for 30 min at 4° C in a 0.01 M Tris-HCl buffer pH 7.8, containing 1% (v/v) NP-40, 0.15 M NaCl, 1 mM PMSF and 0.02 M trypsin inhibitor. A lectin-bound ELISA (12) was performed by coating the lectin wheat germ agglutin (Pharmacia, Uppsala, Sweden) into microtiter well plates, followed by incubation with NP-40 lysates and the subsequent reaction with MAbs. Binding was detected using goat anti-mouse peroxidase complex as has been described in the CELL ELISA.

Radioiodination

Iodination of K 928 and K 984 was performed according to the one vial method described by Haisma *et al.* (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 50 µg Iodogen. After 10 minutes at room temperature a sample was taken to determine the amount of incorporated iodine. One ml of AG1-X8 resin (Bio Rad, Richmond, CA) previously mixed with PBS containing 1% 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 were obtained from Amersham (Rainbow^{TM}, Amersham UK). Proteins were transferred from gel to nitrocellulose with a Multiphor II Nova Blot System (LKB, Bromma, Sweden) 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 overlayered with ^{125}I-labeled K 984 or K 928 (1 x 10^5 cpm/ml) for 2 hours. The sheets were then autoradiographed by exposure to X-ray film with a Kodak X-Omatic intensifying screen for 18-24 hr at -80°C.

Immunoelectronmicroscopy

Oral mucosa tissue was frozen in liquid nitrogen. 10 µm thick cryosections were prepared and mounted on poly-L-lysine coated glass slides. Sections were immediately fixed in PBS containing 1% formaldehyde and 0.1% glutaraldehyde for 20 min at 4° C. The sections were subsequently washed in PBS and incubated overnight with supernatants of MAb K 984 or control antibody JSB-1. The latter is directed against the P-glycoprotein associated with multi-drug resistance and not present in oral mucosa (10). Next, sections were incubated with rabbit anti-mouse immunoglobulins peroxidase conjugated (DAKO) for 3 hr and after subsequent washing in PBS, 3,3'-Diamianobenzidine (DAB, Sigma) was added. Stained slides were fixed in 1% OsO_4 in 1 M Cacodylatebuffer pH 8.2 and dehydrated in ethanol. Subsequently the sections were embedded in epoxy resin (EPON 812, Fluka, Switzerland), ultrathin sections examined by a JEOL electronmicroscope.

Competitive and double immunoperoxidase staining with other antisera

Blocking experiments were performed using several MAbs with overlapping staining patterns in normal or tumour tissue. Histological sections from six different tissue specimens (including normal mucosa and SCC) were preincubated with one of the following antisera: anti-EGF receptor (Bio-Yeda, Rehovot Israel), anti-EGF receptor antibodies donated by Dr. B. Defize Utrecht)(15) and the anti-transferrin receptor antibody OKT-9 (16,17), in concentrations 50 times higher than usual to saturate the defined antigen. MAb K 984 and K 928 were purified using a Protein A column (Pharmacia, Uppsala, Sweden) and subsequently biotinylated. Biotinylated K 984 and K 928 were allowed to react with the preincubated sections. Finally, streptavidine horseradish peroxidase (Sigma) was applied and the complex was visualized using DAB as substrate. Also double staining experiments were performed, in which alkalin phosphatase was used to detect the first incubated antibody and streptavidin horse radish peroxidase to detect the second, essentially as described by Mullink *et al.* (18).

RESULTS

Selected MAbs

Two hybridomas, showing selective binding with frozen sections of SCC, were stabilized and designated K 984 and K 928. MAb K 984 belongs to the IgG 1 subclass and K 928 is an IgG 2b immunoglobulin.

Reactivity of MAbs with normal tissues

In normal stratified squamous epithelia MAb K 984 reacts with the basal cells (Fig.1). Immunoelectron- microscopic studies revealed that the antigen was present on the cell surface. No reactivity was observed with cytoplasmic or nuclear components (Fig. 2 and 3). Further, labelling was seen with spermatogonia in testis, the endocrine pancreas and tubular epithelium in the kidney (Table I). Reactivity in these organs was also seen on the surface with little or no cytoplasmic staining. Binding was also observed with Kupffer cells in the liver and histiocytes.

In contrast to K 984, MAb K 928 reacts with membranes of suprabasal cells in stratified squamous epithelium (Table I). Basal cells did not show surface reactivity but instead slight cytoplasmic staining (Fig. 4). Immunoelectronmicroscopic studies failed to localize the antigen, probably due to loss of the epitope during fixation. Among

TABLE I-REACTIVITY OF MAb K 984 AND K 928 WITH NORMAL HUMAN TISSUES AS DETERMINED ON FROZEN SECTIONS BY IMMUNOPEROXIDASE

ORGAN	K 984	K 928
Oral mucosa		
basal cells	++	- (slight cytoplasmic
suprabasal cells	-	++ reactivity)
Epidermis		
basal cells	++	- (slight cytoplasmic
suprabasal cells	-	++ reactivity)
sebaceous glands	-	+
sweat glands	-	-
Lung		
trachea	-	++
bronchus	-	++
pneumocytes	-	+
Mammary gland		
acinar cells	-	+
duct cells	-	+
Pancreas		
acinar cells	-	+
duct cells	-	+
islet cells	+	-
Liver		
hepatocytes	-	-
bile canicuti	-	+
Kupffer cells	+	-
Kidney		
glomerulus	-	-
Bowmans capsule	-	-/+
tubuli (type 1)	+	+
Colon, jejunum and duodenum		
absorptive cells	-	-
goblett cells	-	-
Brunner's gland	-	-
Ovary		
Oocytes	-	-
oviduct cells	-	+
Testes		
spermatogonia	++	-
Bladder	-	-
Thyroid		
follicular cells	-	-
C-cells	-	-
Lymph node		
lymphoid cells	-	-
histiocytes	+	-
Peripheral neuron		
Schwann cells	-	+

For each organ at least 3 specimens were tested.

other tissues tested K 928 was found to be present in pneumocytes in normal lung, duct and acinar cells of salivary glands, bile ducts and caniculi in liver, oviduct epithelium, all epithelial cells in mamma and urinary bladder and in cells of renal tubuli (type I). Mesenchymal tissues and nervous tissues did not react with K 928 with the exception of peripheral nerves in which Schwann cells stained positive (Table I).

Reactivity of MAbs with human carcinomas

K 984 reacted with a vast group of human neoplasms tested. Also tumours, derived from antigen negative non-neoplastic tissues such as lung or mamma expressed the antigen (Table II). In contrast, colonic carcinomas did not bind the antibody. K 928 showed a more restricted binding to the tumours tested. (Table II).

TABLE II-REACTIVITY OF MAbs K 984 AND K 928 WITH HUMAN CARCINOMAS AS DETERMINED ON FROZEN SECTIONS BY IMMUNOPEROXIDASE

	positive/tested	
	K 984	K 928
Squamous cell carcinomas		
Head and neck	59/59	50/59
Lung	6/6	5/6
Small cell carcinoma		
Lung	0/2	2/2
Undifferentiated large cell	1/2	1/2
Lung		
Adeno carcinoma		
Lung	4/6	6/6
Breast	4/6	6/6
Colon	0/6	0/6
Ovaries	1/6	5/6
Melanomas	0/3	0/3
Lymphomas	0/2	0/2

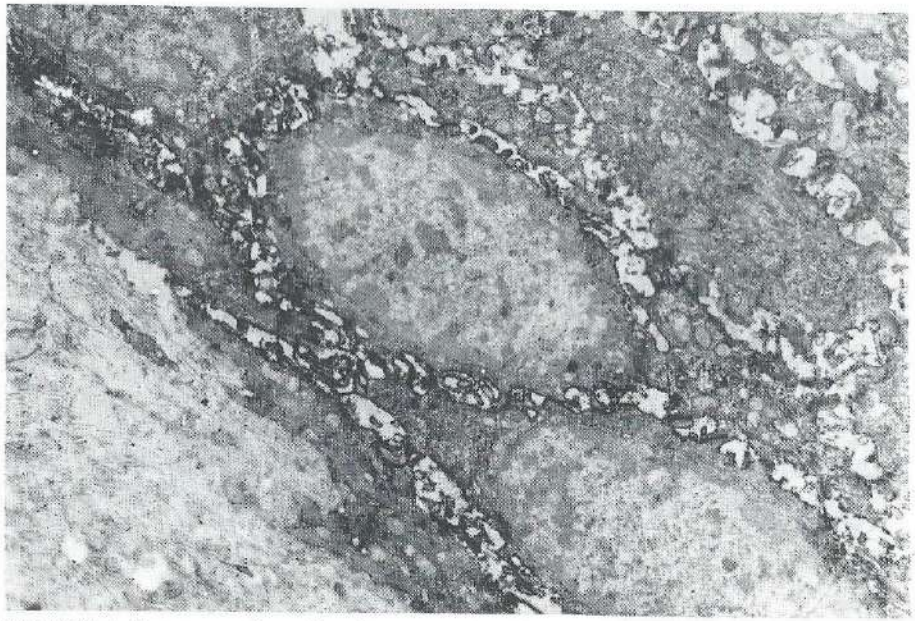

FIGURE 1. Frozen section of oral mucosa showing reactivity of MAb K 984 with the basal cell layer. Immunoperoxidase staining.

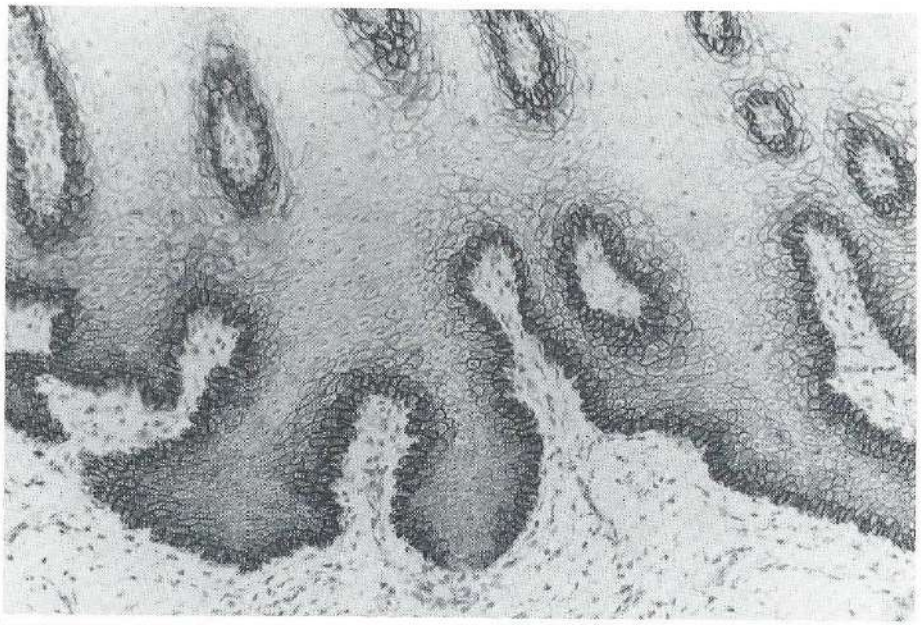

FIGURE 2. Ultrastuctural localization of MAb K 984 in cells of oral mucosa. DAB staining is localized along the cell surface of basal cells x 8750.

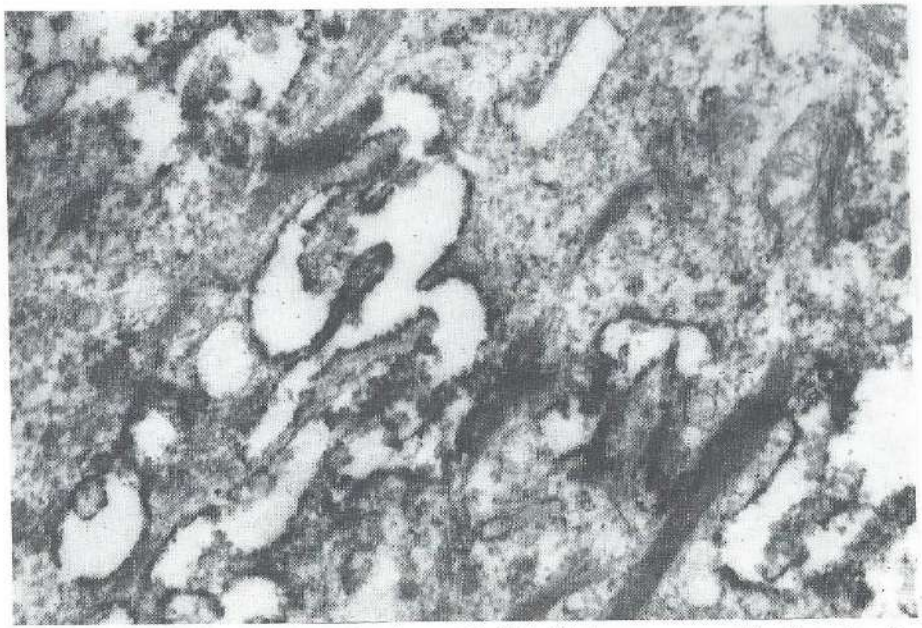

FIGURE 3. Ultrastructural localization of K 984. High power magnification showing the distinct surface labelling. X 50,400.

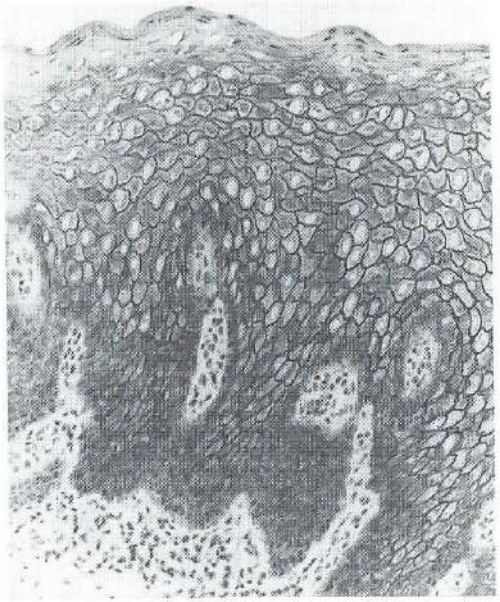

FIGURE 4. Frozen section of oral mucosa showing reactivity of MAb K 928 with the membranes of the suprabasal cell layer. Immunoperoxidase staining.

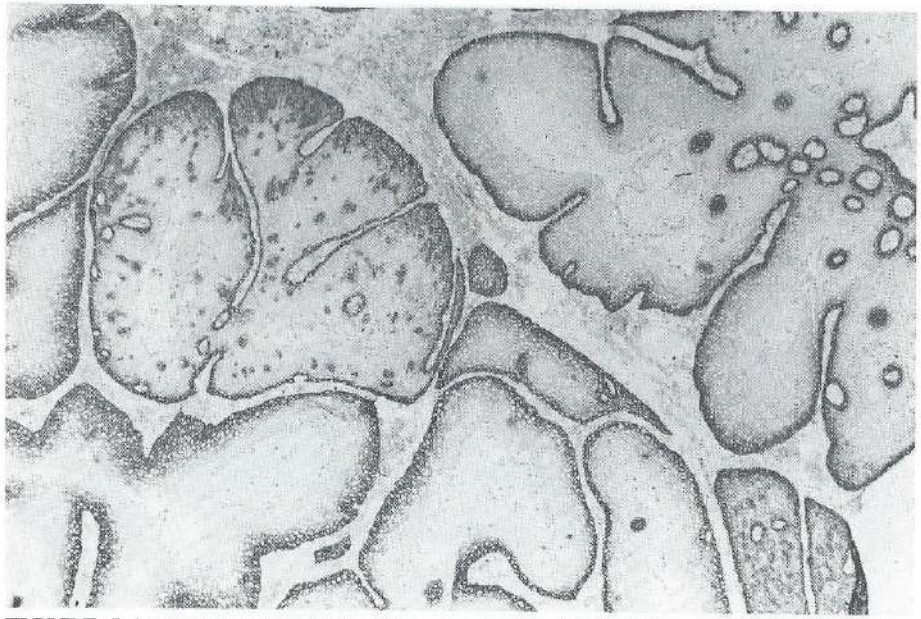

FIGURE 5. Reactivity of K 984 with a verrucuous SCC of the maxillary sinus. Note the reactivity with a single basal layer.

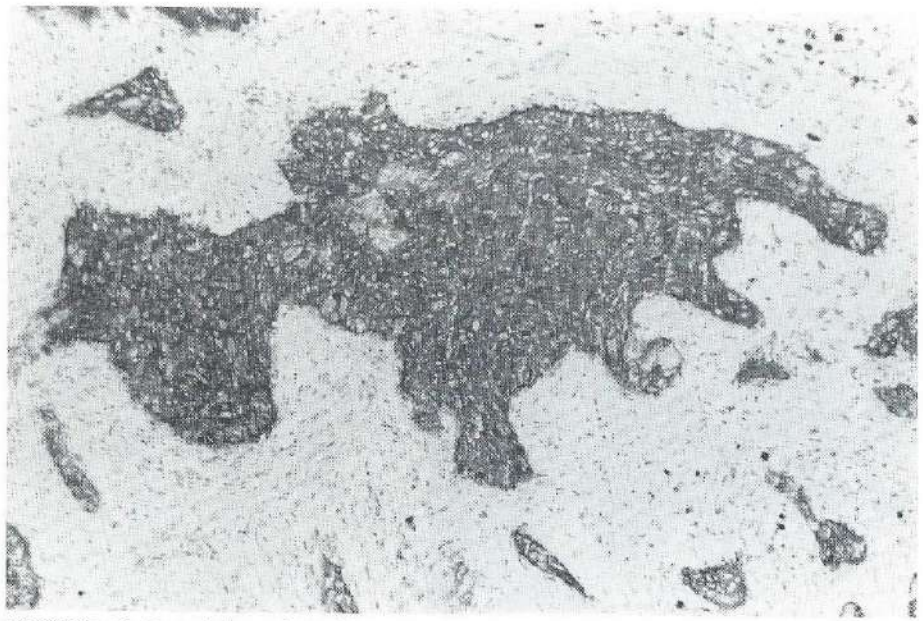

FIGURE 6. Reactivity of K 984 with a poorly differentiated SCC of the larynx. Staining can be observed in all layers.

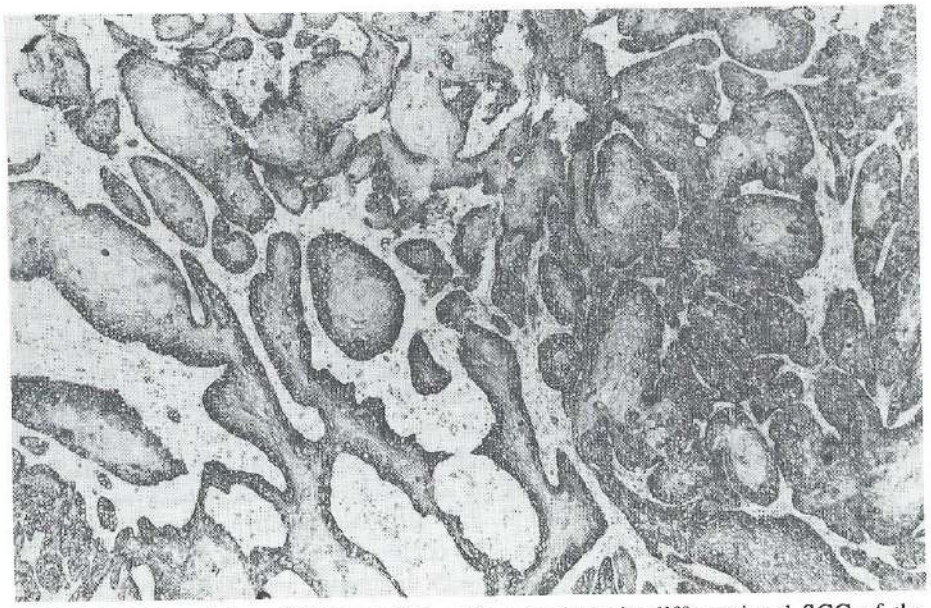

FIGURE 7. Reactivity of MAb K 984 with a moderately differentiated SCC of the floor of the mouth. Binding can be seen in multiple layers.

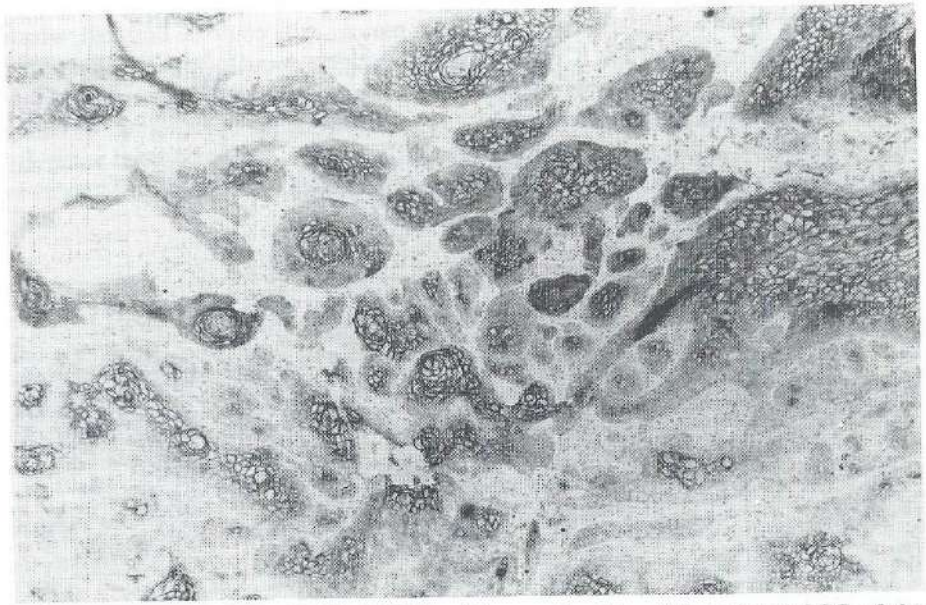

FIGURE 8. Reactivity of MAb K 928 with a moderately differentiated SCC of the floor of the mouth. Peripheral cells in tumour nests are not bound by the antibody, in contrast to the central layers.

Reactivity with SCC of the head and neck

Since MAbs K 984 and K 928 recognize apparently differentiation related antigens in normal squamous cell epithelium, the reactivity of the antibodies was further investigated with a number of squamous cell carcinomas of the head and neck in order to study the correlation between antigen expression and the histological degree of differentiation as determined by light microscopic evaluation. Basically two staining patterns could be observed with MAb K 984 on frozen sections: 1. staining of a single layer ie. the basal cell layer in normal squamous epithelium or the most peripheral cell layer in SCC (representing the light microscopically poorly differentiated cells) (Fig. 5). This staining pattern was seen in 14 out of 22 well differentiated SCC; 2. Staining extended from the most peripheral layer to central layers, thus multiple or all layers stained positive for K 984 (Fig.6). This labelling pattern was seen with all poorly (n=6)(fig. 6) and moderately (n=12) differentiated SCC (fig. 7) and in a minority (8 out of 22) of the well differentated SCC. In well differentiated SCC in which several layers reacted with K 984, those layers were composed of poorly differentiated squamous cells as determined by the lack of individual cell keratinization and a high nucleus/cytoplasm ratio. For MAb K 928 also two patterns of reactivity were observed. 1. No binding to the tumour. This occurred in all poorly differentiated SCC (n=6). 2. Reactivity with central cell layers in tumour nests (cells showing light microscopically features of differentiation), which can be compared with reactivity of suprabasal cells in normal squamous cell epithelia. This occurred in moderately and well differentiated SCC (Fig. 8). Double staining procedures with K 928 and K 984 revealed that in several tissue sections of SCC, tumour cells could be detected, which simultaneously expressed the K 984 and K 928 antigen in contrast to the non-transformed keratinocyt in which this phenomenon was not observed. Direct evidence that the epitopes recognized by the antibodies was accessible on the outer surface of viable tumour cells was found in the results of the CELL ELISA. We noted good reactivity with all human SCC cell lines tested in this assay, although quantitative differences in reactivity were observed as can be seen in table III.

TABLE III-REACTIVITY OF MAbs K 984 AND K 928 WITH IN VITRO GROWING HUMAN SCC CELL LINES

CELL LINE	14A	14B	14C	22A	22B	A 431
K 984	1.10	1.50	1.90	1.50	1.60	1.40
K 928	1.90	1.40	1.00	1.30	1.30	1.50
K 112	0.13	0.15	0.12	0.15	0.16	0.12

Absorbance was red at 492 nm. MAb K 112 served as a negative control.

Identification of K 928 antigen in cultured cell lines by immunoblotting

Using immunoblot analysis of 3 different cell lines derived from patients with SCC, we have identified a single band of almost identical molecular weight of 50-55 kDa under non-reducing conditions (Fig. 9). Quantitative differences could be observed between UM-SCC-14A and UM-SCC-14C. Upon reduction of the samples the recognition of the 50-55 kDa band was abolished. Total membranes were extracted by Triton X-100 and NP-40, and the K 928 antigen was found quantitatively within the detergent phase (Fig. 9). K 984 failed to react with a discrete band in immunoblotting.

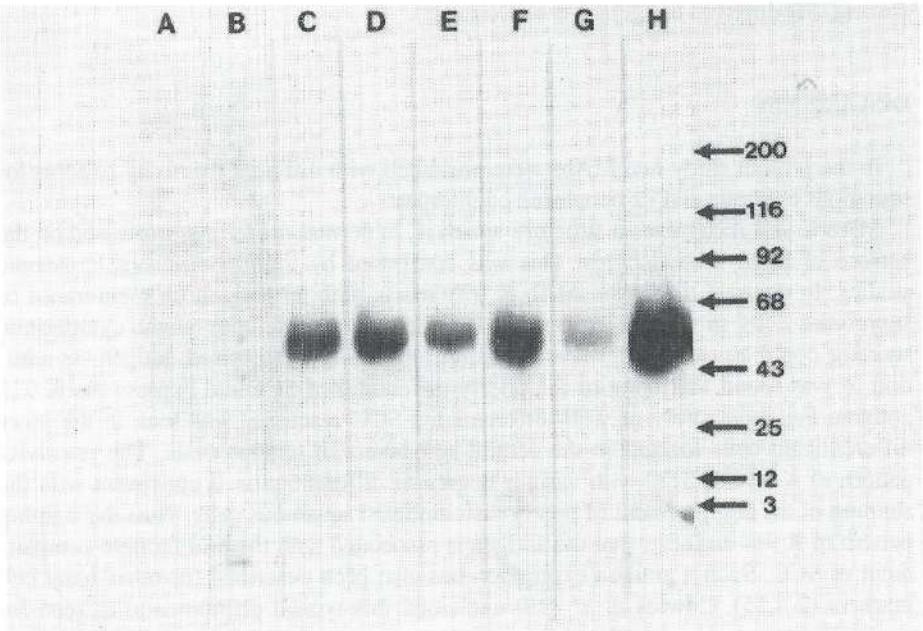

FIGURE 9. Immunoblot analysis of cell lysates of SCC cell lines with radiolabelled MAb K 928. Lane A: control cell line HL 60 not reactive with K 928; Lane B: UM-SCC 14A in the presence of β-mercaptoethanol (reducing conditions); Lane C: UM-SCC-38; Lane D: UM-SCC-38 upon extraction with NP-40; Lane E:UM-SCC-38 upon extraction with Triton X-100; Lane F: UM-SCC-14B;Lane G:UM-SCC-14C; Lane H:UM-SCC-14A.

Enzymatic sensitivity

The relative sensitivity of the structures recognized by K 984 and K 928 was assayed by an immunoperoxidase technique. Pretreatment of the sections with neuraminidase did not influence the labelling, indicating that no sialic acid residues were part of the epitope. In contrast, when sections were treated with periodate, staining disappeared-for K 984 but not for K 928, indicating that the K 984 determinant is located on a car-

bohydrate chain. Also, K 984 reacted with NP-40 lysates of tumour cells which were bound on wheat germ agglutin (WGA) in a lectin-bound ELISA, suggesting that K 984 recognizes a glycoprotein. K 928 gave no detectable signal in this assay.

Competition experiments

Within the limits of immunoperoxidase detection, none of the antisera tested (anti-EGF- and anti-transferrin receptor MAbs) affected either the intensity or the staining pattern of K 928 and K 984. Also in case of double labelling experiments, there were considerable differences in the staining patterns of K 984 and the tested antisera, suggesting that different antigens were recognized.

DISCUSSION

In the present study two MAbs were presented with different reactivity patterns for squamous epithelia and its neoplastic counterpart.

MAb K 984 recognizes a structure which is, in normal epidermis, expressed on the surface of basal keratinocytes. This was confirmed by immunoelectronmicroscopic studies. In contrast to K 984, MAb K 928 reacts with an antigen on membranes of suprabasal cells in normal epidermis. In basal cells sometimes slight cytoplasmic staining could be observed, maybe representing newly synthesized antigen. In addition, it was found that none of the poorly differentiated SCC did express the K 928 antigen. For moderately or well differentiated SCC reactivity was seen in the more differentiated cells located in the central cell layers in tumour nests. The reactivity pattern of K 984 in SCC with various degree of differentiation is consistent with the staining of the compartment of poorly differentiated squamous cells. Thus the staining pattern of K 984 suggests that the antigen is associated with the proliferative compartment of SCC. Such a antigen expression has also been described for other basal cell markers (2-4,15). Cowley *et al.* (19) explained this typical phenomenon as seen for the EGF-receptor. He speculated that as a consequence of transformation the differentiation could be blocked and for that reason the receptor levels remain high.

In immunoblotting analysis, it was found that K 928 reacts with a 50-55 kDa molecule under non-reducing conditions. The loss of reactivity under reducing conditions suggests that one or more disulfide bands are directly or indirectly involved in the formation of the epitope recognized by K 928. The strong expression of the K 928 antigen by UM-SCC-14A in comparison to UM-SCC-14C, as monitored in the CELL ELISA, was also seen in immunoblotting. The fact that the antigen was found quantitatively in the detergent phase upon extraction of total membranes with Triton X 100 or NP-40, indicates that the antigen is not a keratin polypeptide, since these are found in the NP-40 insoluble pool (20). The antigen structure recognized by MAb K 984 could not be identified in immunoblot analysis, since the antibodies failed to react with a distinct band in this assay. The relative sensitivity of the antigen for periodate, indicates that the epitope is located on a carbohydrate chain, in contrast to K 928 antigen which was not sensitive towards this treatment. The K 984 antigen could be

detected in the Lectin bound ELISA after lysis of cells with NP-40, suggesting that it is presumably a glycoprotein rather than a glycolipid since the latter would not be recovered upon treatment with non-ionic detergents. Since selective reactivity with basal cells in squamous cell epithelium has been documented for antibodies against the well described glycoproteins, the transferrin receptor and the epidermal growth factor (15-17), we considered the possibility that the K 984 antigen is one of these receptors. K 984 did not precipitate the phosphorylated epidermal growth factor receptor in a radio immunoprecipitation assay (data not shown) nor did preincubation with anti-EGF receptor antibodies compete with K 984 reactivity on histologic sections, making it unlikely that K 984 recognizes this molecule. With regard to the transferrin receptor, competition experiments with OKT-9 did not reduce the staining of K 984 in histological sections. Double staining experiments with anti-transferrin- and anti-EGF receptor antibodies and K 984 showed for a variety of tumour specimen a different staining pattern, suggesting that different antigens were recognized.

Although further studies are required to identify the antigens described here, the data of this study indicate that K 984 and K 928 recognize surface antigens associated with proliferating respectively more differentiated (neoplastic) squamous cells. In addition, preliminary results have shown that MAb K 984 is able to inhibit the growth of SCC lines in an antibody concentration dependent manner. Together with the strong expression of the K 984 antigen on the majority of SCC, this can make the K 984 antibody valuable for radioimmunoimaging and therapy of SCC. Such studies are now in progress.

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ADDENDUM TO CHAPTER IV.

GROWTH INHIBITORY EFFECT OF MONOCLONAL ANTIBODY K 984 ON *IN VITRO* CULTURED HEAD AND NECK SQUAMOUS CELL CARCINOMA CELL LINES.

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INTRODUCTION.

In chapter IV we demonstrated that the K 984 is present on cells which are supposed to represent the proliferative compartment of SCC. This expession raised the idea that the K 984 antigen may be involved in the regulation of growth of SCC. To test this hypothesis, the effect of MAb K 984 on *in vitro* cultured head and neck squamous cell carcinoma cell lines (HN-SCC) was assayed. A significant dose dependent reduction of growth was observed, indicating that the K 984 antigen is indeed functionally active in regulation of proliferation of SCC.

MATERIAL AND METHODS.

The origin of tissues and cells have been described in chapter IV. For here described experiments, additional cell lines were a gift of Dr. A. van den Eynden-van Raay, Hubrecht Laboratory Utrecht (NRK: normal rat kidney cell line) and Dr. J. Kipp, Laboratory for Radiobiology, University of Amsterdam (R1: a rat rhabdomyosarcoma cell line). Cells were routinely grown at 37^0c as subconfluent monolayers in 25 cm^2 plastic Nunclon flasks. The medium consisted of Dulbecco's modified Eagle's medium (DMEM) buffered with 15 mM Hepes and 16 mM $NaHCO_3$, 5% CO_2, pH 7.5 and supplemented with 10% heat inactivated calf serum (FCS).

Growth inhibition assay

Suspensions of single cells were seeded on 8 cm^2 plastic dishes (30 X 10^3 cells/dish) in 2 ml DMEM plus 10% FCS and 24 hr later the medium was exchanged every three days. Cell counts were performed using a hematocytometer and growth curves were constructed.

RESULTS

Growth inhibitory effects of MAb K 984 on in vitro cultured SCC cell lines

To examine whether K 984 has any effect on the growth of SCC, monolayers of cell line UM-SCC-14C were exposed to MAb K 984 and cells were counted at various time intervals after antibody addition. As shown by Figure 1 there was a significant reduction of growth of UM-SCC-14C cells treated with K 984.

Growth inhibiting effects became apparent within the first day after antibody addition and resulted in an extension of the lag phase. During the next two days of culturing, growth commenced in the presence of antibody and after that period cells entered the logarithmic growth phase. K 984 extended the doubling time of UM-SCC-14C cultures in logarithmic growth phase from 1.9 to 3.1 days. Under the same conditions, there was no reduction of growth by another MAb, K 931 (see also Chapter 5), which also binds to high extent to the cell membrane of this cell line (data not shown).

To evaluate the growth inhibitory effect of MAb K 984 further, UM-SCC-14C cells were exposed to various concentrations of K 984 (figure 2).

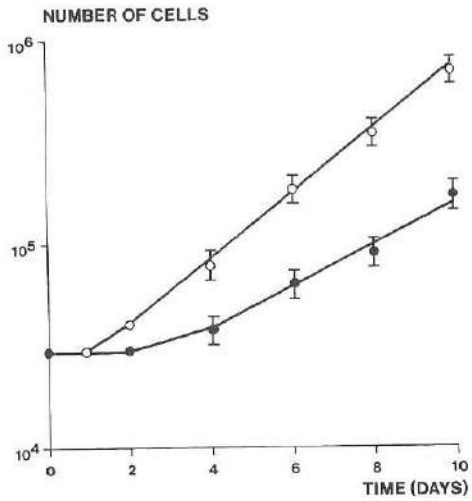

FIGURE 1 - Effect of MAb K 984 on growth of SCC cell line UM-SCC-14C. Cells were seeded on 8 cm^2 plastic dishes (30 X 10^3 cells/dish) in 2 ml DMEM/10% FCS, and 24 hours later the medium was exchanged for 2 ml DMEM/10% FCS with or without MAb K 984 (10 µg/ml). The number of cells was counted at various time intervals after seeding. Points: mean of triplicate determinations.

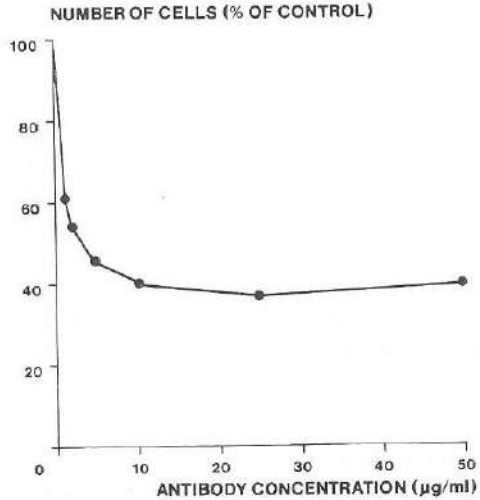

FIGURE 2 - Dose-response curve for growth inhibition of SCC cell line UM-SCC-14C induced by MAb K 984. Cells were seeded on 8 cm^2 plastic dishes (30 X 10^3 cells/dish) in 2 ml DMEM/10% FCS, and 24 hours later the medium was exchanged for 2 ml DMEM/10 % FCS with or without MAb K 984. The number of cells was counted 4 days after seeding. Points: mean of triplicate determinations. The experiment was repeated two times. Standard errors were 10% or less.

Antibody preparations were tested over a concentration range from 1 to 50 µg/ml, but dose dependent inhibitory effects were only observed for concentrations up to 10 µg/ml. Higher doses had no further effect. When evaluated at day 4 (3 days after antibody addition) UM-SCC-14C exhibited a 50% reduction of cell counts (IC50) when grown in the presence of 3 µg MAb K 984 per ml.
Inhibition of growth by K 984 was not limited to UM-SCC-14C but was also observed for 3 other SCC lines tested, UM-SCC-11B, UM-SCC-22B and A 431 (figure 3).

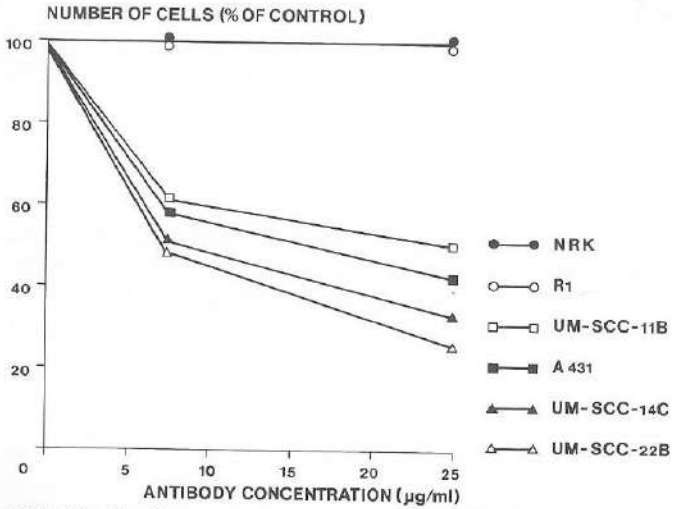

FIGURE 3 - Dose response curves for K 984 induced growth inhibiton of the head and neck SCC lines UM-SCC-22B, UM-SCC 11B, UM-SCC-14C and the vulva SCC cell line A 431. As control, the antigen-negative normal rat kidney (NRK) and rhabdomyosarcoma (R1) cell lines were included. Cells were seeded on 8 cm^2 plastic dishes (30 X 10^3 cells/dish) in 2 ml DMEM/10% FCS, and 24 hours later the medium was exchanged for 2 mlDMEM/10 % FCS with or without MAb K 984. The number of cells was counted 4 days after seeding. Points: mean of triplicate determinations. The experiment was repeated two times. Standard errors were 10% or less.

Growth inhibitory effects of K 984 tested at a concentration of 7.5 and 25 µg/ml was comparable for all 4 cell lines tested. In contrast, under the same conditions K 984 did not reduce cell counts of the antigen-negative cell lines NRK and R1.

DISCUSSION

In this addendum we describe our first efforts to elucidate the physiological function of the K 984 antigen. It was demonstrated in Chapter IV that 6 SCC cell lines grown as subconfluent monolayer cultures on plastic, all showed strong reactivity with MAb K 984. When four of these lines were cultured in the presence of MAb K 984, a significant reduction of growth rate could be observed. Inhibition of growth seems specific because (i) no reduction of growth was observed with another, isotype matched antibody which also binds to a high extent to the cell membrane, (ii) the growth rate of antigen negative cell lines was not reduced and (iii) $F(ab)_2$ fragments had a comparable growth inhibitory effect at an equimolar concentration (data not shown). No cell detachment was observed, indicating that the antibody has a cytostatic rather than a cytotoxic effect. Neither a viability staining with trypan blue gave evidence for antibody mediated cell killing. The mechanisms by which K 984 inhibits growth is unclear. That growth inhibtion of SCC cells might be the result of impaired cell adhesion seems unlikely. Irrespective the antibody was added during seeding or 1 day thereafter, growth kinetics were the same (data not shown). Under both conditions no apparent delay of cell attachment was observed. Further characterization of the antigen will be necessary to reveal the mechanism by which K 984 inhibits the growth of SCC. This growth inhibitory effect of K 984 makes the antibody even more suitable for radioimmunotherapy of SCC.

CHAPTER V

PRODUCTION OF A MONOCLONAL ANTIBODY (K 931) TO A SQUAMOUS CELL CARCINOMA ASSOCIATED ANTIGEN IDENTIFIED AS THE 17-1A ANTIGEN.

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ABSTRACT

During our efforts to develop monoclonal antibodies (MAbs) to tumour associated surface antigens of squamous cell carcinomas of the head and neck, monoclonal antibody K 931 was produced. The antibody showed reactivity with 58 out of 62 squamous cell carcinomas of the head and neck. In contrast normal squamous epithelium as found in epidermis, oral cavity, epiglottis, pharynx, larynx and esophagus did not express the antigen. All further tested epithelial (simple and transitional) tissues did express the antigen, but non-epithelial tissues were negative. Further characterization revealed that the antigen represented the 17-1A antigen. A, not earlier reported, enhanced expression of the 17-1A antigen was observed among some primary and all metastatic SCC.

INTRODUCTION.

Monoclonal antibodies (MAbs) to tumour associated antigens (TAA) are potentially powerful tools in diagnosis and treatment of cancer. Despite the theoretical possibility to produce antibodies to any desired antigen, so far only a resticted number of TAA have been identified. Among these are the MAM-6 antigen (1), the transferrin receptor(2), the Carcinoembryonic antigen (CEA)(3) and the 17-1A antigen(4).

In an attempt to produce MAbs to tumour associated surface antigens of head and neck squamous cell carcinomas (HN-SCC), mice were immunized with viable tumour cells of the laryngeal carcinoma cell line UM-SCC-11B. Upon screening, one hybridoma was selected because of selective reactivity with HN-SCC and not with normal squamous epithelium of oral mucosa. Further characterization revealed that the antigen represented the 17-1A antigen. The expression of the 17-1A antigen in primary HN-SCC and their metastases, as monitored by the newly produced MAb K 931, as well as the apparent immunogenicity of the antigen are discussed.

MATERIALS AND METHODS.

Immunization and hybridoma production

Balb/C mice were injected intraperitoneally with 10^6 viable tumour cells of the *in vitro* growing cell line UM-SCC-11B, derived from a SCC of the larynx. Four weeks later an intrasplenic booster was given under general anesthesia (5). Three days later the spleen was removed and the dissociated spleen cells were fused with the non-producing cell line SP-2/0. Hybridomas were cultured with RPMI-1640, penicillin, streptomycin, 15% Fetal Calf Serum (FCS) and 1% Hybridoma Growth Factor (HGF, Janssen, Beerse Belgium). Growing hybridomas were screened on ELISA for reactivity with UM-SCC-11B cells and for lack of reactivity with red blood cells. Selected antibodies were further screened upon frozen sections from oral mucosa, submandibular gland and a SCC. Hybridomas showing selective binding were stabilized by limiting dilution.

Isotypes of the MAbs

Isotype determination was performed with the use of an 96-wells ELISA plate which had been coated with affinity purified rabbit anti-mouse subclass specific antibodies (IgG1, IgG2a, IgG2b, IgG3 and IgM, Miles, Belfast IN)

Tissues and cell lines

Tumour and normal tissues were obtained from surgical procedures and they were also derived from autopsies. Tissues were stored in liquid nitrogen.

Human SCC cell lines with the prefix UM-SCC were kindly provided by Dr. T. Carey, University of Michigan, Ann Arbor MI. 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% FCS.

Cell ELISA

The Cell ELISA was performed essentially as has been described (5). In short, monolayers of SCC cells were growing in 96-wells ELISA plates. The plates were washed with PBS and incubated with supernatant of MAb K 931 for one hour. In this way only surface binding was detected and no cytoplasmic or nuclear reactivity. MAb K 112, which reacts with a 43 kDa nuclear antigen present in the nuclei of the tested cell lines, served as a negative control (6). Subsequently the plates were washed, incubated with goat anti-mouse peroxidase conjugated immunoglobulins (DAKO, Copenhagen Denmark) washed and as chromogen ortho phenyl diamine (OPD, Sigma Chemical Co, ST Louis MO) was added. Color development was stopped by adding 2 N H_2SO_4. Absorbance was red at 492 nm.

Immunoperoxidase staining

Four to six micrometer thick sections of frozen tissue blocks were prepared on a cryostat microtome, mounted on poly-L-lysine coated glass slides, air dried and acetone fixed for 10 minutes at room temperature. Immunoperoxidase staining was performed as has been described in detail elsewhere (5).

Biochemical characterization

SDS-PAGE was carried out as described by Laemmli (7). Proteins were transferred to a nitrocellulose filter essentially according to Townbin *et al.*(8). Antigens were detected using the immunoperoxidase method according to Wood (9). 17-1A antigen was purified from SW 1116 cells using a Sepharose column coupled with anti-17-1A antibodies, essentially as has been described by Herlyn *et al.* (10).

RESULTS

Selection and isotype

Approximately 1100 hybridomas were generated from one fusion. Antibodies secreted by these hybridomas were screened by binding assays against cultured cells.

MAbs wich bound to squamous cell carcinoma cell lines but not to red blood cells were further screened for reactivity on frozen sections of squamous cell carcinomas, normal oral mucosa and a submandibular gland. One hybridoma cell line designated K 931, secreting an IgG_1 antibody, was established on the basis of its preliminary response on test cell lines and on normal and SCC tissues.

Reactivity of K 931 with normal human tissues

Non-keratinizing stratified squamous epithelia, as found in mucosa of oral cavity, pharynx, larynx and esophagus did not react with K 931. In stratified squamous epithelium (epidermis) reactivity was seen with sweat glands, but not with sebaceous glands and keratinocytes. Squamous cell metaplasia, as present in a large excretory duct of the floor of the mouth, did not react with the antibody in contrast to the adjacent simple and columnar epithelia (Fig. 1). Extensive screening of the antibody on frozen sections of human tissues revealed that the antigen, recognized by K 931 was present on almost all epithelial tissues (Table I).

Reactivity of K 931 with human SCC tissues and cell lines

Immunoperoxidase staining was performed on both frozen and formalin fixed paraffin-embedded tissue sections. Whereas in some cases good binding of K 931 was seen with formalin fixed tissues, in other specimen there was reduction of staining as compared to the frozen sections. Therefore all further experiments were performed on frozen sections. Of 62 squamous cell carcinomas of the head and neck, 58 were found to be positive. In the majority of cases K 931 exhibited heterogenous staining. Reactivity was notably observed within the cytoplasm of light microscopically differentiated cells in the central parts of tumour islets (Fig 2). A different reactivity of K 931 was seen in lymph node and hematogenous metastases of SCC of the head and neck (n=6). In these specimens binding was clearly observed with the surface of the poorly differentiated cells in the peripheral cell layers in tumour islets. Also the number of cells in such specimens showing reactivity was enhanced as compared to that of sections prepared of primary tumours. This reactivity pattern was seen for both poorly differentiated as well for well differentiated SCC metastases. A small group of advanced primary (stage III and IV) tumours exhibited a similar reactivity pattern as the lymph node and distant metastases (Fig. 3 and 4). Among poorly differentiated primary SCC, 2 showed the enhanced expression pattern, while 4 were non-reactive. The CELL ELISA results demonstrated that the K 931 antigen was present on the outer surface of cells of SCC cell lines. K 931 bound to 13 out of 15

TABLE I - REACTIVITY OF K 931 WITH FROZEN SECTION OF NORMAL HUMAN TISSUES

Tissue	positive/tested	type of cell reactive
epidermis		
keratinocytes	0/6	
sweat gland	6/6	
sebaceous gland	0/6	
oral mucosa (buccal)	0/8	
tongue	0/2	
pharynx	0/2	
layngeal epithelium	0/3	
esophagus	0/3	
tonsil (cryptepithelium)		
salivary glands	8/8	serous and mucus acinar cells, intercalated, excretory ducts
lung	3/3	pneumocytes, epithelium lining bronchi
mamma	2/2	all epithelial cells except myoepithelial cells
stomach	2/2	all epithelial cells
colon	3/3	all epithelial cells
jejunum	2/2	all epithelial cells
liver	3/3	bile ducts and caniculi
kidney	2/2	tubular epithelia
thyroid	2/2	all epithelial cells
ovary	2/2	oocytes and oviduct epithelium
testes	0/2	
bladder	3/3	epithelial cells
spleen	0/3	
lymph node	0/4	
peripheral neuron	0/2	
cerebellum	0/2	

Exceptions were seen only among normal keratinocytes as described above and hepatocytes. All other epithelial tissues stained positive for K 931. In contrast non-epithelial tissues or cells did not exhibit any reactivity.

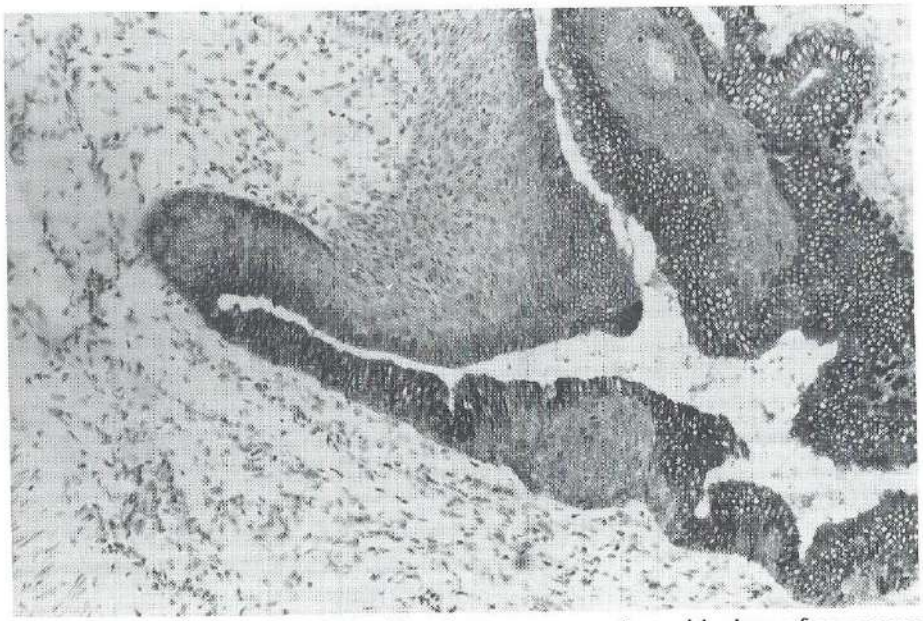

FIGURE 1. Reactivity of K 931 with a large excretory duct with signs of squamous metaplasia.

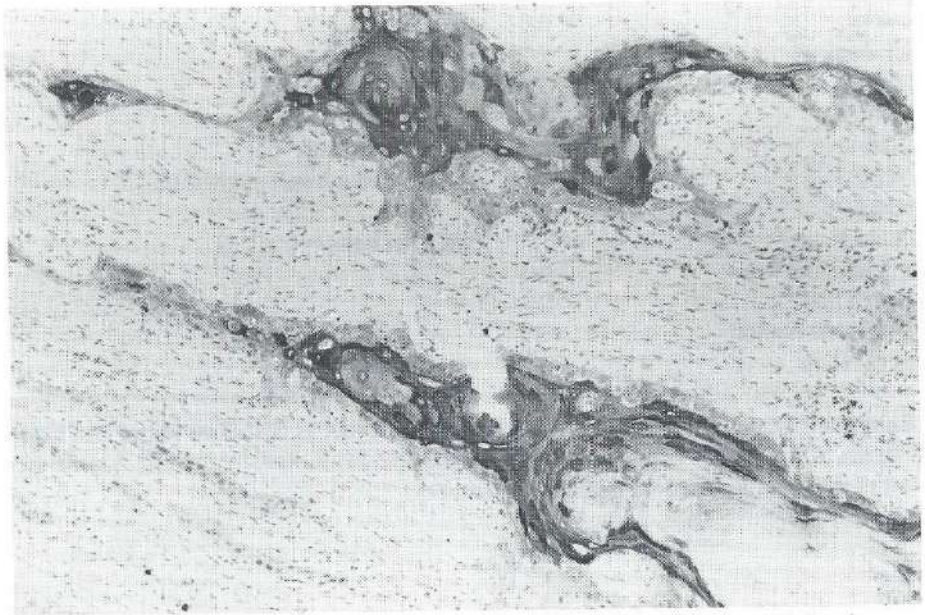

FIGURE 2. Reactivity of K 931 with a primary SCC of the larynx: staining is confined to the central areas of the tumour

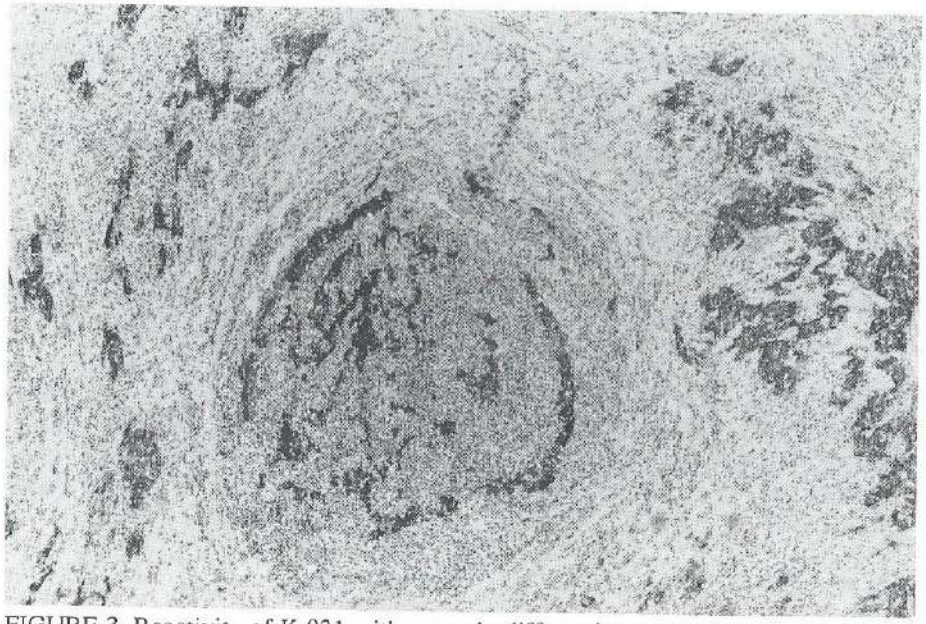

FIGURE 3. Reactivity of K 931 with a poorly differentiated SCC lymph node metastasis. Note the homogenous labelling of the cells

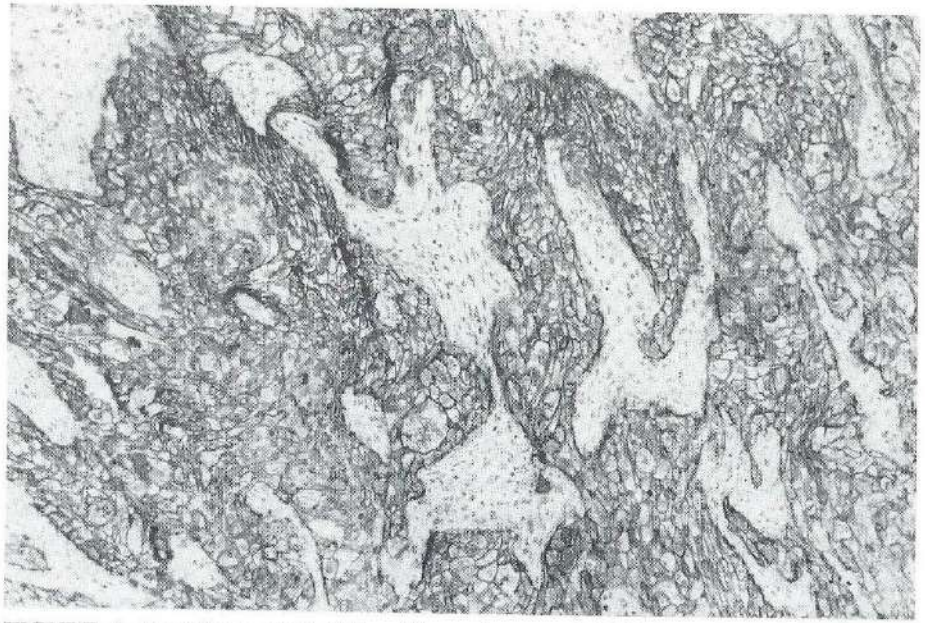

FIGURE 4. Reactivity of K 931 with a pharyngeal SCC metastasis. Labelling is notably seen on the surface of the tumour cells.

SCC cell lines. No reactivity was seen with UM-SCC-75, UM-SCC-21B and only weak reactivity was observed with UM-SCC-10B. Non-epithelial tumours and fibroblasts did not react. Weak reactivity was observed with normal keratinocytes, which had been in culture for 3 passages. When these cells were tested in the hemadsorption assay (11) to determine the number of cells positive, about 10% were found to be bound by the antibody (Table II).

TABLE II - REACTIVITY OF K 931 WITH IN VITRO CULTURED SCC CELL LINES

CELL LINE	ORIGIN – POSITIVE BINDING
UM-SCV-2	vulva
UM-SCC-11A	larynx
UM-SCC-11B	lymph node metastasis larynx
UM-SCC-14B	lymph node metastasis floor of the mouth
UM-SCC-14C	skin metastasis floor of the mouth
UM-SCC-1	floor of the mouth
UM-SCC-21A	ethmoid sinus
UM-SCC-22A	hypofarynx
UM-SCC-22B	lymph node metastasis hypofarynx
UM-SCC-5	larynx
UM-SCC-10A	larynx
UM-SCC-10B	lymph node metastasis larynx
WEAK OR NO BINDING:	
UM-SCC-10B	larynx
UM-SCC-75	maxillary sinus
UM-SCC-21B	lymph node metastasis ethmoid sinus
NL FIBROS	fibroblasts
UM-MEL-1	melanoma
UM-FS-3	fibrosarcoma
NL-SQ-2	*in vitro* cultured keratinocytes

Reactivity of K 931 in solid tumours other than SCC

A panel of 54 carcinomas and 19 other malignant tumours was tested for reactivity with K 931. All carcinomas showed strong and homogenous reactivity with the antibody, which was independent of the histological type or the grade of differentiation. Only one serous ovarian carcinoma did not react. The preferential staining type was a strong membrane associated reactivity with no or a faint cytoplasmic reactivity. In contrast to the carcinomas, no reaction was observed with any of the sarcomas, malignant lympohomas and melanomas tested (Table III).

TABLE III - REACTIVITY OF MAB K 931 WITH FROZEN SECTIONS OF HUMAN NEOPLASTIC TISSUES.

Squamous cell carcinomas	
head and neck	58 /62
lung	10/10
Adenocarcinomas	
lung	15/15
mamma	12/12
colon	10/10
pancreas	2/2
Ovarian carcinoma	
cysteadeno muceus carcinoma	3/3
serous adeno carcinoma	1/2
melanoma	0/3
sarcoma	0/8
non-Hodgkin lymphoma	0/8

K 931 detects a 37 kDa antigen

Immunoblots prepared from whole cells of SW 1116 (colon carcinoma cell line), which were directly lysed in sample buffer, demonstrated a distinct 37 kDa band (Fig 5). This band was also seen when samples were prepared with 17-1A purified material and was almost identical to the band which resulted when the nitrocellulose sheets were probed with MAb 323-A3, an antibody described to recognize the 17-1A antigen (10).

DISCUSSION

In this study we documented the production of a new MAb to the 17-1A antigen, a well defined general epithelial marker, as well its distribution in normal simple epithelium and SCC. The lack of expression in normal squamous epithelium of 17-1A antigen has been described by other authors as well (14). The binding of anti-17-1A antibodies in SCC has not been reported before, perhaps since sofar all these MAbs were generated after immunization with gastro-intestinal tumours.

For histopathological detection of epithelial neoplasms the antigen appeared to be a suitable target, since only one serous ovarian carcinoma out of 54 carcinomas did not react. A major advantage is the apparently homogenous distribution of the antigen among carcinomas, independent of the degree of differentiation and origin of the tumour.

Despite the absence of the K 931 antigen among normal stratified squamous epi-

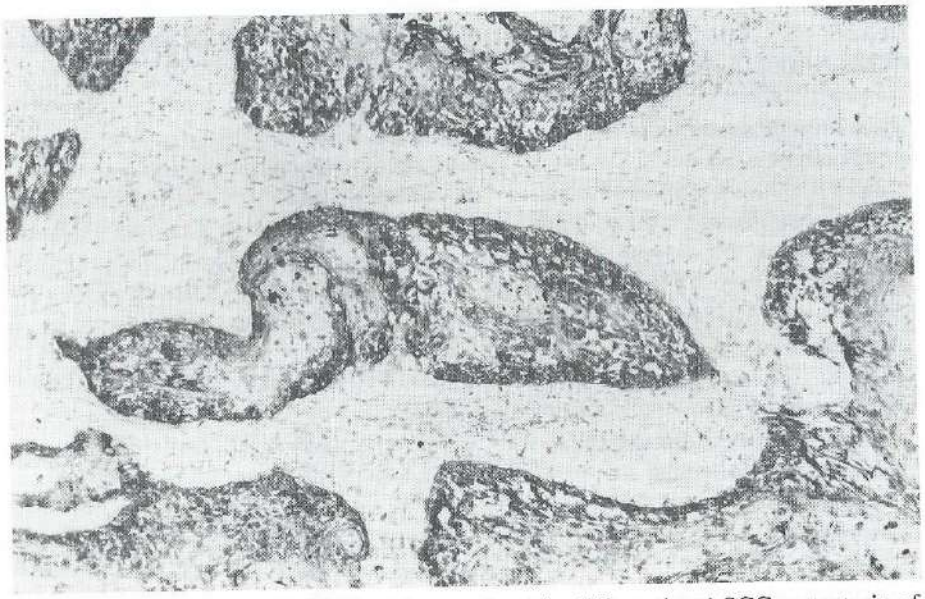

FIGURE 5. Reactivity of K 931 with a moderately differentiated SCC metastasis of the larynx.

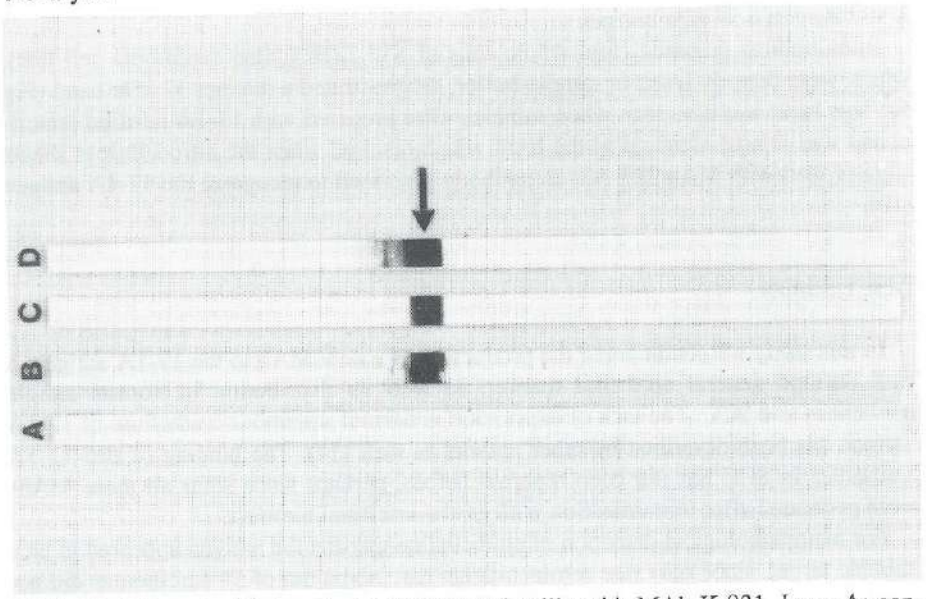

FIGURE 6. Immunoblot analysis of SW 1116 cellls with MAb K 931. Lane A: control antibody, not reactive with SW 1116; Lane B: SW 511 cells directly lysed in sample buffer probed with K 931; Lane C: SW 1116 cells probed with MAb 323-2a;Lane D: purified 17-1A antigen and K 931. Arrow marks 38 kDa.

thelia, we noted a strong expression of the antigen in SCC. Out of 62 primary squamous cell carcinomas tested sofar, 58 reacted with the antibody. Staining was heterogenously and notably confined to tumour cells located in the central parts of tumour nests. A small group of advanced primary SCC and all lymph node and hematogenous SCC metastases exhibited another pattern of expression. Labelling was enhanced and pronounced on the surface of poorly differentiated cells in the peripheral cell layer of tumour nests. The strong surface labelling was also observed among tumour cell lines, originally derived from primary and metastatic SCC. Unfortunately, we were unable to test if the K 931 antigen is only associated with SCC since frozen sections of premalignant lesions were not available. However, since *in vitro* cultured keratinocytes do bind K 931 , it is likely to expect that K 931 shows reactivity with dysplastic lesions. The expression of 17-1A on dysplastic and neoplastic squamous cells can be regarded as an abnormal direction of differentiation. This has also been demonstrated with other antisera eg. keratins which are in normal tissues only expressed in simple epithelia, can be frequently identified in premalignant and SCC (12,13).

The antibody reacted with a 37 kD molecule in immuno-blotting. This molecular weight as well the antigenic expression in normal tissues suggested that the identified antigen represented the 17-1A antigen. Direct evidence was found in immunoblot analysis with purified 17-1A antigen, in which MAb K 931 showed indeed reactivity with the 17-1A antigen.

The 17-1A antigen has been identified as a 37 kDa glycoprotein containing sialic acid residues and N-linked glycans (14). The relative high frequency of MAbs to this molecule suggests an immunogenic nature of the antigen(4,10,14-17). There are apparently several epitopes present on the antigen, since K 931 differs from the original described MAb 17-1A in that gastric mucosa is strongly labelled by K 931, but not by MAb 17-1A. Moldenhauer *et al.* (18,19) produced a MAb to a 34 kDa glycoprotein containing N-linked glycans, which they called Egp 34 (epithelial glycoprotein 34). Comparison between this MAb and K 931 shows an identical tissue distribution, suggesting that Egp 34 represents 17-1A. Minor tissue distribution and molecular weight differences can be delusive in revealing the nature of the antigen. In order to avoid such problems, workshops should be organized for all human neoplasms to characterize and cluster tumour associated antigens recognized by MAbs, analogous as to those already existing for leukemia and small-cell lung cancer (20,21).

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CHAPTER VI

IDENTIFICATION OF A 43 kD NUCLEAR ANTIGEN ASSOCIATED WITH PROLIFERATION BY MONOCLONAL ANTIBODY K 112

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ABSTRACT.

Monoclonal antibody (MAb) K 112 was generated after a single intrasplenic immunization with a recurrent laryngeal squamous-cell carcinoma. The antibody detects a 43 kD nuclear antigen which is expressed in proliferating cells only. Expression is typically seen in a granular pattern excluding the nucleoli. During mitosis the bulk of the antigen is diffusely distributed in the cytoplasm. Identical reactivity was observed for tissues or cells of all mammalian species tested. Evidence was found that the antigen is associated with the nuclear matrix. These data indicate that MAb K 112 recognizes a protein belonging to the class of proliferating cell nuclear antigen (PCNA)like molecules.

INTRODUCTION

The identification of proteins that are predominantly synthesized in proliferating cells is essential for understanding the mechanisms underlying growth regulation and cellular transformation. Recently several nuclear antigens have been recognized that become preferentially expressed in proliferating cells, including tumour cells, but are present in relatively low or undetectable amounts in normal, resting cells. The best characterized protein of this group of proliferating cell nuclear antigens (PCNA's) is cyclin(1), recently identified as a 38 kD auxiliary protein of DNA polymerase α (2) Most of the other known PCNA's represent nucleolar proteins (3-5) Here we present the MAb K 112 recognizing a PCNA that has to our knowledge not been described before. The K 112 antigen shows a granular distribution in the nucleoplasm of stimulated cells from various animal species. During metaphase the K 112 antigen is diffusely distributed throughout the cytoplasm. In contrast to most other PCNA's the K 112 reactive antigen is not found in nucleoli. It is also distinct from cyclin, since its relative molecular weight (43 kD) is clearly higher. Moreover, it seems to have different spatial distribution within the nucleus. The K 112 antigen remains detectable upon *in situ* nuclease digestion of cells followed by high salt extraction, suggesting that it is associated with the nuclear matrix.

MATERIALS AND METHODS

Immunogen

A radiotherapy resistant T1N0 squamous-cell carcinoma (SCC) of the larynx was, after surgical removal, used as immunogen. The tissue was cut into pieces and treated with a mixture of 0.1% collagenase (Sigma Chemical Co, St. Louis, MO) and 0.03% DNase type 1 (Sigma) in Hank's Buffered Salt Solution. The single cells were subsequently used for immunization. Details about immunization and screening have been described elsewhere (6).

Immunization and hybridoma production

Balb/C mice were immunized with 10^6 viable cells intrasplenically under general anesthesia. Three days later the spleen was removed and the dissociated spleen cells were fused with the non-producing cell line SP-2/0. Growing hybridomas were screened on ELISA for reactivity with red blood cells and for immunoglobulin production. Selected antibodies were further screened upon reactivity with frozen sections from squamous-cell carcinomas and salivary glands. Hybridomas producing antibodies with selective binding were stabilized by limiting dilution.

Tissues and cell lines

Tumour and normal tissues were obtained from surgical procedures or autopsies. Tissues were stored in liquid nitrogen. Cell lines with the prefix UM-SCC were a gift of Dr. T. Carey, Ann Arbor, University of Michigan, MI. A 431, derived from a vulva epidermoid carcinoma, was a gift of Dr. B. Defize, Hubrecht laboratory, Utrecht, the Netherlands. P19 END-2 cells (7) were kindly made available by Dr. C.L. Mummery, Hubrecht Laboratory, Utrecht, the Netherlands. Unless otherwise stated, cells were cultured in RPMI-1640 containing 10% Fetal Calf Serum (FCS) Lymphocytes were isolated from the peripheral blood of healthy donors by Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) centrifugation. Lymphocytes were placed in tissue culture wells and stimulated with PHA (Sigma, 10 μg/ml). Proliferation was measured by determining [^{3}H]thymidine incorporation. Stimulated PHA blasts incorporated usually 10,000 - 12,000 cpm per well, background incorporation was about 135 cpm per well.

Immunofluorescence and immunoperoxidase staining

Cells to be tested were washed in PBS, suspended in PBS at a concentration of 2 x 10^7 cells/ml, spread on a glass slide and dried. The cells were fixed in acetone for 10 minutes or in formalin (2% v/v). The formalin fixed cells were permeabilized with 0.5% Triton X-100. The slides were incubated with MAb K 112 for 1 hr. at room temperature. Cells were washed 5 times in PBS and incubated with a second antibody either FITC-conjugated or horse radish peroxidase-conjugated rabbit anti mouse (Dakopatts, Copenhagen, Denmark). The slides were washed in PBS and mounted in glycerol saline or counterstained with hematoxiline, dehydrated and embedded in depex.

Confocal scanning laser microscopy (CSLM)

The confocal scanning laser microscopy has been described in detail by Brakenhoff *et al.* (8). Routinely 16 optical sections of 256 x 256 pixels each, were made. Fluorescence stained specimen were excited at 476 nm using a Crypton ion laser. Typical optical resolutions were 0.6 μm to 0.8 μm, perpendicular and parallel to the optical axis respectively. The signal to noise ratio in the images of the optical sections was improved by using a 2-3 dimensional median filter. Furthermore the central range in each serie of section was optimized with respect to the sensitivity of the photographic film.

Preparation and isolation of nuclei

Nuclei for immunoblotting were prepared by lysis of cells in TSM buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) as protease inhibitor and 0.5 % Nonidet NP-40. The sample was subsequently separated into a crude nuclear pellet and a cytoplasmic supernatant by centrifugation at 10,000 g for 15 minutes. The pellet was washed 5 times in the same buffer. For SDS-PAGE, preparations were dissolved in sample buffer (0.0625 M Tris-HCl pH 6.8, 2.3% SDS and 10% glycerol),either in presence or absence of 5 % β-mercaptoethanol, and incubated at 96•0C for 2 minutes prior to loading on stacking gel.

For *in situ* preparation of nuclear matrices from A 431 cell lines we used the protocol of Chaly *et al.* (9) with minor modifications. A 431 cells were grown on coverslips. Cells were first treated with STM buffer (0.25 M sucrose, 10 mM Tris-HCl pH 7.5, 2mM $MgCl_2$, 25 mM KCl) for 10 minutes. Then the cells were washed with STM and incubated for 1 hour with STM buffer containing 0.5 mM sodiumtetrathionate. After the incubation the cells were washed three times with STM buffer Subsequently the cells were incubated with STM containing DNAse I (Sigma Chemical Co, St. Louis MO, 50 µg/ml) and RNAse A (Sigma, 50µg/ml) for one hour and again washed with STM buffer. Finally the nuclease digested cells on cover slips were incubated in a low salt buffer (10 mM Tris/HCl pH 7.4, 0.2 mM $MgSO_4$, 0.5 M NaCl) followed by the slow, dropwise, addition of a high salt buffer (10 mM Tris-HCl pH 8.4, 0.2 mM $MgSO_4$, 2.0 M NaCl) untill a final concentration of 1.6 M NaCl was reached. Finally, preparations were washed three times with a 1.6 M NaCl buffer.

SDS-PAGE gel electroforesis and immunoblotting

SDS-PAGE was carried out as described by Laemmli (10), using 5 to 15% linear acrylamide gradient slab gels. Molecular weight markers (Rainbow^{TM}, Amersham UK) ranged from 200 kD (myosin), 92.5 kD (phosphorylase A), 69 kD (bovine serum albumin), 46 kD (ovalbumin), 30 kD (carbonic anhydrase), 21.5 kD (trypsin inhibitor) to 14.3 kD (lysozyme). Proteins were transferred from gel to nitrocellulose with a Multiphor II Nova Blot System (LKB, Bromma, Sweden) for semi-dry electrophoretic transfer using a discontinuous buffer system. Antigen was detected using the immunoperoxidase method according to Wood (11).

RESULTS

Antibody selection

Immunoglobulin producing hybridomas were screened upon frozen sections of a squamous-cell carcinoma and a normal parotid gland. K 112 was selected because of selective reactivity with a proportion of the tumour cell nuclei. The clone is stable for more than 1 year and produces an immunoglobulin of the IgM isotype.

Immunolocalization of the antigen

Immunoperoxidase staining of tumour cells grown *in vitro* on cover slips revealed that the antigen recognized by K 112 is selectively detectable in the nucleoplasm. The spatial distribution of the antigen in the nuclei was studied more closely by confocal scanning laser microscopy (CSLM) in optical sections of P19END-2 cells.

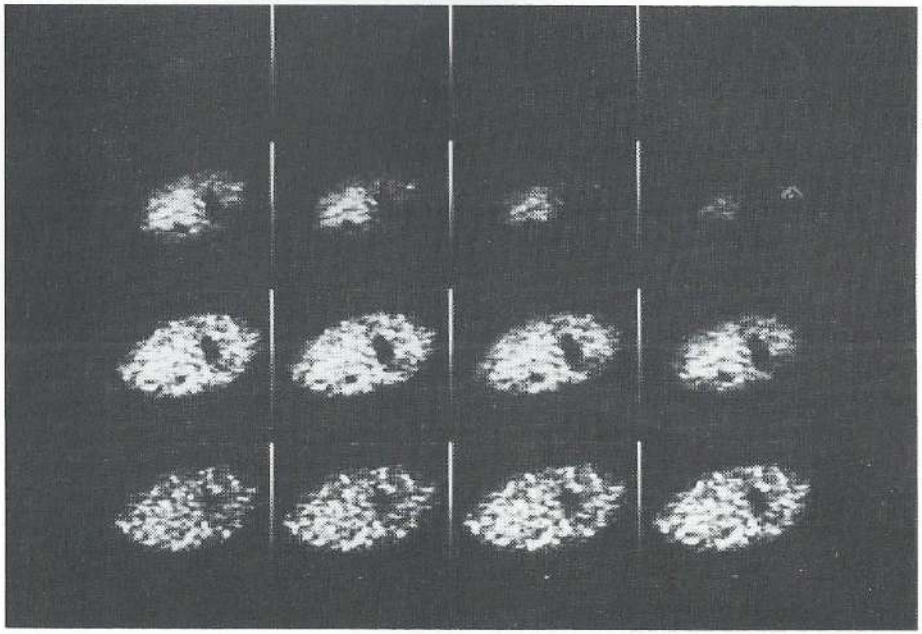

FIGURE 1 - Detection of K 112 in cultured P19END-2 cells using confocal scanning laser microscopy. P19END-2 cells were grown in monolayers in tissue culture flasks. Cells were fixed permeabilized and incubated with K 112 and FITC-conjugated second antibody as described in materials and methods. Note the reactivity in subnuclear compartments.

The granular pattern observed (Fig.1) suggests that the antigen is present in distinct nuclear compartments. During mitosis the antigen is diffusely distributed throughout the whole cytoplasm, apparently leaving the chromatine unstained (Fig.2).
We could not exclude the possibility, however, that small amounts of the antigen remained associated with the chromosomes. In order to investigate the possible association of the K 112 antigen with the nuclear matrix, exponentially growing A 431 cells, cultured on cover slips, were subsequently permeabilised with Triton X-100, treated with DNAse and RNAse, extracted with 1.6 M NaCl, and fixed with formaldehyde. A strong immunoperoxidase staining was observed indicating that the K 112 antigen is associated with this *in situ* prepared nuclear matrix.

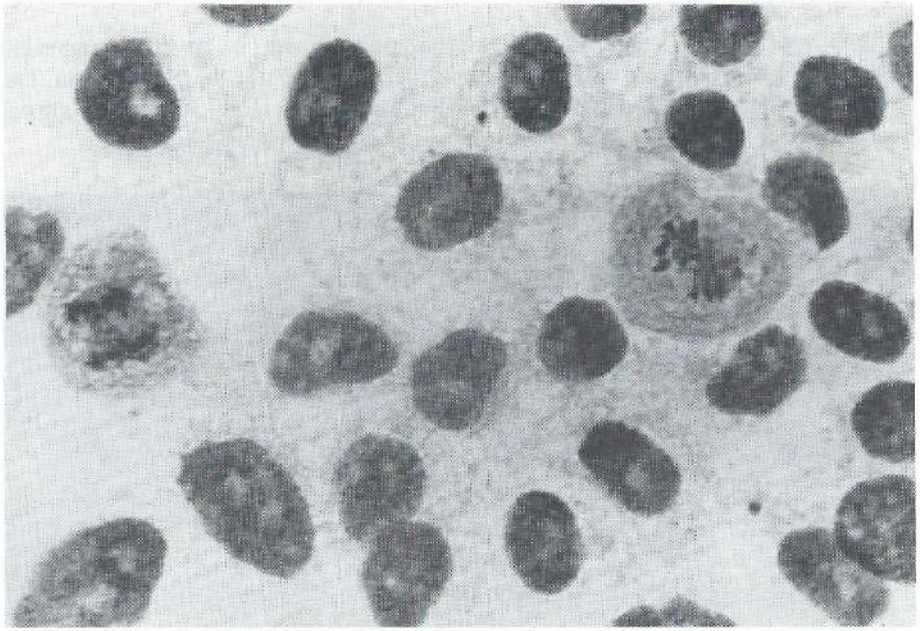

FIGURE 2 - Detection of K 112 in A 431 cells growing on cover slips. Note the cytoplasmic reactivity with mitotic cells.

TABLE I - DETECTION OF THE K 112 ANTIGEN IN NORMAL TISSUES BY INDIRECT IMMUNOPEROXIDASE STAINING.

	positive/tested
Epidermis	4/4 basal cells only
Parotid gland	0/4
Mamma	0/2 *
Lung	0/2
Colon	3/3 basal cells only
Liver	0/2
Kidney	0/2
Thyroid	2/2 *
Myometrium	0/2
Endometrium	2/2 #
Muscle	0/4

*occassionally positive cells were seen
#only during the proliferative stage

Expression of the K 112 antigen in normal and neoplastic tissues and cell lines

Resting cells of normal non-proliferating tissues were negative for K 112 (table I). In contrast to this, a proportion of cells within normal proliferating tissues exhibited strong labeling. In epidermis staining was seen of basal cells while the non– dividing suprabasal cells were negative. Myometrium was negative while simultaneously obtained endometrium in the proliferative stage strongly expressed the antigen.

Further, a variety of tumours was tested as shown in table II. In all tumours positive nuclei were seen, although considerable differences were noted in intensity and number of positive cells. In general, stroma surrounding tumour cells did not react (Fig. 3). Strong reactivity was found with *in vitro* growing human tumour cell lines of various origin (table II).

Proliferating transformed cells were found to stain positive with K 112 irrespective their species origin. Mouse cells (SP2/0), rat (HF2, Insulinoma), goat (thyroid cells derived from goats with hyperthyroidism), bovine epithelia (basal cells in bovine muzzle), guinea pig (line 10 tumour), all showed nuclear staining patterns similar to those exhibited by human cells from in vitro cultured carcinoma cell lines or on frozen sections.

TABLE II - DETECTION OF THE K 112 ANTIGEN IN NEOPLASTIC TISSUES AND IN VITRO CULTURED CELLS BY IMMUNOPEROXIDASE

Tumour	positive/tested
squamous-cell carcinomas of the head and neck	32/32
adenocarcinoma of the lung	4/4
adenocarcinoma of the breast	3/3
adenocarcinoma of colon	6/6
ovary carcinoma	7/7
sarcoma	3/3

The following tumour cell lines revealed a strong expression of the K 112 antigen when tested in the indirect immunoperoxidase assay:

line	origin
UM-SCC-11B	derived from a laryngeal lymph node metastasis
UM-SCC-14A	oral cavity carcinoma
UM-SCC-14B	lymph node metastasis of an oral carcinoma
UM-SCC-14C	skin metastasis of an oral carcinoma
UM-SCC-22B	lymph node metastasis of a pharyngeal carcinoma
A 431	vulva epidermoid carcinoma
HL 60	leukemia cell line
Line 10	Guinea pig tumour line
HF2	Rat insulinoma
SP-2/0	Mouse myeloma

K 112 detects A 43 kD nuclear antigen

Immunoblots prepared from whole cells with the use of TSM buffer or directly lysed in sample buffer demonstrated a distinct 43 kD band under non-reducing conditions (Fig. 3). Typically a pattern was seen, suggestive for a double band. In order to obtain a better separation for molecules in this range (40-60 kD), samples were loaded on a 15% gel. This did not result however in more distinct bands (Data not shown). When cells were lysed in TSM buffer and subsequently fractionated by differential centrifugation reactivity was found only in the crude nuclear fractions, the cytoplasm containing no detectable K 112 reactive material. Pretreatment of the nuclear fractions with DNAse 1 and RNAse A did not influence this reactivity, making it unlikely that a DNA or RNA structure was recognized. Murine myeloma cells (SP-2/0) gave rise to an identical 43 kD band in the nuclear preparations.

Expression of K 112 in PHA activated blood lymphocytes

The K 112 antigen was not detectable in normal resting blood lymphocytes. When the lymphocytes were stimulated by PHA for 48 hours the antigen became visible in the blast cells, but not in the small lymphocytes (determination of [^{3}H]thymidine incorporation confirmed the proliferation). To determine whether the expression of

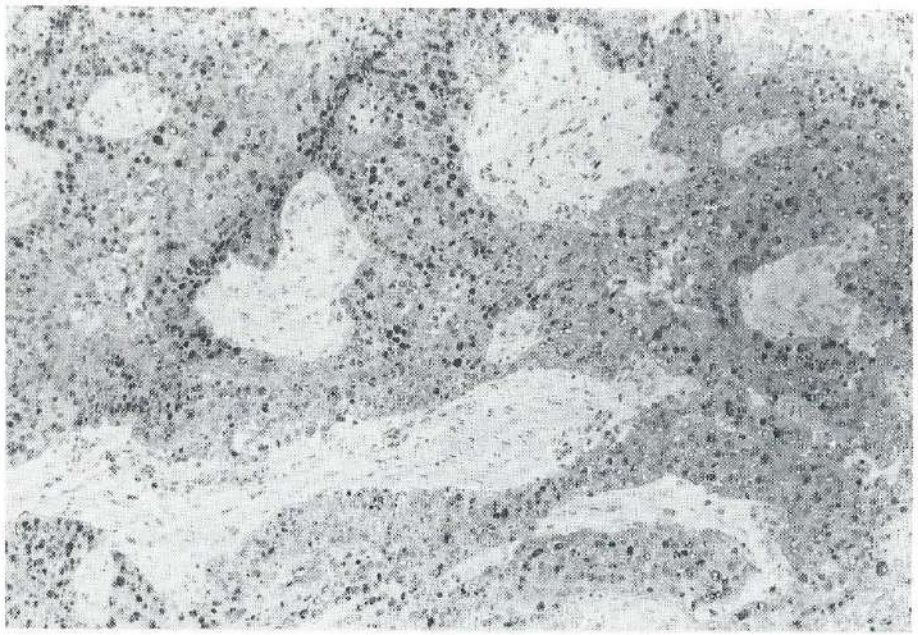

FIGURE 3 Detection of K 112 in frozen sections of squamous-cell carcinomas of the head and neck using an immunoperoxidase technique. No reactivity is seen with the stroma surrounding the tumour, whereas several tumour nuclei show strong staining.

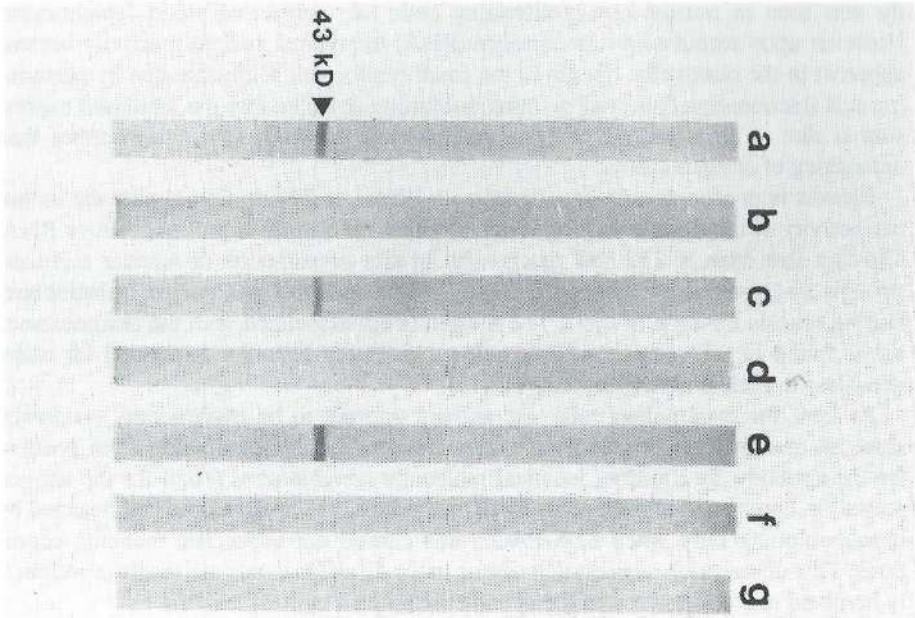

FIGURE 4 Immunoblot reactivity of MAb K 112. Lane a: Nuclear fraction of UM-SCC-14C cells, B: cytoplasmic fraction of UM-SCC-14C cells, C: Nuclear fraction of mice cells (SP-2/0) D: cytoplasmic fraction mice cells, E: Leukocytes 24 hours after PHA stimulation, F: Leukocytes without stimulation G: Nuclear fraction UM-SCC-14C with a control antibody.

the 43kD antigen induced by PHA was due to an alteration of gene expression or to an unmasking of antigenic sites, we analysed nuclear extracts prepared from non-stimulated and PHA stimulated lymphocytes by immunoblotting. A clear 43 kD band was seen in the nuclear preparations from stimulated lymphocytes, but not in the fractions from non-stimulated lymphocytes (Fig.4).

DISCUSSION

The data presented in this report indicate that Mab K 112 recognizes a 43 kD PCNA molecule. Both immunoperoxidase staining and immunoblotting analysis revealed that a nuclear antigen was recognized. Staining was observed typically in a granular pattern localized throughout the whole nucleus excluding the nucleoli. The results also made clear that the antigen is associated with proliferation. Within sections of normal tissues staining was only observed in layers known to contain the proliferative compartment of cells (basal cell layers of epidermis and colon). In contrast no reactiv-

ity was seen in normal non-proliferating cells like peripheral blood lymphocytes. However upon stimulation with mitogen (PHA) the typical nuclear reactivity became apparent in the blast cells, but not in the small lymfocytes. Data obtained by performing cell fractionation followed by immunoblotting revealed that the increased expression is due to an alteration of gene expression upon PHA stimulation rather than unmasking of antigenic sites.

Pretreatment of immunoblot samples with RNase or DNase did not alter the immunereactivity of the sample making it unlikely that the antigen is part of DNA or RNA. The fact that Mab K 112 still reacts with in situ preparations of nuclear matrices, strongly suggest that the antigen is tightly associated with this nuclear substructure. During mitosis the bulk of the K 112 antigen is not associated with the chromosomes but is found in the cytoplasm. This cell-cycle related behavior is noticed for many non-histone nuclear matrix proteins (12,13).

At least for mammalian cells, the antigen appears to be evolutionary conserved since all mammalian cells under proliferative conditions, tested sofar, stain positive for the antibody. In addition, identical molecular weights were found for the antigen present in human and murine cells. In all cases the K 112 antigen could be detected by immunoblotting only when SDS-PAGE was carried out under non-reducing conditions. This observation suggest that one or more disulfide bands are direct or indirectly involved in the formation of the epitope recognized by K 112.

Several PCNA 's have been identified, including the Ki 67 antigen.(1-5,9,14) Based on their molecular weight and their subcellular localization (the nucleolus), they seem to be different from the K 112 antigen. MAb K 112 shares a number of characteristics of the recently detected PCNA as described by Philipova *et al*(15), such as the speckled distribution, association with the nuclear matrix and mitotic behavior (16) but this antigen has a considerable higher molecular weight (125 kD). Three lines of evidence indicate that the K 112 antigen described here is also different from cyclin (1). (i) There is a difference in the relative molecular weight of cycline and the K 112 antigen 36-38 kD vs 43 kD respectively. It should be noted that the K 112 antigen could only be detected by immunoblotting after SDS-PAGE under non reducing conditions, which may lead to an abnormal migration behavior of proteins containing one of more disulphide bands.(ii) The nuclear distribtion of cyclin shows characteristic changes during the cell cycle. At the onset of the S phase a fine granular distribution evolves into a clear punctate pattern, colocalizing with replications sites (217,18). We could not detect a similar punctate pattern upon examination of large numbers of asynchronously growing A 431 or UM SCC 14C cells, all cells in interphase showed the same granular pattern. (iii) Cyclin is also present in quiescent cells, although at a lower a level than in growing cells (19). In contrast our data indicate that the K 112 antigen is only detectable in proliferating cells.

Interest in nuclear markers for proliferation has increased since it has become clear that some of these markers play an essential role during malignant transformation (20,21). Whereas in normal squamous epithelium K 112 was exclusively expressed in the basal cell layer, irregular expression was seen in squamous-cell carcinomas. Tumours in which several layers stained positive were seen as well as tumours with

labelling restricted to small clusters of cells. Whether there is a relation between enhanced expression and clinical behavior is an intriguing question. Several studies, using monoclonal antibodies as markers for cycling cells, have indicated a relation between proliferative capacity of tumours and their clinical course (22-24). These reports suggest that tumours with high proliferative activity may tend to a more aggresive clinical behavior.

The capability of MAb K 112 to recognize the proliferative fraction in tumour specimen may contribute to test this hypothesis. More fundamental studies may elucidate the role of the K 112 antigen during the molecular events leading to proliferation and malignant transformation.

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CHAPTER VII

LOCALIZATION AND IMAGING OF RADIOLABELED MONOCLONAL ANTIBODY AGAINST SQUAMOUS-CELL CARCINOMA OF THE HEAD AND NECK IN TUMOUR-BEARING NUDE MICE

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ABSTRACT

Nude mice carrying human squamous-cell carcinoma xenografts were given i.v. injections of radiolabelled monoclonal antibodies (MAbs). MAb E 48, which reacts with squamous-cell carcinomas was labelled with ^{131}I, while a second control MAb of similar immunoglobulin subclass was labelled with ^{125}I. Both antibodies were injected simultaneously, then the mice were scanned with a gamma camera or their tissues were removed and antibody uptake was calculated as a percentage of the injected dose. Uptake of E 48 reached a peak value of 16 % /g on day 3, while uptake of the control antibody was less than 1,8 % /g. By 24 hr after injection tumour could be visualized without subtraction techniques. At days 3 and 7, only xenografts were visible on imaging. These findings suggest that E 48 is capable of high specificty in tageting radioisotopes to squamous-cell carcinomas in an experimental setting.

INTRODUCTION

Squamous-cell carcinomas (SCC) represent the vast majority of all malignant tumours of the head and neck, and they account for approximately 5% of all malignant neoplasms in Europe and the United States (Blitzer, 1988). The early stages of disease are usually treated with surgery or radiotherapy alone, and advanced stages with combined surgery and radiation therapy. With these modalities local-regional control can at present be achieved in the majority of patients (Snow, 1989). In the past, failure of treatment at distant sites has not been a major problem in patients with head and neck cancer. However, a changing pattern of failure is being observed: as fewer patients die from uncontrolled disease in the head and neck, more are exposed to the risk of disseminated disease below the clavicles (Vikram, 1984). Chemotherapy so far has not been successful in eradicating metastases (Tannock and Browman, 1986). Therefore, we have looked for other means of treating metastatic disease. Since squamous-cell carcinomas are relatively sensitive to radiotherapy, (Marks *et al*, 1985) our attention has been focused upon the possibility of preferentially delivering high amounts of radiation preferentially to the tumour. Hybridoma technology has made it possible to generate large quantities of MAb with high specificity for tumour cells. When tagged with isotopes, these antibodies may be used for diagnosis and treatment of malignancies, as has been demonstrated in a variety of tumours, in both experimental and clinical studies (Mach *et al*, 1988).

At present, only a few antibodies to SCC of the head and neck are available (Kimmel and Carey 1986, Boeheim *et al*. 1985). However, these antibodies show considerable cross reactivity to normal tissues. We have developed a MAb E 48 which shows strong and selective reactivity to squamous epithelia and their neoplastic derivatives (Quak *et al*, 1989). In this study we evaluate the ability of radiolabelled E 48 MAb to localize and visualize xenografts of a squamous-cell carcinoma of the head and neck in nude mice as a first step in determining its *in vivo* value for radioimmunoimaging.

MATERIAL AND METHODS

Monoclonal antibodies

MAb E 48 (IgG_1) detects a 22-kD surface antigen which, in normal tissues is exclusively expressed in stratified squamous and transitional epithelia (Quak *et al*,1989). Out of 76 SCC of the head and neck, lung, cervix and skin, tested sofar, 75 reacted with the antibody, while out of 42 non-SCC tumours, 40 showed no binding. The isotype-matched control antibody (IgG_1) JSB-1, not reactive with the xenograft in our study, has been described in detail elsewhere (Scheper *et al*,1988). It is directed against the P-glycoprotein related to multi-drug resistance.

Hybridomas were grown either in tissue culture or as ascites in BALB/c mice. To purify the antibodies, the ascites was filtered through a 0.2 µm filter and then loaded on a protein A Sepharose column (Pharmacia, Uppsala, Sweden). Eluted fractions were tested for immune reactivity and dialysed against PBS. Protein concentration was determined in the Bio-Rad (Richmond, CA) microassay procedure.

Nude mice and xenografts

Female nude mice (NMRI, 25-32 g Harlan Olac CPB, Zeist, The Netherlands) were 8-10 weeks old at the time of the experiments. Tumour tissue was derived from a 54-year old female patient with a T4N2 squamous-cell carcinoma of the base of the tongue. After surgical removal, the tumour was cut into fragments measuring 3x3x1 mm, then one of these fragments was implanted s.c. on both sides of nude mice in the lateral thoracic region. Tumours growing and measuring over 800 mm^3 were serially transplanted in a similar way. In the experiments described below, tumour xenografts were used from the 5th passage.

Radioiodination

Iodination of E 48 and JSB-1 was performed essentially as described by Haisma *e t al* (1986). In short, 500 µg of antibody in phosphate buffer pH 7.4 were mixed with 1 mCi ^{125}I or ^{131}I in a vial previously coated with Iodogen. After 10 min of incubation at room temperature, a sample was removed to determine the amount of incorporated iodine. One ml. of AG1-X-8 resin (Biorad) previously mixed with PBS containing 1 % BSA was then added to the vial to absorb unbound iodine. This reaction mixture was then filtered through a 0.22 µm filter to remove the resin and to sterilize the product. Biological activity after labelling was tested in an ELISA (Lansdorp *et al*., 1980) system using viable tumour cells of a squamous-cell carcinoma cell line.

Biodistribution

In vivo tissue distribution was studied in nude mice bearing squamous-cell carcinoma xenografts of the head and neck, following i.v. administration of 10 µCi ^{131}I E 48 and 10 µCi ^{125}I JSB-1 in sterile saline. Mice were bled, killed and dissected 1, 2, 3 and 7 days after i.v. injection. For each day 3 or 4 mice were used. Organs were immediately removed, rinsed with PBS, and placed in 12 x 75 mm plastic tubes and

weighed. Samples were taken from blood, urine, tumour, liver, spleen, kidney, heart, stomach, jejunum, colon, bladder, sternum, muscle, lung, skin and tongue. After weighing, all organs were counted in a dual isotope gamma counter. The antibody uptake in the tumour and other tissues was calculated as a percentage of the injected dose per gram of tissue. The specificity index of tumour localization was calculated by dividing the uptake of the specific antibody (E 48) by the uptake of the non-specific antibody (JSB-1) into tumour.

Radioimmunoscintigraphy

Mice were anesthezised with Nembutal and immobilized under the camera. Scanning was performed with an Ohio gamma camera (Sigma 410 S); 30,000 counts were obtained and data were stored in a computer (PDP 1134 computer system) for further analysis and production of color images.

RESULTS

Immunohistochemistry

The presence of antigen on the tumour was assessed by immunoperoxidase staining. Frozen sections of the original tumour from which the xenograft had been derived were incubated with protein-A-purified E 48 and JSB-1. E 48 showed strong

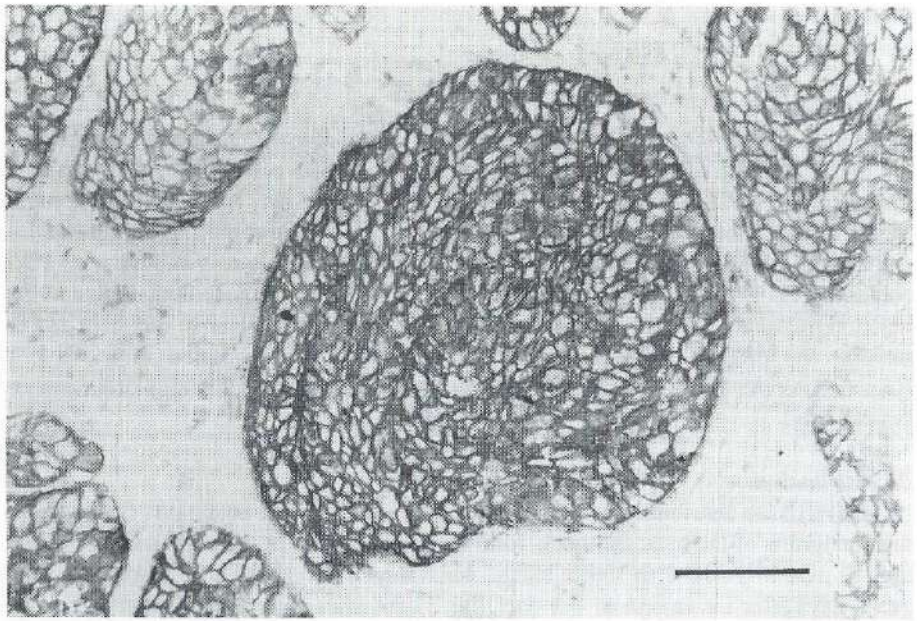

Figure 1. Xenograft section, stained with Mab E 48 by indirect peroxidase method.

binding in concentrations of up to 100 ng/ml (Fig. 1). No reactivity was seen with JSB-1 in concentrations of up to 35 µg/ml.

Radiolabelling of MAbs

Aliquots of 400 - 500 µg of E 48 were labelled with 1 mCi ^{125}I. Specific activity ranged from 1.3 - 1.8 mCi/mg. Labelling of 400- 500 µg of antibody with 0.5 mCi ^{131}I resulted in a specific activity of 0.6 mCi/mg. JSB-1 (500 µg) was labelled with 1 mCi ^{125}I. Specific activity was 0.8 mCi/mg. TCA precipitation revealed that more than 98 % of the iodine was bound to the immunoglobulin.

Pharmokinetics of E 48

Serum samples were collected on day 1, 2, 3 and 7. TCA precipitation revealed that less than 5% free iodine was present in the blood. Clearance was relatively slow as is typical for intact IgG molecules following i.v. injection.

Biodistribution

Tumour uptake. The amount of E 48 ^{131}I in the xenograft and the various organs is shown in Figure 2.

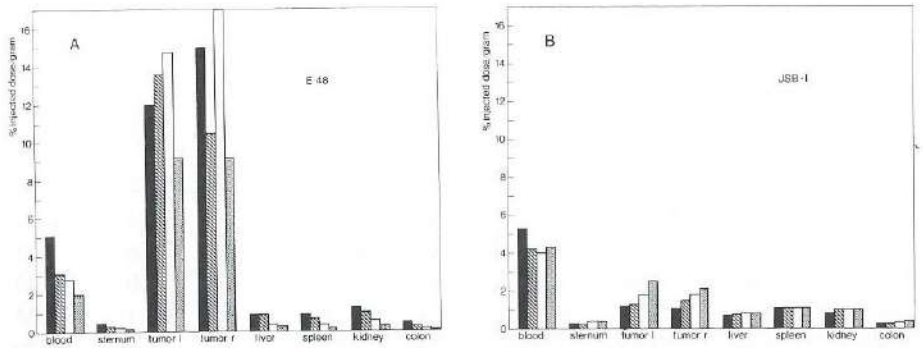

FIGURE 2. Biodistribution data for (a)10 µCi ^{131}II-labelled E 48 and (b)10 µCi ^{125}I JSB-1 in athymic nude mice bearing SCC xenografts. At 1(open), 2(hatched), 3(black) and 7(speckled) days following i.v. injections tissues were dissected and counted and the concentration (%ID/g) in tissues was calculated. Each day 3-4 mice were dissected.

The concentration is depicted as the average percentage of radioactivity of the injected dose/g tissue (ID/g). Table I shows the tumour to tissue ratio on various days, as calculated by dividing the uptake/g of tumour tissue by the uptake/g of non-target tissues. For each day 3-4 mice were used.

TABLE I-COMPARISON OF TUMOUR TO TISSUE RATIOS IN MICE BEARING SCC XENOGRAFTS: COADMINISTRATION OF ^{131}I E 48 AND ^{125}I JSB-1 MABS

Tumour to tissue ratio MAb E 48

	Days							
	1		2		3		7	
	ratio		ratio		ratio		ratio	
Blood	2.75	0.10	3.18	0.03	7.01	1.22	4.75	0.02
Sternum	23.6	2.35	36.66	4.91	76.34	9.80	56.39	3.41
Liver	11.33	0.88	13.69	3.13	39.13	6.90	22.61	1.43
Spleen	13.89	3.04	15.77	2.55	44.49	9.45	31.25	0.75
Kidney	10.02	0.95	11.06	0.92	28.46	7.17	19.15	1.05
Colon	27.22	1.37	39.45	3.54	77.07	11.0	51.5	7.82

Tumour to tissue ratio MAb JSB-1

	Days							
	1		2		3		7	
	ratio		ratio		ratio		ratio	
Blood	0.36	0.05	0.4	0.04	0.47	0.04	0.41	0.17
Sternum	3.76	0.52	6.46	1.66	4.36	0.60	3.6	1.21
Liver	1.61	0.20	2.15	0.10	2.44	0.55	2.07	0.93
Spleen	1.93	0.09	2.61	0.64	3.21	0.42	3.16	1.34
Kidney	1.44	0.25	1.76	0.30	1.85	0.16	1.64	0.56
Colon	5.56	1.14	7.46	1.11	5.73	0.95	4.12	1.5

Tumour to tissue ratio was calculated as by dividing the uptake /gram tumour tissue by the uptake /gram of non-target tissues. For each day at least 3 mice were used. STD is also given.

At 24 hr after injection, 13% ID/g of E 48 was found in the tumour. This value increased to 16 % ID/g on day 3. We noted a higher accumulation of radiolabelled antibody E 48 in smaller tumours (Table II).

To exclude the possibility that uptake might be due to non-specific protein trapping because of higher vascularisation of the xenograft, an isotype-matched antibody was included for control. Specificity index of tumour localization was calculated by dividing the uptake/g of the specific antibody (E 48) by the uptake/g of the control antibody (JSB-1) into the tumour. As indicated by the specificity indices, which ranged from 4 to 11, localization was specific for E 48 (Table III). The decrease in the specificity index was mainly due to an increase in the amount of the non-specific antibody present in the xenograft, whereas the concentration of E 48 also diminished after day 3.

TABLE III SPECIFICITY INDEX ON VARIOUS TIMES

Day 1	Day 2	Day 3	Day 7
11.76 (0.4)	8.39 (0.4)	.41 (0.5)	4.16 (0.2)

Specificity index of tumour localization was calculated by dividing the uptake/g of the specific antibody (E 48) by the uptake/g of the control antibody (JSB-1) into tumour. Standard deviation is given in brackets.

TABLE II - ANTIBODY UPTAKE IN RELATION TO TUMOUR WEIGHT

DAY 1		DAY 3	
TUMOUR WEIGHT	% UPTAKE	TUMOUR WEIGHT	% UPTAKE
60	28.0	190	20.7
420	11.6	302	19.2
445	12.3	572	16.9
477	15.2	583	16.5
627	8.4	897	14.1
1008	7.1	1249	11 0

Tumour weight is in mg. Antibody uptake was calculated as the percentage of the injected dose / gram tumour tissue.

Uptake in the other organs. Uptake, expressed as the average injected dose/g tissue of the various organs is presented in Figure 2. Only the most relevant organs are shown, the uptake in heart, stomach, jejunum, muscle, lung and tongue being comparable to or less than the uptake in colon. Sternum is shown here because of the presence of blood forming. Control antibody JSB-1 did not show preferential localization in any organ (Table I and Figure 2).

Radioimmunoscintigraphy

Mice were scanned at specific time intervals following i.v. injection of radiolabelled antibody. After scanning, mice were killed and biodistribution data were collected and compared to immunoscintigraphic images. Each picture shown in Figure 3 a-d represents thus a different mouse. Subtraction techniques were not performed.

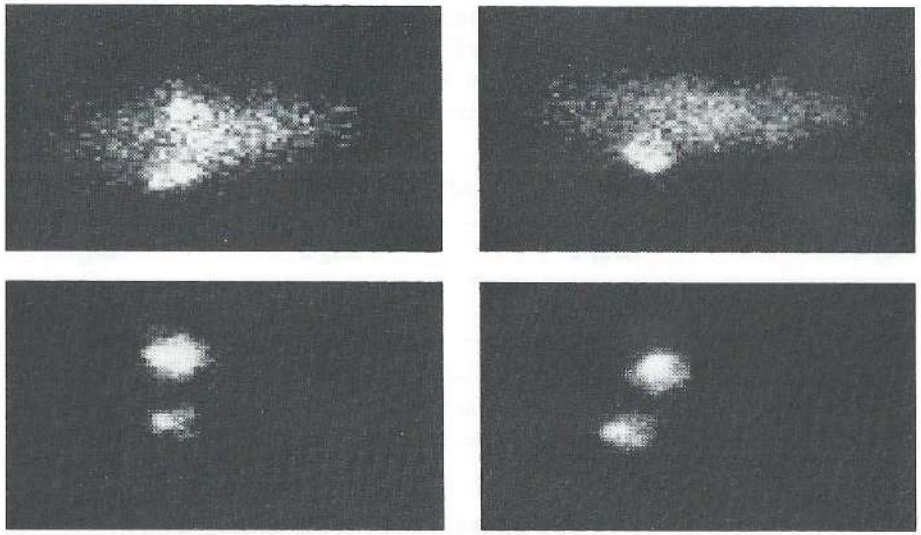

FIGURE 3. Whole body scintigraphic images of an athymic nude mouse bearing two subcutaneous squamous-cell carcinomas given an injection of 10 μCi ^{131}I-labelled E 48. Images were taken at 1 day(A), 2 days (B), 3 days (C) and 7 days (D)

Figure 3a shows the results obtained 22 hr after injection of 10 μCi E 48 antibody. At this time a 420-mg tumour (11.6% ID/g) could be visualized. Background levels markedly disturbed too much the aspect of a 60-mg tumour despite enhanced antibody uptake by this xenograft (28% ID/g). Tumour to blood ratio in this specific mouse was 2.85. Antibody uptake in large organs such as liver or spleen (1.4 % and 1.15 % ID/g respectively) was too low for visualization of these organs. On the second day, 2 tumours, both of 220-mg (14.1 and 15.0 % ID/g) became visible, though blood activity remained moderate (tumour to blood ratio: 3.20). On day 3, a 190-mg and a 897-mg xenograft are depicted, while the antibody uptake is 20.7 and 14.1 %ID/g respectively. Tumour to blood ratio was 6.5. Figure 3d represents the situation 7 days after injection of E 48, revealing 274-mg (11.2 %ID/g) and 1216-mg (7 % ID/g) xenografts. Tumour to blood ratio was 4.85.

DISCUSSION

Our study demonstrates the feasibility of radioimmuno- localization of SCC xenografts in nude mice using ^{131}I E 48. Whereas extensive studies using radiolabelled MAbs have been performed for ovarian or colorectal carcinomas (Colcher *et al*, 1988; Epenetos *et al*, 1986) there is only limited experience in the use of MAbs for detection of SCC. Wahl *et al.*(1987) used the A9 antibody with moderate succes to image

SCC xenografts, obtaining tumour to blood ratios of 1.84 at day 7 after i.v. injection. However, this antibody shows cross reactivity with normal human endothelium in immunohistochemical studies, thus limiting its clinical applications (Kimmel and Carey, 1986).

In order to generate antibodies with limited reactivity to normal tissues, mice were immunized with a metastasis of a laryngeal SCC. One of the producing clones, E 48, was selected because of its strong reactivity with SCC. Among normal tissues it was only found in normal stratified squamous and transitional epithelium (Quak *et al*,1989).

In our study only one type of xenograft was used, immunoperoxidase staining with E 48 revealing strong reactivity with all SCC (n=8) available in our laboratory. Immunohisto- chemical experiments on frozen sections also revealed that 75 out of 76 SCC reacted with the antibody, regardless of the degree of morphological differentiation or origin (lung, head and neck, cervix) of the tumour. Staining was observed in all or almost all neoplastic cells.

The biodistribution data demonstrate the preferential accumulation of MAb E 48 in the xenografts. The control antibody did not accumulate in the xenografts, as shown by the specificity index which ranges from 4-11, indicating that E 48 MAb uptake was not due to better blood supply to xenografts. Furthermore, E 48 did not localize in liver and spleen, both of which are particularly prone to non-specific antibody uptake after intravenous injection (Moseley *et al*, 1988).

Immunoscintigraphic studies were performed in a small number of animals prior to obtaining biodistribution data. Images show distinct tumours on days 3 and 7, whereas on days 1 and 2 blood pool activity is also present. A small (60 mg) tumour is even masked by the amount of ^{131}I E 48 in the blood. If the aim of using radiolabelled antibodies in man is detection of tumour extension, immunoscintigraphy should be more sensitive than conventional techniques such as magnetic resonance imaging (MRI) or computed tomography and thus be capable of imaging small tumour deposits. Increased uptake by small tumours as compared to larger ones is indeed encouraging, as reported also by Matzku et al. (1988) and Hagan *et al.*(1986). On days 3 and 7 the plasma has been cleared from most of the free circulating ^{131}I E 48 resulting in a higher resolution of images. On these days only relatively large tumours (ranging from 191 to 1259 mg) were present, further studies are thus required to determine whether radiolabelled Mab E 48 can be used for detection of minute tumour xenografts.

Experiments in nude mice are a prerequisite in evaluating the properties of a radiolabelled MAb. Within the limitations of our model, we have shown that radiolabelled E 48 preferentially reactive with SCC can specifically accumulate within tumours and allows xenograft imaging with a high level of resolution.

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GENERAL DISCUSSION

When this research line was started 1986, there were only a limited number of antibodies available to squamous-cell carcinomas (SCC). These few MAbs, however, had such characteristics that they could not be considered as suitable for radio immunolocalization or therapy purposes. Therefore we had to develop an immunization protocol and a screening system to produce appropriate antibodies. Much emphasis was given to the selection of high affinity IgG antibodies directed to SCC surface antigens, which were not present on blood cells. Using an ELISA-system of viable tumour cells and red blood cells as well as screening on frozen sections, hybridomas producing interesting antibodies could be identified at an early stage. Twenty to thirty MAbs have been selected out of a total of more than 10,000 producing hybridomas, indicating that monoclonal antibody production is a laborious procedure with a low efficiency. Of these MAbs, 4 showed promising features for our study goals and were further investigated. These 4 antibodies (K 984, K 931, K 928 and E 48) recognize different antigens as based on immunohistochemical and biochemical data. Four other antibodies have also been selected, 2 of these show K 984 characteristics and 2 are K 928 like. In addition a MAb, K 112, was produced with reacts with proliferating cell nuclei. Although this antibody does not appear suitable for imaging purposes and therefore will not be discussed further on this chapter, the potential of this antibody for predicting biological tumour behavior as well as response to treatment will be further investigated in our laboratory.

In the introduction of this thesis, parameters which are of importance for the success of the magic bullet theory have been reviewed. These parameters are summarized in table I and we have added the characteristics of the 4 different MAbs mentioned above. Referring to this table, the potential of the 4 different antibodies will be discussed for tumour localization in patients with squamous-cell carcinoma (SCC) in general and in patients with SCC of the head and neck (HN-SCC) in particular.

MAb K 928 reacts only with well and moderately differentiated SCC and within these tumours only with differentiated cells as present in the central layers (suprabasal) of tumour nests and therefore has to be considered as less suitable for tumour imaging as these areas are usually not penetrated by the systemically administered antibody. The antibody may serve as a differentiation marker in SCC.

Biochemical characterization revealed that K 931 reacted with the 17-1A antigen. MAb K 931 was found to react within the cytoplasm of the more differentiated cells in primary tumours, however, in some primary SCC, lymph node and hematogenous metastases an enhanced and distinct surface labelling was noted. This has not been reported before for the 17-1A antigen. If this preferential reactivity with metastases holds true for larger series, it can make the antibody valuable for radio-immunolocalization purposes. The antibody is now subject of imaging studies in the nude mouse model. Antibodies to the 17-1A antigen are among the most extensively studied in patients with gastro-intestinal malignancies (1). Toxicity was not observed, despite the expression in almost all human epithelia, suggesting that normal tissues were not reached by the antibody, for reasons as stated in the introduction of this thesis (CHAPTER I,section 1.3). An major disadvantage of these antibodies, however, is their rather low affinity (I). Preliminary studies indicated that the dissociation con-

TABLE I.

Antibody/antigen characteristics suitable for tumour imaging.

parameter	E 48	K 984	K 928	K 931
ANTIBODY RELATED				
IgG isotype	+	+	+	+
immune reactivity $F(ab')_2$ fragments	+	+	NT	NT
biological activity of antibody	+	+	NT	-
ANTIGEN RELATED				
expression on blood cells	-	-	-	-
expression on peripheral tumour cell layer	+	+	-	+/-
antigen/antibody internalization	+	+	NT	NT
antigen shedding	-	-	-	-

NT:not tested.

stant of K 931 is higher: 4×10^{-9} M (Gerretsen *et al.*, unpublished data).

MAb K 984 recognizes an antigen expressed in all SCC at the outermost layer of tumour nests, which are composed of light microscopic poorly differentiated cells. Furthermore, binding of the antibody to tumour cells *in vitro* resulted in growth inhibition of SCC cell lines (CHAPTER 4, addendum). These data are indicative that the antibody recognizes the proliferating SCC cells. The antibody shows reduced immunoreactivity after iodination, but despite this handicap specific tumour localization in nude mice occurred when ^{131}I was injected intravenously (Gerretsen *et al.*: unpublished results). Other iodination methods or other isotopes may be less harmful for the antibody and contribute to its usefulness. Iodine in general is less suitable as label for

tumour detection in the head and neck region, since free Iodine will concentrate into the thyroid and consequently disturb the imaging. Further investigation with this antibody, using alternative isotopes, in the nude mouse model is now performed.

The most promising antibody at this moment, however, is E 48. The identified antigen has a very restricted expression in normal tissues but is present in 89 out of 91 SCC sofar tested. An important aspect is lack of heterogeneity of E 48 expression by head and neck squamous-cell carcinomas (HN-SCC), although a small group of HN-SCC express the antigen only in the light microscopically more differentiated cells in the central layers of the tumour nests. In HN-SCC grown as subconfluent cultures *in vitro* the antigen expression was low. However, a dramatic increase of the expression was found when the cells were cultured to confluency. A similar high expression was found in all lines grown as colonies within collagen gels. Presence of MAb E 48 during culturing of HN SCC cells in this latter *in vitro* sytem apparently disturbed the formation of cell-cell contacts. Instead of spherical colonies, groups of single cells arose. These data indicate that the antigen recognized by MAb E 48 be involved in the structural organization of HN SCC (van Dongen *et al*, unpublished results). Together with the immunolocalization of the E 48 antigen along the cell surface and within the interdesmosomal cleft (CHAPTER 3), these data are highly suggestive that the E 48 defined antigen is a cell surface protein belonging to the desmosomomal core glycoproteins (2), and that the recognized epitope is actively involved in cell-cell adhesion. The fact that the epitope represents a peptide structure is in concordance with the findings of Overton (3), who demonstrated that the carbohydrate moiety of surface glycoproteins is not responsible for cell-cell recognition, but serves stabilizing purposes. It was also noted that E 48 becomes internalized upon binding to the tumour cell (van Dongen *et al.* unpublished observations). The antigen is not found at detectable levels in sera of HN SCC bearing patients (nor is any of the antigens recognized by the MAbs described here; Davis and Quak, unpublished results).

Based on these data radio immuno localization imaging studies with MAb E 48 were started after it was found that the antigen expression in xenografts was similar to those in frozen sections from surgically excised patient material. Indeed, the feasibility of the antibody for specific delivery of radiochemicals could be demonstrated in the nude mouse model (CHAPTER 7). In later experiments Gerretsen *et al.* extended these studies to several xenografts and obtained even better tumour to non-tumour tissue ratios with $F(ab')_2$ fragments (4). Although the nude mouse model is an attractive system prior to clinical investigations, one should keep in mind that there are several factors which make tumour localization with antibodies in this sytem more successful than in the human situation. Tumours have in general a relatively large size as compared to the total body weight of the animal (100 - 1000 µg tumour vs. 20-25 g. total body weight) and a different vascularization. The experience in the human situation with MAbs directed to a variety of human cancers, except SCC, in general shows that approximately 0.006 to 0.017 % of the injected dose/gram tissue can be found after intravenous injection of monoclonal antibodies, which is much lower than was found in animal models (1,5). Secondly, only a minority of all transplanted tumours starts to grow in the nude mouse and therefore xenografts may not be representative for HN SCC. Also, none of the here mentioned antibodies was found to react with normal murine tissues, so that it was not possible to determine antibody penetration into these tissues. Despite these limitations studies within the nude mouse model are generally considered a prerequisite prior to clinical studies and within this model good tumour localization with MAb E 48 could be demonstrated. A phase I/II study with E 48 for the detection of SCC in patients with squamous-cell carcinomas of the head and neck, lung and cervix will be started shortly.

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SUMMARY

This thesis presents preclinical studies on the production of monoclonal antibodies (MAbs) to squamous-cell carcinomas of the head and neck (HN-SCC), with the aim to use antibodies for radio- immunolocalization and immunotherapy of these malignancies.

The need for such antibodies is caused by the inability of the current treatment possibilities to cure patients with local regional recurrence, after initial therapy, or those with metastatic disease throughout the body.

In Chapter 1, the concept of the use of MAbs in clinical oncology is discussed, referring to Paul Ehrlich, who was the first to predict the potential use of antibodies for the above mentioned purposes.

Chapter 2 describes different immunization protocols to produce MAbs to tumour associated surface antigens. It is concluded from these experiments that the murine immune response shows preferential reactivity to a resticted group of, apparently highly immunogenic, antigens. This phenomenon is a major cause of the limited number of MAbs to tumour associated antigens (TAA). One immunization protocol was advocated, since it was shown to have a number of advantages over the other assayed protocols. In chapter 3 to 6, five MAbs are described which have been generated with this protocol.

Chapter 3 documents the production of MAb E 48, which is the most valuable antibody produced in this study. The antibody binds among normal tissues only with stratified squamous and transitional epithelia. Malignancies derived from these tissues maintain the antigen expression and therefore the antigen is considered as an attractive target for radio immunolocalization purposes. Biochemical characterization revealed that the E 48 antigen has a molecular mass of 22 kD. With the aid of immunoelectron microscopy, the antigen could be traced along the cell surface as well on desmosomes. It is speculated that the antigen may represent the 22-kD polypeptide, which can be found in biochemical purifications of desmosomes. It is concluded that MAb E 48 is of value to discriminate poorly differentiated squamous-cell carcinomas and transitional epithelial malignancies from other undifferentiated neoplasms.

In chapter 7 the feasibility of MAb E 48 for radioimmuno- localization purposes is demonstrated. Nude mice, carrying squamous-cell carcinoma xenografts were injected iv with radiolabelled MAb E 48. Specific localization occurred for E 48, but not for a radiolabelled control antibody. Tumour imaging was possible 24 hours after E 48 was injected. These findings suggest that E 48 is capable of high specificty in targeting isotopes to squamous-cell carcinomas in an experimental setting.

Chapter 4 describes 2 antibodies, K 984 and K 928, which selectively identify cell populations within normal squamous epithelium and SCC. K 984 was demonstrated to recognize a glycoprotein present on the surface of basal cells in squamous epithelium and poorly differentiated cells in SCC. K 928 detects an antigen with a molecular mass of 50-55 kD, which becomes expressed by suprabasal cells in squamous epithelium and the more differentiated cells in SCC. The expression of the identified antigens in SCC is discussed in relation to the histological diagnosis of SCC. In the addendum of Chapter 4, the ability of MAb K 984 to inhibit the growth of 4 different SCC carcinoma cell lines for a period of time is demonstrated. Together with the immunohistochemical results, these data are highly suggestive that the K 984 antigen is associated with proliferating cells in SCC and therefore may be an attractive target for radio-immunolocalization and therapy purposes of SCC.

In chapter 5, a MAb is presented which shows no reactivity with normal squamous epithelia, but was found to bind 58 out of 62 SCC. Further biochemical characterization revealed that the antigen represents the 17-1A antigen. A, not earlier reported, differential reactivity was observed among several SCC. We noted that some SCC, including all metastases tested so far, showed an enhanced expression as compared to most primary SCC.

Chapter 6 shows the characteristics of MAb K 112, an antibody which was selected because of its strong labelling of tumour nuclei. It was shown to recognize a 43 kD antigen, associated with the nuclear matrix, and which becomes detectable only in cells under proliferative conditions. The antigen shows an identical expression in all mammalian cellls tested and its presence in transformed tissues is discussed.

The potential use for clinical purposes of any of the described MAbs is discussed in chapter 8. Based on the results presented in this thesis a phase I/II will be started shortly with radiolabelled MAb E 48 for the detection of SCC in patients with tumours of the head and neck, lung and cervix.

SAMENVATTING

In dit proefschrift worden studies beschreven welke leiden tot de productie van monoklonale antilichamen tegen plaveiselcel carcinomen uit het hoofd/hals gebied. Het uiteindelijke doel van deze monoklonale antilichamen is hen te gebruiken voor radio- immunolocalizatie en therapie van deze tumoren.

De vraag naar dit soort antilichamen wordt veroorzaakt door de beperkingen in de huidige therapeutische mogelijkheden om patienten te genezen die een loco-regionaal recidief ontwikkelen, na een aanvankelijk succesvolle behandeling, en bij patienten met een hematogeen gemetastaseerd plaveiselcel carcinoom.

In hoofdstuk 1 wordt het concept van monoklonale antilichamen voor toepassing in de klinische oncologie besproken, refererend aan Paul Ehrlich, die reeds in het begin van deze eeuw, de mogelijkheden van antilichamen voor de bovengenoemde doelstellingen voorspelde. Er wordt verder ingegaan op de verschillende parameters, die van belang zijn voor de penetratie van antilichamen in tumor weefsel.

Hoofdstuk 2 beschrijft verschillende immunizatie protocollen om monoklonale antilichamen te produceren tegen tumor geassocieerde antigenen. Uit deze studie bleek dat het muizen immuunsysteen een voorkeursrespons laat zien tegen een beperkte groep, klaarblijkelijk sterk immunogene, antigenen. Dit verschijnsel is er waarschijnlijk een van de oorzaken van dat er zo weinig antistoffen zijn tegen tumor geassocieerde antigenen. Van een bepaald immunizatie protocol kon worden aangetoond dat het wezenlijke voordelen bezat boven anderen. In hoofdstuk 3 tot en met 6 worden vijf monoklonale antilichamen beschreven die op deze manier opgewekt zijn.

Hoofdstuk 3 documenteert de productie van het antilichaam E 48, hetgeen de meest waardevolle is welke in deze studie is geproduceerd. In normaal weefsel bindt het antilichaam alleen aan gelaagd plaveisel epitheel en overgangs epitheel van de blaas. Maligniteiten, die zich vanuit deze weefsels ontwikkelen blijven het door E 48 herkende antigen in hoge mate tot expressie brengen op de cel membraam en om die redenen wordt het antigen als een geschikt doelwit gezien voor radio-immunolocalizatie doeleinden Biochemische karakterisatie maakte duidelijk dat het E 48 antigen een relatief molecuul gewicht heeft van circa 22 kD. Immunoelectromicroscopische studies toonden aan dat het antigen zowel langs de cel membraam te vinden was als ook in de interdesmomale ruimten. Er wordt daarom gespeculeerd dat het antigen een 22-kD polypeptide voorstelt, welke gevonden kan worden bij biochemische analyse van desmosomen. Het wordt geconcludeerd dat het monoklonale antilichaam E 48 van waarde is om weinig gedifferentieerde plaveisel cel carcinomen en overgangsepitheel carcinomen van de blaas te onderscheiden van andere ongedifferentieerde carcinomen.

In hoofdstuk 7 wordt de mogelijkheid van E 48 voor radio- immunolocalizatie van tumoren aangetoond. Naakte muizen, waarin menselijke plaveiselcel carcinomen groeiden, kregen intraveneus E 48 toegediend welke gekoppeld was aan een radioactieve stof ter tracering. Tumoren konden op deze manier binnen 24 uur na toediening zichtbaar gemaakt worden. Wanneer niet-relevante (controle) antilichamen werden gebruikt, was dit niet mogelijk.

De resultaten suggereerden verder dat E 48 in staat is om radio-isotopen met een hoge specificiteit naar de tumor te targetten.

Hoofdstuk 4 beschrijft 2 antilichamen, K 984 en K 928, welke met verschillende cel populaties reageren in normaal plaveiselepitheel en plaveiselcel carcinomen. Het wordt duidelijk gemaakt dat K 984 reageert met een een glycoproteine, welke aanwezig is op het oppervlakte van basale cellen in plaveisel epitheel en de weinig gedifferentieerde cellen in plaveiselcel carcinomen. K 928 herkent een 50-55 kD antigen, welke daarentegen tot expressie komt op suprabasale of licht microscopisch meer gedifferentieerde cellen in plaveiselcel carcinomen. De expressie van de herkende antigenen wordt bediscussieerd in relatie tot de histologische differentiatie graad van plaveiselcel carcinomen. In het addendum van hoofdstuk IV wordt aangetoond hoe K 984 groei remming van 4 plaveiselcel carcinoom cel lijnen bewerktstelligd voor een bepaalde periode. Tesamen met de immuunhistochemische aankleuringen, wijzen deze gegevens erop dat het K 984 antigen geassocieerd is met proliferende cellen in plaveiselcel carcinomen en daarom een attractief doelwit kan zijn in radio-immunolocalizatie en therapie studies.

In hoofdstuk 5 wordt een antistof beschreven welke geen reactiviteit bezit met normaal plaveisel epitheel, maar welke wel reageerde met 58 uit 62 plaveiselcel carcinomen. Biochemische analyse maakte duidelijk dat het antigen het 17-1A antigen voorstelde. Een nog niet eerder gedocumenteerd verschil tussen het aankleurings patroon werd geconstateerd van de diverse tumoren, waarbij met name metastasen sterk aankleurden. De preferentiele reactiviteit met metastasen kan de antistof geschikt maken voor radio-immunolocalizatie doeleinden.

In hoofdstuk 6 wordt het antilichaam K 112 beschreven, die werd geselecteerd vanwege sterke aankleuring van tumor cel kernen. De resultaten maakten duidelijk dat K 112 een 43 kD antigen herkent, welke geassocieerd met de nucleaire matrix en alleen in cellen onder proliferende condities tot uiting komt. Verder bleek het antigen op een identieke wijze tot expressie te komen in alle geteste zoogdiercellen. Tot slot wordt het voorkomen van het K 112 antigen in kwaadaardige nieuw vormingen besproken.

In de slotdiscussie van hoofdstuk 8 worden de mogelijkheden voor elk van de geproduceerde antilichamen voor immuno-detectie en therapie van plaveisel cel carcinomen besproken. De resultaten van deze studie hebben ertoe geleid dat binnenkort een fase I/II zal beginnen naar de detectie van plaveiselcel carcinomen bij patienten met tumoren uitgaande van het hoofd/hals gebied, long en de cervix uteri.

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De schrijver van dit proefschrift werd geboren op 19 maart 1960 te Mijnsheerenland. Na de Gymnasium β opleiding op het dr. FH de Bruyne Lyceum te Utrecht (eindexamen 1978) begon zijn studie Geneeskunde aan de Rijks Universiteit Utrecht (Artsexamen 26 April 1985). Gedurende zijn militaire dienstplicht, die daar aansluitend opvolgde, was hij als reserve-1^{e} Luitenant-arts verbonden aan de Militaire Bloedtransfusie Dienst te Amsterdam. In dezelfde periode werd onderzoek vericht naar de detectie van trombocyten antistoffen op de afdeling Immunohematologie (Dr. AEGKr von dem Borne) van het Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlands Rode Kruis (Amsterdam). Van september 1986 tot september 1988 werkte hij als assistent-in-opleiding (AIO) in dienst van de afdeling Keel- Neus- en Oorheelkunde van het Academisch Ziekenhuis de Vrije Universiteit te Amsterdam aan het onderzoek dat tot dit proefschrift leidde. Sinds september 1988 is hij in opleiding tot KNO-arts in bovengenoemd ziekenhuis (Hoofd: Prof. Dr. GB Snow).