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Morphological Tumor Markers

General Aspects and
Diagnostic Relevance

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Preface

New methods open new insights and provide the basis for new concepts in cancer research. The discovery of monoclonal antibodies and the further development of immunocytochemistry and hybridization techniques has led to a “renaissance” of pathology in the field of tumor classification and prognosis as well as in oncotherapy. The application of the new marker methods may result in a more intensive integration of morphological findings into clinical practice.

In recent years an increasing battery of commercially available antibodies has made a progressively more detailed classification of tumors possible using a broader spectrum of immunocytochemical methods. All scientific journals publish papers about the results of these new applications in tumor diagnosis. Principally, we must differentiate between biological and morphological tumor markers.

In this monograph the importance of morphological tumor markers is the focus of the presentation by an international group. As an introduction, two chapters offer accounts of the application of monoclonal antibodies to human tumor antigens and the important role of biochemical markers in human cancer. A special chapter refers to the different immunocytochemical methods for the demonstration of tumor markers and their relevance in diagnostic pathology.

The so-called tumor markers are very often cell or tissue markers which provide data related to the problems of cytogenesis, cell function or cell differentiation. In contrast with the evidence from biological markers, the methods of immunocytochemistry or hybridization techniques permit the demonstration of tumor markers precisely, on the cell membrane, in the organelles of the cytoplasm or in the nucleus. In the very heterogeneous group of morphological tumor markers the intermediate filaments of the cytoskeleton, glycoconjugate antigens of the cell membrane and special proteins such as enzymes play the major role in routine diagnostic work.

From the histogenetic point of view, markers of different tissues can be distinguished. This fact is the organizing principle of the majority of chapters. The epithelial tumor markers include widely varying substances (cytokeratins, tissue polypeptide antigen, epithelial membrane antigen, oncofetal antigens, markers of glandular differentiation). Mesenchymal markers consist of some intermediate filaments (e.g. vimentin, desmin etc.), special proteins and enzymes. Other markers are

suitable for demonstration of the intercellular matrix (proteoglycans, basal membrane antigens), blood group substances, lectins or neuroendocrine products. Further chapters demonstrate the importance of tumor markers in neuro-oncology and viral-associated tumors. The conclusion of the monograph is an overview on the theme of "cell, tissue and organ specific tumor markers".

Altogether the monograph is a review of methods and practical application of the different morphological tumor markers in the field of tumor diagnosis and prognosis. Organ specific diagnosis has a very great clinical relevance for treatment and prognosis. Therefore, this volume brings all pathologists and oncologically orientated clinicians up to the state of the art in cancer detection and diagnosis.

I would like to thank very much all contributors for their excellent cooperation in timing and arrangement of the chapters. I am also grateful for the outstanding work on this volume by Springer-Verlag, in particular Mrs. H. Herion and Mrs. U.S. Davis.

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G. SEIFERT

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Monoclonal Antibodies to Human Tumor Antigens

G.L. WRIGHT JR. and A.D. COX

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1 Introduction

The introduction of monoclonal antibody (Mab) technology (KÖHLER and MILSTEIN 1975) revolutionized the serological and biochemical analysis of human cancer. By fusing spleen cells from immunized mice with myeloma cell partners, followed by cloning of the resulting hybrids, it is possible to produce monoclonal cell populations, each of which has acquired both the property of immortality and the ability to produce a specific antibody. The power of this technology results from the ability to generate large quantities of stable, sensitive, specific Mab probes which can detect but a single epitope on a complex molecule. Tumor markers may be qualitatively or quantitatively different from those expressed on normal cells; Mabs can detect such markers even if they are present

only in very small quantity or differ only slightly from other molecules. Although human tumor-specific antigens have continued to prove elusive, and indeed may not exist, many tumor-associated antigens (TAA) from a wide variety of human malignancies have been identified and characterized using Mab probes. Tumor markers detected by Mabs to date include oncofetal antigens, differentiation antigens, growth factors and oncogene products, hormones, receptors, enzymes, and many novel antigens for which no function has yet been described.

1.1 Specificity of Monoclonal Antibodies

Although the class/subclass, affinity and biological effector functions of a Mab clearly affects its utility, the careful, thorough analysis of Mab specificity is the most essential aspect of monoclonal antibody characterization. Since heterogeneity within and among tumors is the rule rather than the exception (*vide infra*), the use of multiple assays (e.g. immunoperoxidase (IP), immunofluorescence (IF) and radioimmunoassay (RIA)) on frozen or fixed tissue sections and on live and fixed cells, as well as the use of multiple tumor and normal tissue sources is crucial to determining the true distribution of antigen specified by the Mab under study.

Although Mabs react with their antigens in a highly specific manner, the recognition of the same epitope borne on an unrelated antigen is a common source of lack of specificity, or cross-reactivity. In a typical example, Mabs directed against a glycolipid antigen found on prostate carcinoma were found to cross-react with red blood cells (LINDGREN *et al.* 1986). The findings suggested that the carbohydrate moiety was attached to ceramide in the prostate-associated antigen but to a protein core in the red blood cell.

1.2 Murine Versus Human Monoclonal Antibodies

A theoretically major advantage of human Mabs is the increased potential to detect subtle antigenic differences between cells of different human malignancies or between malignant and normal human cells.

Unfortunately, despite considerable effort, only limited progress has been made in generating useful human anti-tumor Mabs. Human monoclonal antibodies to tumors of such diverse origin as breast, lung, gastric, colorectal and urogenital carcinomas, as well as melanomas, gliomas and leukemias, have been produced by a variety of interspecies fusion, human/human fusion and Epstein-Barr virus transformation techniques (reviewed in STRELKAUSKAS 1985). Most of the Mabs are not yet well-characterized, but their quality has been largely disappointing. In general, most have been characterized by low affinity (particularly since many are IgM) and by low levels and unstable antibody production. Interestingly, and perhaps most disappointingly, none has detected a human tumor-specific antigen: most react with normal membrane or cytoplasmic components of the tumor cell. It is possible that many interesting TAA may not be immunogenic in humans.

A critical appraisal of current progress thus suggests that many technical problems need to be resolved before clinical application of human Mab becomes a reality. Some of these formidable problems may be more immediately amenable to solution than others. For example, *in vitro* immunization, perhaps using splenic lymphocytes, is unlikely to be more successful without improved understanding of the growth and differentiation factor requirements of antigen-responsive cells. However, recent advances such as electrofusion methodology (LO et al. 1984) may improve fusion frequencies, while DNA transfer techniques (TAKEDA et al. 1985) may make fusion unnecessary altogether.

As an alternative to wholly human Mabs, several investigators have generated human/mouse chimeric antibodies, in which the antigen-binding region is from a murine Mab and the constant region is of human origin (MORRISON et al. 1985; TAKEDA et al. 1985; SUN et al. 1986). In a marriage of hybridoma, recombinant DNA and gene transfer technologies, the genes of interest are cloned and transfected into a myeloma cell line for expression. Such a "transfectoma" approach permits the construction of antibodies with desired specificity, as can be produced in the mouse, but which are less immunogenic than murine Mabs and which possess human effector functions for use in human hosts. These attributes are desirable for the *in vivo* diagnostic and therapeutic use of anti-tumor Mabs, as discussed below.

1.3 Tumor Antigens, Growth Factors and Oncogenes

A wide range of antigens expressed in tumor cells have been detected and characterized by monoclonal antibodies (for reviews see WRIGHT 1984; SELL and REISFELD 1985; SCHLOM and WEEKS 1985). Most Mab-defined TAA are generally not confined to tumors of one type, many are expressed on normal cells, and "preferential tumor expression" is often identified in quantitative terms. Properties of potential tumor markers which must be examined before strategies for their use can be developed are: 1) their biochemical nature, 2) number of epitopes, 3) cellular location, 4) stability, 5) distribution on normal/tumor tissue types (public vs. private specificity), 6) distribution within and among tumors (heterogeneity), and 7) biological function.

One class of TAA deserves special mention as a field of active inquiry to which Mabs are being newly applied. Aberrant expression of cellular oncogenes has been found in carcinomas, melanomas, sarcomas, neuroblastomas and hematopoietic malignancies. They appear to be associated with abnormalities of growth and differentiation, and some have been shown to encode cellular growth factors or their receptors. There is evidence for greater amplification and enhanced expression of oncogenes in the advanced stages of lung carcinomas, neuroblastomas, and leukemias/lymphomas (*myc*) (reviewed in COLB and KRONTIRIS 1986), and (controversially, see below) in more aggressive forms of breast, colon and prostatic carcinoma (*ras*) (reviewed in THOR et al. 1986). If this is so, then simple methods to detect specific oncogene products would be of enormous clinical utility.

Mabs have now been produced to members of several families of oncogenes: *ras*, *myc*, *erbB* and *neu*, enabling the detection and characterization of oncogene

products by antibody binding in immunoblotting or immunohistochemical techniques. For example, Mabs directed at the *erbB* product, i.e. the EGF receptor, have shown that overexpression of EGFR is a property of squamous, but not other, tumor types (OZANNE et al. 1986), and these Mabs have been shown to have anti-tumor effects (MASUI et al. 1986).

The apparent presence of activated or otherwise aberrant *ras* genes in approximately 20% of solid tumors is enticing, although the role of *ras* in malignancies is unclear at present. The p21 protein product of the *ras* family, which may be an obligate intermediate in the transduction of growth factor signals (GOUSTIN et al. 1986), has been extensively studied using anti-p21 Mabs. The Rap 1-5 series of Mabs (HORAN HAND et al. 1984) and the Y13-259 Mab (FURTH et al. 1982) detect both normal and activated forms of the *ras* proteins; at this writing, controversy exists over the significance of tissue binding by Rap-5, since IP studies (THOR et al. 1986) have shown a much higher presence of *ras* than can be accounted for by oncogene transcripts. It has been suggested that the additional reactivity to tissue sections seen in IP studies may be due to cross-reactivity of the anti-p21 Mabs with both normal and activated *ras*; however, the Mabs identified by CARNEY et al. (1986) detect only mutated p21 yet also show more cross-reactivity in IP studies than in vitro specificity would predict. Work by ROBINSON et al. (1986) indicates that Y13-259, but not Rap-5, can discriminate between cell lines with and without transforming *ras* genes. It is therefore possible that Mabs such as Rap-5 bind to cancer tissues at high levels at least in part through cross-reaction with an epitope also found on an unrelated antigen, as described above in Section 1.1. Further studies will have to be performed to settle the controversy. Such experiences are common when Mab technology is applied to any new area of tumor immunology, and illustrate the necessity to apply rigorous control systems to the specificity analysis of anti-TAA Mabs. Whatever the outcome of this particular case, Mabs directed against oncogenes and their products appear to show great promise in tracking the etiology, classification, and prognosis of human tumors.

1.4 Antigenic Heterogeneity

Heterogeneity of antigenic expression on tumor cells is a major potential problem for the development of strategies for the immunological diagnosis and therapy of cancer. The increased use of highly specific, non-crossreacting monoclonal antibodies has both highlighted the presence of antigenic heterogeneity and accentuated the problems due to it.

Immunohistochemical studies using Mabs have shown both qualitative and quantitative heterogeneity, such that (1) multiple antigenic phenotypes can exist in tumors of a given type, ranging from cells expressing all the antigens under study to cells expressing none; (2) variable phenotypic expression can occur within a given tumor, showing a patchwork distribution of antibody-binding and non-antibody-binding cells or areas of cells; (3) a particular antigen may be expressed in the cytoplasm of cells in one part of the tumor and in the membrane of cells in a different part; (4) different metastases from the same

patient may show different antigenic phenotypes and (5) individual cells of the same phenotype may differ significantly in antigen density (reviewed in EDWARDS 1985).

The multiple genotypic and phenotypic causes of the heterogeneity described above have been reviewed in SCHLOM (1986). Most intriguing is the possibility that different antigenic expression may sometimes reflect biological behavior: certain Mab-defined TAAs have been shown to correlate with more (SUTER et al. 1985) or less (XIANG and KIMURA 1986, personal communication) aggressive behavior in melanomas. It might be possible to exploit such variation in antigen expression for diagnostic and prognostic purposes.

The problem of antigenic heterogeneity becomes clear and vexing when one considers that a primary advantage of using Mabs lies in their exquisite sensitivity and specificity and their ability to discriminate between morphologically similar cells. Unfortunately, cells which do not, for whatever reason, express the appropriate antigen are rendered "invisible" to the Mab. Since TAA-directed Mabs tend to react not with 100% of tumors or tumor cells, but rather with a subpopulation of those tumors or cells, the use of a single Mab thus inevitably will mean missed diagnoses, unrecognized recurrences and metastases, and tumor cells resistant to Mab-directed therapy.

One answer to the problem of variable tumor marker expression is therefore the use of panels of Mabs or defined mixtures (cocktails) of Mabs that react with distinct epitopes on the same or different antigens. Such panels have improved the detection of TAA in serum by RIA and ELISA, in urine by flow cytometry, in tissue sections by IP, and *in vivo* by radiosciintigraphy, and are discussed in the appropriate sections below.

Another interesting approach is the enhancement of marker expression in a tumor population by the use of biologic response modifiers. For example, interferon has been shown to enhance the expression of TAA and HLA-DR antigens on the surface of human colon or breast carcinoma, but not normal, cells (GREINER et al. 1984; ROWLINSON et al. 1986). In the latter study, HLA-DR antigens were induced on the surface of 80% of formerly HLA-DR-negative breast carcinoma cells, where they then served as targets for radioimmunoimaging in a nude mouse xenograft model.

2 Diagnostic Applications of Monoclonal Antibodies to Human Tumor Antigens

2.1 Serum Assays

Tumor markers may be released into the serum by antigen secretion, antigen shedding or tumor cell death, where they may serve as markers of residual or recurrent disease, or aid in the assessment of response to therapy.

Although CEA and AFP will be covered in detail in other chapters, they are the prototypical seroassays for tumor-associated antigens; therefore a few

salient points relating to monoclonal antibody technology will be discussed below.

2.1.1 *Alpha-Fetoprotein (AFP)*

AFP is an important marker for the detection and monitoring of primary hepatocellular carcinoma (HCC) and germ cell tumors. In the past its use, particularly in populations at high risk for HCC, has been plagued by cross-reactivity of conventional polyclonal antisera with degradation products of albumin. BELLET et al. (1984) produced 2 high affinity Mabs to different epitopes on the AFP molecule that are not shared with albumin, and used these Mabs in a double determinant RIA on a very large series of sera. This impressive study showed that 80% of patients with hepatitis B antigen-positive (HBsAg) HCC had strikingly high levels of AFP, >200 ng/ml, while all normal donors, and 99% of disease controls had AFP levels of less than 20 ng/ml. These encouraging results are substantially different from those seen with conventional polyclonal antisera, in which 31–40% of patients with hepatitis and 8–33% of patients with cirrhosis show elevated levels of AFP. The ability of the Mabs to specifically detect AFP-specific, and possibly tumor-associated, epitopes therefore appears to have greatly enhanced the potential of this assay for screening for AFP-producing tumors. The Mabs themselves may be potential candidates for immunotherapy regimens.

2.1.2 *Carcinoembryonic Antigen (CEA)*

Several studies have shown the advantage of panels of anti-CEA Mabs directed against CEA-specific epitopes. One of the major drawbacks of the use of anti-CEA Mabs in immunodiagnosis, monitoring and therapy has been their cross-reactivity with several normal adult tissues, particularly polymorphonuclear leukocytes (PMN). Since "CEA" appears to be a family of isoantigens with differing properties, investigators have attempted to improve the discrimination of their Mabs by developing sets or series of Mabs detecting different epitopes of CEA (PRIMUS et al. 1983; HERLYN et al. 1983). One series of Mabs, COL 1-15 (MURARO et al. 1985), selected early in the screening process for lack of reactivity to PMN, react with at least five epitopes on the CEA molecule and are highly selective for reactivity to colon carcinomas compared to normal tissues, other carcinomas and benign gastrointestinal disease. The use of such improved Mabs may thus enhance both the sensitivity and the specificity of seroassays for CEA and other TAAs.

2.1.3 *Mab 19-9*

Mab 19-9 is directed against the sialylated Lewis^a antigen, also called GICA, a large mucin-containing antigen found in many gastrointestinal cancers. In a longitudinal study of 100 colorectal cancer patients by serum RIA, persistent or rising antigen levels preceded recurrence of their disease by 3 to 18 months

(SEARS et al. 1982). Elevated levels of Mab 19-9 were also found to be associated with pancreatic, gastric and hepatic carcinomas (79%, 57% and 49% positive, respectively), but not with benign lesions (3%) or healthy individuals (0.5%) (MAGNANI et al. 1983). The correlation between elevated levels and disease states indicates that Mab 19-9 will be useful for the serum monitoring of tumor burden in gastrointestinal cancer patients. The inability of this Mab (like others) to detect all cases of known disease indicates that it may benefit from being included in a panel of Mabs to TAA known to be expressed on such tumors. A new Mab, Co29.11, isolated by the same workers (HERLYN et al. 1985), detects a different epitope on the same antigen as 19-9. A large prospective study is now underway using a panel of Mabs to detect the 19-9, Co29.11, LNFIII and CEA antigens in serum.

2.1.4 *Mab OC125*

A highly useful new Mab is OC125, which reacts with a glycoprotein antigen (CA125) found in most nonmucinous epithelial ovarian tumors. Several large studies have shown elevated levels of this antigen by RIA and ELISA in the serum of 85–96% of ovarian cancer patients, but only 1–7% of healthy controls (BAST et al. 1983). In two separate studies, CA125 levels were found to correlate well with progression or regression of ovarian cancer (42/45 patients (BAST et al. 1983) and 18/20 patients (RICOLLEAU et al. 1984)). Unfortunately, many patients with benign diseases also may show elevated levels of CA125; in one study, 24% of patients with benign pancreatic, biliary or hepatocellular disease had such levels (HAGLUND 1986). Finally, although pancreatic, colon and breast carcinomas have also been shown to shed CA125 antigen into serum (BAST et al. 1983), the CA125 assay appears to be of limited diagnostic or prognostic importance in such cases.

2.1.5 *Other Anti-TAA Monoclonal Antibodies*

Several other antitumor Mabs, although less extensively tested, also appear to have potential clinical application. The DU-PAN-2 Mab reacts to a large mucin-like molecule distinct from the 19-9 antigen. Elevated levels of the DU-PAN-2 antigen have been demonstrated in 89% of patients with pancreatic cancer (METZGAR et al. 1984). The DF3 Mab has detected elevated antigen levels in 76% of patients with metastatic breast cancer compared to 8% of normal controls (HAYES et al. 1985).

The B72.3 Mab detects yet another large, mucin-like molecule, designated TAG-72; TAG-72 antigen expression appears to be widespread in carcinomas. In a pilot study, elevated levels of TAG-72 were found in sera from some ovarian, advanced colorectal, pancreatic and lung carcinoma patients (PATERSON et al. 1986). TAG-72 was elevated in only 35% of these patients, perhaps indicating the existence of TAG-72-positive and -negative subpopulations of these tumors. Interestingly, although B72.3 was raised against a breast carcinoma and reacts with 50% of breast cancer tissues (NUTI et al. 1982), no shed TAG-

72 antigen could be detected in sera of breast cancer patients in the study cited above. The results of this study therefore emphasize once again the need to anticipate antigenic heterogeneity within and among tumor types.

2.2 Cytology of Body Fluids

2.2.1 Flow Cytometry

Patients with residual or recurrent bladder carcinoma shed malignant transitional epithelial cells (TCC) into their urine. DNA flow cytometry (FCM) can detect aneuploid (tumor) populations, but will give a falsely low value in low grade malignancies, which have a near diploid DNA content, or if a large number of inflammatory cells is present. In patients with cystitis, or after BCG treatment for TCC, the presence of inflammatory cells in the urine poses a serious obstacle to the accurate differential diagnosis of TCC. ALEXANDER et al. (1986) have used TCC-directed Mabs in indirect IF procedures and FCM to selectively stain TCC cells, but not inflammatory cells, collected from voided urine or bladder washings. The TAA-positive TCC cells could then be sorted for further purification and study. These investigators found that the immunoselection process improved the sensitivity of TCC detection, and also that the use of TCC-directed Mabs represents an improvement over tissue-specific markers such as cytokeratin and HMFG/EMA (ALEXANDER, personal communication).

Flow cytometric techniques are also highly useful for the subclassification of leukemic phenotypes according to the expression of CALLA and other tumor markers, and have been of major importance in determining prognosis and appropriate treatment strategies for leukemic patients (reviewed in BERNARD et al. 1984).

2.2.2 Immunocytochemistry

Malignant cells in serous effusions of pleura, pericardium and peritoneum are often exceptionally difficult to differentiate from reactive mesothelial cells; once identified, the source of the malignancy may remain obscure. The ability of Mabs to detect even a single cell expressing Mab-defined markers offers the possibility of improving the detection and classification of these cells. For example, the F36/22 Mab has been used to detect malignant adenocarcinoma, but not reactive mesothelial cells, in ascites from ovarian adenocarcinoma patients (CROGHAN et al. 1984). Additionally, a pool of breast cancer Mabs could distinguish breast, ovarian and lung carcinoma cells from reactive mesothelial cells in an IF assay (TAGLIABUE et al. 1986).

Despite the growing number of cases of malignant mesothelioma, there is currently no positive Mab marker available for this disease, and the best method for its diagnosis is controversial (reviewed in SHEIBANI et al. 1986). The immunological diagnosis of such tumors now largely rests on their failure to react with Mabs directed against lung, breast, ovarian or other carcinomas. The B72.3 (SZPAK et al. 1986) and HMFG Mabs (WRIGHT, unpublished observations) seem

especially suited for this purpose. Again, because none of the Mabs react with 100% of carcinomas, the use of a panel of Mabs has the potential to improve the sensitivity of discrimination. Such Mabs might also be used for the immunocytochemical differential diagnosis of cells in sputum.

2.2.3 Immunohistopathology

Severe morphological distortions can make interpretation of fine needle aspiration biopsy material difficult. The use of Mabs such as B72.3 have been shown to be of value in distinguishing between adenocarcinomas and benign lesions of breast, lung and pancreas (JOHNSTON et al. 1986). It is probable that the application of other TAA-directed Mabs will have similar potential to improve the accuracy of diagnoses based on fine needle aspiration biopsy material.

The most prominent use of Mabs to tumor markers today lies in the immunohistopathological detection of malignant cells in tissue sections. Recent reviews have described the use of Mabs to tumor markers for this purpose (BOROWITZ and STEIN 1984; WRIGHT 1984), and several of the chapters in this volume describe current work with both monoclonal and polyclonal antibodies in detail. The brief discussion below describes the use of Mabs in prostate cancer to exemplify 1) turning cross-reacting epitopes to advantage and 2) the use of a panel of Mabs to improve the detection of malignant cells in tissue sections.

WAHAB and WRIGHT (1985) have shown that the Leu-7 marker, although directed against natural killer cells, also reacts with an antigen present on cells of normal, benign and malignant prostate origin. Unlike other organ-specific markers such as prostate-specific antigen, the Leu-7 marker was not lost on any of the metastatic tumors examined. Therefore, anti-Leu 7 may be useful in the diagnosis and prognosis of prostate cancer.

Since no tumor-selective anti-prostate Mabs are available, the routine immunohistochemical diagnosis of prostate cancer currently relies on reactivity with the organ-specific prostate markers prostatic acid phosphatase (PAP) and prostate-specific antigen (PA). However, not all tumors express these antigens, particularly poorly differentiated tumors, and heterogeneity of staining is observed. WAHAB (1985) examined a series of normal prostate, benign prostatic hyperplasia and primary and metastatic prostatic carcinomas, using a panel of 7 anti-prostate Mabs in IP studies on formalin-fixed, paraffin embedded tissues. The panel included PAP, PA, CEA, NCA, Leu-7, and two new anti-prostate Mabs designated TURP-27 and TURP-73 (STARLING et al. 1986). No single Mab in the panel could detect all cases of prostate carcinoma; however, the collective use of TURP-27, Leu-7, anti-PAP and anti-PA permitted the correct identification of all specimens.

2.3 Radioimmuno-detection (RID) of Cancer

The creation of site-specific radiopharmaceutical probes by coupling antibodies to radionuclides has the potential to improve the diagnostic capabilities of conventional imaging techniques in the detection of very small tumors, in determin-

ing the tissue origin of a mass, or in distinguishing tumor recurrence from post-operative changes. Further, there is the very real possibility of individually tailoring immunotherapy regimens, by using the same Mab to track a tumor and to treat it (see Sect. 3.2.3).

It has been clearly established that Mabs detecting human tumor markers can specifically localize in human tumor xenografts in model systems. Mabs to tumor markers found in carcinomas of the breast, colon and rectum, lung and ovary, as well as melanoma and osteogenic sarcoma have been intensively investigated and some have now gone on to clinical trials. Considerable clinical experience has been gained with Mabs HMFG-2, 791T/36, B72.3, 19-9, Col17-1A (specificities given elsewhere in text), T-101 (anti-T cell), and several anti-CEA and anti-melanoma Mabs (for review, see PRIMUS et al. 1984; LARSON 1985; BALDWIN and BYERS 1985).

In the few short years since MACH et al. (1981) successfully detected colorectal cancer sites in patients scanned with ^{131}I -anti-CEA Mabs, understanding of the complex interactions between Mab, antigen, tumor, host and imaging technology necessary to produce useful tumor imaging *in vivo* has advanced tremendously. As an example of the complexity involved, a recent multicenter study used $\text{F(ab}')_2$ fragments of the anti-melanoma Mab 225.28S in RID of 254 patients. These investigators confirmed the influence of tumor size, site, blood flow and antigen density, and route of Mab injection, and also demonstrated effects of the isotope ($^{99\text{m}}\text{Tc}$ vs. ^{111}In) and the clinical stage of the patients on the results of the scans (SICCARDI et al. 1986). This study also provides a typical illustration of both the power and the problems associated with the use of RID: occult lesions were visualized in many patients, and most of these were confirmed to be melanoma by other means; however, nonspecific radioactivity accumulated in bone marrow, spleen, liver and kidneys, in some cases limiting the detection of tumor sites.

Some problems which have yet to be satisfactorily resolved are 1) high blood background, 2) nonspecific uptake in normal organs, 3) low absolute levels of labeled Mab in the tumor, and 4) the fact that not all antigen-positive tumors can be imaged by a given Mab directed to that antigen. Solving the first two problems is probably a matter of technical improvements, for example the use of second antibody clearance (SHARKEY et al. 1984) to reduce blood background. There is not enough data at present to say whether the third problem reflects a true physiological limit. Unfortunately, the last problem has serious clinical consequences, implying that each patient must be tested individually, and should temper enthusiasm with the realization that not every patient will be able to be imaged and/or treated with any Mab directed against antigen(s) found on his or her tumor.

In a similar vein, various investigators using different systems regularly disagree about whether the optimal isotope to use is ^{111}In , ^{131}I , ^{125}I , ^{123}I or $^{99\text{m}}\text{Tc}$. The failure to agree on a single isotope is a reflection of the cardinal rule in RID: each Mab and tumor system configuration must be individually optimized.

In an exciting recent advance in instrumentation, the advent of a hand-held gamma probe has now made it possible to detect tumor cells intraoperatively,

for example in the selective detection of ^{125}I -Co17-1A in subclinical tumor masses compared to surrounding normal tissues (MARTIN et al. 1986).

In an exciting advance in Mab and labeling technology, bifunctional Mabs have been generated which have one combining site specific for antigen and the other site specific for haptan. GOODWIN et al. (1986) have used such a bifunctional antibody to label Mab in vivo. By separately administering Mab and isotope, this new technique has the potential to decrease blood background and to allow the use of short-lived radionuclides such as $^{99\text{m}}\text{Tc}$ to be used with Mabs which take 24–96 hours or more to localize at the tumor site.

3 Immunotherapeutic Applications

One of the most difficult problems in conventional cancer therapy is how to eradicate malignant cells while sparing normal ones. The advent of highly specific monoclonal antibodies has brought with it the possibility of greatly improving the selective targeting of cytotoxic agents to cancer cells, and thus decreasing their systemic toxicity. Some of the issues in Mab-based immunotherapy which must be addressed are: Mab specificity, cross-reactivity, and immunogenicity; antigenic heterogeneity; target cell access; conjugate stability; delivery of the toxic effector(s) within appropriate range of the target, and the susceptibility of the target to cytolysis by the effector.

3.1 Cytotoxic Mabs

Many Mabs have the ability to kill targets expressing their antigen in vivo, either directly or by the activation of host effector mechanisms. In clinical trials, regression of metastatic malignant melanoma lesions were observed in 4 of 21 patients following therapy with a complement-fixing IgG3 Mab, R₂₄, which recognizes the GD3 ganglioside antigen (HOUGHTON 1986). Inflammation and complement deposition at tumor sites were seen (HOUGHTON et al. 1985). The R₂₄ Mab also activates human effector cells, i.e. is active in antibody-dependent cell-mediated cytotoxicity (ADCC) and binds to a subpopulation of T8⁺ lymphocytes (HERSEY et al. 1986). The cytodestruction of tumors in vivo by this Mab appears to result from all these activities, and might be enhanced by the addition of a second antibody directed at a distinct epitope on the same (GD3) or a closely related (GD2) antigen (HELLSTRÖM et al. 1983); such studies are underway.

Murine IgG3 and IgG2a Mabs are particularly efficient activators of ADCC (HERLYN et al. 1985). An IgG2a Mab, Co17-1A, directed against gastrointestinal adenocarcinomas, has been shown to mediate ADCC in vitro and in vivo by the activation of mouse/human monocytes and killer cells (HERLYN and KOPROWSKI 1981). Mab 17-1A has now been used in several early clinical trials (for review, see STEPLEWSKI 1986), and some patients have shown objective responses to treatment. WEINER et al. (1986) are currently pretreating colorectal and pancreatic carcinoma patients with gamma interferon, in an attempt to

increase Fc receptor expression on peripheral blood monocytes and thereby potentiate the anti-tumor ADCC activity of Mab 17-1A.

These early clinical trials have clearly shown that, 1) while the administration of a single dose of murine Mab is safe, a significant fraction of patients develop human anti-murine antibodies (HAMA); 2) HAMA may be directed against the murine Fc portion, in which case chimeric or human Mabs would decrease the incidence of HAMA, or against the antigen-binding site (idiotype); 3) HAMA have the potential to cause severe allergic reactions, but some, especially anti-idiotypic antibodies, may bode well for the patient by mimicking the tumor antigen and provoking a beneficial immune response to the tumor (HERLYN et al. 1986); 4) only a very small percentage of administered Mab actually localizes to tumor deposits; 5) the number of binding sites on the tumor occupied by Mab correlates with the effectiveness of Mab-mediated tumor destruction; 6) at least partly due to poor Mab binding and to antigenic heterogeneity, the degree of tumor burden in these patients precludes the possibility of eradication of the tumor by Mab-based immunotherapy, and 7) it therefore seems unlikely that unmodified Mab alone will be successful in immunotherapy. When preliminary trials to establish the performance parameters of Mab alone are farther along, further trials incorporating Mab-based immunotherapy as part of a multimodality treatment regimen would seem to be in order, especially in patients with minimal residual disease.

3.2 Mab Conjugates

Most current immunotherapeutic strategies use Mabs as tumor targeters (war-head) only, letting the Mabs deliver other effectors (missiles) to the site of action. Most effectors now in use are drugs, toxins or radionuclides.

3.2.1 Mab-Toxin Conjugates

The use of toxins has proven to be very difficult to translate from theory into practice. Mabs conjugated with intact ricin and the subunit ricin A-chain are being intensively investigated, and have been shown to be active both in vitro and in animal models. However, at present their in vivo clinical use does not yet appear promising. Intact ricin-Mab conjugates are highly toxic to normal cells and there are problems with the in vivo stability and delivery into target cells of ricin-A chain-Mab conjugates. In contrast, the ex vivo use of immunotoxins for cleansing the bone marrow of tumor cells prior to autologous grafting or of mature T cells prior to allogeneic grafting seems very promising indeed (SAÄRINEN et al. 1985).

3.2.2 Mab-Drug Conjugates

Many biologically active Mab-drug conjugates have been prepared, most notably with adriamycin, daunomycin, vindesine (VDS) and methotrexate (MTX), and these have been analyzed both in vitro and in vivo in nude mouse xenograft model systems. The anti-osteogenic sarcoma Mab 791T/36 has been extensively

tested with different agents and shows great promise in model systems (EMBLETON et al. 1984). VDS conjugates with 791T/36, antimelanoma Mab 96.5 and anti-CEA 11.285.14 and 14.95.55 Mabs (ROWLAND et al. 1985), as well as MTX conjugated to an anti-prostatic acid phosphatase Mab (DEGUCHI et al. 1986), have been able to retard or suppress growth of the appropriate tumor xenografts. In the VDS conjugate study above, effective doses of free drug were highly toxic, whereas conjugation with Mab decreased drug toxicity but not effectiveness. The MTX conjugate study was hampered by conjugate uptake in normal liver and spleen. The results of these studies and others emphasize that: 1) delivery of drug into the target cell is crucial for most current anti-cancer drugs, but most Mabs bind to stable (non-internalized) surface antigens; 2) drug conjugates require very high levels of antigen binding to produce cytotoxic effects; 3) such high levels may often not be achievable due to differences in antigen expression; 4) preferential targeting of drug to tumor by specific Mab does not prevent conjugate uptake in normal organs and tissues; 5) antigenic heterogeneity will continue to pose a problem for tumor eradication, since only cells binding drug conjugates will be killed; and 6) drug resistant populations introduce another form of "antigenic" heterogeneity which must be dealt with. Nevertheless, many of these problems appear amenable to solution, perhaps with novel techniques such as hybrid Mab-toxin conjugates and drug potentiation (WEBB et al. 1986).

3.2.3 *Mab-Radionuclide Conjugates*

Mab-radionuclide conjugates currently appear to have the most immediate immunotherapeutic application. The ability to deliver cytotoxic doses of radiation to cells without entering (internalizing) or even binding to them, i.e. the ability to produce radiocytotoxicity over several cell diameters, suggests that Mab-radionuclide conjugates may prove to be capable of dealing with antigenic heterogeneity. (Of course, it also suggests that non-specific uptake will be a critical limiting factor for this modality, as it is for RID, see Sect. 2.3.) Further, the prospects for using RID with the same Mab to determine 1) prior to therapy, the biodistribution of labeled Mab in that particular patient, and 2) post-therapy, the tumor response, suggest that radioimmunotherapy may be more amenable to individualization than other forms of treatment.

While tumor xenografts have been completely eradicated with radioimmunotherapy (CHEUNG et al. 1986; LANGE et al. 1985), it is too early to properly evaluate clinical trials of Mab-radionuclide conjugates, as these have been conducted on only a small number of patients to date. Nevertheless, the results are encouraging, as objective responses have been seen in melanoma patients treated with ^{131}I -anti-p97 Mabs (CARRASQUILLO et al. 1984) and in lung, ovarian and breast cancer patients with effusions treated with ^{131}I -HMFG2 Mab (PECTASIDES et al. 1986), for example. Intracavitary Mab administration in the latter group of patients was not associated with toxicity, in contrast to bone marrow toxicity reported by the former group following intravenous administration of labeled Mab.

Table 1. Possible solutions to current problems with monoclonal antibody therapy

Problem	Possible solution
Tumor heterogeneity	<ol style="list-style-type: none"> 1. Use a mixture of monoclonal antibodies reacting to different antigens or epitopes. 2. Use monoclonal antibodies conjugated to radionuclides that emit radiation beyond a single cell.
Antigenic modulation	<ol style="list-style-type: none"> 1. Use monoclonal antibodies reacting to stable membrane antigens. 2. Use a mixture of monoclonal antibodies reacting to different antigens or epitopes.
Human anti-mouse antibodies (HAMA)	<ol style="list-style-type: none"> 1. Induce tolerance with large doses of monoclonal antibody. 2. Prevent development of HAMA by use of human or chimeric human/mouse antibodies. 3. Remove HAMA with plasmapheresis and immunoabsorption.
Blocking antigen	<ol style="list-style-type: none"> 1. Remove TAA and TAA-antibody complexes by plasmapheresis and immunoabsorption.
Unmodified monoclonal antibody lacks cytotoxicity	<ol style="list-style-type: none"> 1. Use of monoclonal antibodies conjugated to radionuclides, drugs, enzymes, or toxins. 2. Class switch the monoclonal antibody to IgG_{2a} or IgG₃. 3. Make a chimera with an appropriate Fc region. 4. Make heterobifunctional monoclonal antibodies with cytotoxic properties and original antigen binding site.
Nonspecific monoclonal antibody uptake	<ol style="list-style-type: none"> 1. Block with cold monoclonal antibody.

Extensive experience with ¹³¹I-anti-ferritin polyclonal antibodies has shown dramatic clinical responses in 40–50% of patients with hepatoma and ferritin-positive Hodgkin's disease (ORDER 1985). Interestingly, these workers found monoclonal anti-ferritin to lack efficacy in their hands, due to dehalogenation of antibody. In this regard, beta-emitters such as ⁹⁰Y are being actively investigated, as they bind stably, can be administered on an outpatient basis, and are expected to reduce systemic toxicity.

Problem areas to be addressed and prospects for radioimmunotherapy have recently been discussed (COBB and HUMM 1986; EPENETOS et al. 1986). Possible approaches to improving current techniques in Mab-based immunotherapy in general, directed at specific problems encountered today are listed in Table 1. It will be noted that many of these approaches are currently under investigation, while others are impractical at the moment, given the current state-of-the-art.

4 Conclusions and Outlook

The increased sensitivity, specificity, and reliable, homogeneous binding properties of monoclonal antibodies have contributed to great improvements in many conventional, polyclonal antibody-based diagnostic techniques. In addition, the ability of Mabs to detect a single epitope on complex molecules has allowed

the detection and characterization of many new and useful tumor markers which have served to define, track and manipulate tumor cells. There is every reason to expect that, with a better understanding of the mechanisms of growth, differentiation and carcinogenesis, a new generation of Mabs defining more accurate tumor markers will become available. Meanwhile, heterogeneity in the expression of many of the presently defined tumor markers remains a difficult problem to overcome, but the use of panels of Mabs of differing specificity is a promising approach.

In the near future, the ability to manipulate Mab structure in the production of bifunctional Mabs may allow the generation of families of "all-purpose reagent" Mabs in which the antigenic specificity of the Mab remains the same, but different reagents are linked to it for use in different techniques or to create an additive effect. Examples might include a radionuclide for in vivo diagnosis and an enzyme with a colored substrate for in vitro (IP) diagnosis; or, in a form of "multimodality" immunotherapy, the same Mab could carry both drug and toxin to the cell.

Advances in gene cloning and transfer techniques have already allowed the production of chimeric antibodies, and promise to lead to increasingly engineered forms of Mab reagents. Within the constraints of gene expression, protein folding and antigenic specificity, it should be possible to create sets of Mab probes with differing specificity and function. These statements are made with the caveat that it is important to temper one's expectations, in order to avoid the roller coaster of too-high expectations followed by deep disappointments that has long plagued tumor immunology. The roller coaster effect results from the overly optimistic belief that all things theoretically possible are in fact practically possible, and has proved difficult to resist in the past.

Nevertheless, despite these caveats and the known limitations of today's Mabs, monoclonal antibodies to tumor antigens have opened up new vistas; in all probability new uses for them have yet to be discovered.

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Biochemical Markers for Human Cancer

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1 Introduction

Development of biochemical markers for human cancer in recent years that have been shown to be of some clinical value in management of patients will be presented. Previous approaches of using conventional immunochemical techniques to the measurement of target markers still dominate the area of laboratory investigation and clinical application. However, in addition to radioimmunoassay and enzyme linked immunosorbent techniques, immunohistochemistry, flow cytometry and new molecular biology procedures are increasingly being

utilized. Also, tissue and body fluid other than serum are acquired as the specimens for measurement of these markers.

Tumor markers are ideally to be as the indicator for the presence of preneoplastic and neoplastic diseases. Cancer is described as a disorder of cell proliferation. Neoplastic cells basically are different from their normal counterparts in its failure to respond to growth control. At the present stage of development no qualitative tumor markers, so called tumor-specific markers, have been identified. Rather, quantitative difference between normal and neoplastic cells is used as the index.

Almost without exception, monoclonal antibody is the choice of reagent in immunochemical techniques, although it should be kept in mind that polyclonal antiserum reagent of proved value and quality can be equally effective. Typical advantages of monoclonal antibody are standardization of test reagent and consistency of quality control. At present no marker for general screening of cancer is yet in existence. Most of the clinical utilizations are in patient management of post-diagnosis, with a few that are potentially applicable in early detection of cancer for high risk population. During recent years, with the use of monoclonal antibodies, many new tumor markers have also been recognized, characterized and evaluated for clinical applications.

This chapter aims primarily to discuss and emphasize recent development of tumor markers in *in vitro* clinical applications.

2 Tumor Associated Antigens

2.1 Carcinoembryonic Antigen

Although carcinoembryonic antigen (CEA) was first reported more than twenty years ago (GOLD and FREEDMAN 1965), it is still the most extensively investigated tumor marker with the largest number of publications. Recent review articles are readily available (ROGER 1983; STEELE and ZAMCHECK 1985; BEARD and HASKELL 1986; FLETCHER 1986), only a few recent aspects will be discussed.

Complete chemical structure of CEA is still unknown. Using conventional biochemical and chemical techniques, CEA molecule has been characterized partially. Elucidation of the total amino acid sequence of the peptide portion has been tried but to no avail. Modern techniques of molecular biology to sequence the nucleotides encoded for the amino acids would be a more effective approach. The microheterogeneity in carbohydrate portion of the molecule still would be a formidable problem. A recent interesting piece of information regarding the molecular structure of CEA is that CEA is composed of two identical subunits (LISOWSKA et al. 1983). Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and cross-linking experiments, CEA has been shown to be a dimer consisting of two identical or closely similar non-covalently bound subunits. CEA is dissociated upon heating in the presence of sodium dodecyl sulfate, and the dissociation is reversible upon the removal of the detergent. Subunits of CEA are not linked by way of the disulfide bonds, but rather by the hydrophobic interactions between the subunits and among peptide chains.

Should this result be confirmed it may simplify the work on characterization of the entire CEA molecular structure. In spite of the extensive clinical evaluation of CEA, biological function, if any, of CEA still remains totally unknown.

More than twenty years since CEA was first reported, clinical usefulness and limitation of CEA have been mostly assessed with very few outstanding clinical issues. The role of CEA in second-look surgery is one that awaits definitive information. Although information is available concerning this critical subject, with some describing favorable data and some expressing reservation; none has been really systematically and statistically analyzed. One report describing an extensive investigation just became available recently (MINTON et al. 1985). This study was initiated by the Society of Surgical Oncology in 1978. It involved 400 patients in a nonrandomized prospective study to evaluate the usefulness of CEA as an indicator of recurrent colon and rectum cancer, as conducted by 37 surgical oncologists in 31 institutes across the USA. Patients were evaluated post-operatively with customary follow-up procedures and serial CEA determinations. During follow-up, 130 patients had recurrence (21 Dukes' B, 8 B₂, 31 C₁, 58 C₂ and 12 unknown), and 75 were reported on, with 43 reoperations were CEA-directed and 32 clinically directed. Two of these 75 patients died within one month after the second surgery. Twenty-two second-look patients remained disease-free five years after their second operation. The highest resectability of recurrent cancer occurred in patients with a CEA level less than 11 ng/ml, in whom the CEA was assayed at intervals of one to two months.

Four important guidelines for follow-up management were proposed from this study. For Dukes' A carcinoma, there may be no justification for a CEA follow-up when the tumor is resected, since no patient in this group (a total of 17 patients) developed recurrent disease. CEA follow-up is recommended for patients with Dukes' stages B₁ to C₂ patients, since about 25% of B₁ (21/91), B₂ (18/31) and C₁ (31/119) carcinomas and 50% of C₂ patients (58/122) developed recurrent disease. CEA determinations should be performed at one- or two-month intervals, since this practice offers the best opportunity for early discovery of resectable recurrent cancer during the first and second years of post-operative follow-up. Reoperation should be performed before the CEA exceeds 11 ng/ml, since patients with CEA less than this level have shown the greatest resectability rate (63%) and the best five-year disease-free survival results (68%).

Of interest to note is the observation that there is no significant difference in overall cumulative surviving between CEA directed and clinically directed second-look surgeries. A previous report has described similar observation regarding disease-free interval and survival (HINE and DYKES 1984). This may be due to the fact that there is still no effective therapy for colon/rectum cancer. Therefore, the most effective use of CEA in management of patients will be in those where potentially effective therapeutic manipulation is available, such as small cell carcinoma of the lung.

Investigations on other subjects of CEA, such as assays, screening, diagnosis, prognosis and monitoring, including that of *in vivo* radioimmuno-detection, have provided no dramatically new information in recent years. With the use of monoclonal antibodies, at least five different antigenic determinants (epitopes) have been identified in CEA molecule (MURARO et al. 1985). Whether these CEA-reactive monoclonal antibodies could improve the clinical effectiveness of CEA assay remains to be determined.

A refined CEA assay was reported a few years ago that showed an improvement in the specificity of serum CEA for carcinomas (HEDIN et al. 1983). Using two monoclonal antibodies specific for two separate CEA epitopes, an enzyme-linked immunosorbent procedure was developed and shown to be quite specific in detecting CEA levels in serum specimens from patients with solid tumors.

So far, no further reports on a large scale specimens including patients with early cancer have been available.

2.2 Alpha-Fetoprotein

In comparison with CEA, we know much more about α -fetoprotein (AFP) (HIRAI 1982). The primary structures including the complete sequence of 590 amino acids of human AFP have been elucidated (MORINAGA et al. 1983; SAKAI et al. 1985). Its clinical use in primary hepatocellular carcinoma and in testicular tumors of germ-cell origin (along with human chorionic gonadotrophin) is well known (NORGAARD-PEDERSEN 1979). AFP also is a potential prenatal diagnostic tool for neural-tube defects. For reasons that are not scientifically justified, full diagnostic application of this clinically useful marker unfortunately has not been achieved (SELL 1981).

The most important clinical utilization of biochemical marker for cancer is its use as a screening test for cancer, either in general populations or in a high risk population. No such marker is yet available. The best candidate marker potentially for such a use in fact is AFP. An elevated serum AFP in apparently healthy adult (non-pregnant) is the single most discriminating marker indicative of cancer (SELL 1981). AFP has been a well known diagnostic marker associated with primary hepatocellular carcinoma for over twenty years. Simple laboratory tests are available with the use of either polyclonal or monoclonal antibody reagent.

Several studies using AFP as a screening tool for high risk population have been conducted in China, Southeast Asia and Africa, where the incidence rate of primary hepatocellular carcinoma is highest in the world (SELL 1981). When a tumor marker is used for cancer screening, the factor of cancer prevalence as well as specificity and sensitivity of the assay should be considered (WAGENER 1984). As far as AFP is concerned, infection with hepatitis B virus also needs to be taken into account, since highest rates of hepatitis B infection and primary hepatocellular carcinoma are commonly found in the same populations. Therefore, should an AFP test be of screening value, it should be able to discriminate, at least quantitatively, primary hepatocellular carcinoma from patients with hepatitis B infection and other non-malignant hepatic diseases, such as cirrhosis.

In one study conducted in China, where almost a half million asymptomatic individuals were screened with AFP test, 57 were identified to have a serum AFP of greater than 500 ng/ml. All 57 of these apparently healthy individuals were subsequently diagnosed to have liver cancer. In a later study involving 115,000 Chinese, the results were not so drastically definitive. Although 20 individuals having high AFP levels were identified to have primary hepatocellular carcinoma, another 270 individuals had slightly elevated levels of AFP and none was found to have evidence of cancer. It is not clear whether these two studies were conducted in the same region or different pocket areas where incidence rate of liver cancer could be different.

As indicated previously, when AFP is used as a screening cancer test, the prevalence of cancer is a critical factor. This can be best illustrated by a study conducted in Germany, where liver cancer is not common. A highly elevated serum AFP level, greater than 2,000 ng/ml, was found in 10 of 18 patients with primary hepatocellular carcinoma, who also had cirrhosis of the liver. In addition, among 18 patients with primary hepatocellular carcinoma, 13 were found to have an AFP of greater than 400 ng/ml; but also were 14 of the 385 patients who had cirrhosis of the liver without liver cancer (AFP ranged 400–2,000 ng/ml) (LEHMANN and WEGENER 1979).

In a recent report screening an entirely different population for early detection of primary hepatocellular carcinoma, the results were quite encouraging (HEYWARD et al. 1985). The subjects of screened population were Alaskan Natives who had high rates of primary hepatocellular carcinoma and hepatitis B virus infection. In addition to screening, it was also to determine if primary hepatocellular carcinoma could be detected at an early stage at the time when tumor could be surgically removed. Serum AFP was tested semiannually by an ELISA (AUSZYME II) among 1,394 Alaskan Natives who were infected with hepatitis B virus tested by an RIA (AUSRIA) for hepatitis B surface antigen (HBsAg). A total of 3,387 AFP tests were performed among these natives during a 26-month period (1982–1984). Of 126 persons with elevated AFP (> 25 ng/ml), nine males were found to have primary hepatocellular carcinoma and all had an AFP of > 350 ng/ml. Six of these nine persons were asymptomatic and four had small tumors (< 6 cm) that were surgically resected.

It is interesting to note that of all 602 HBsAg-positive females, no cases of primary hepatocellular carcinoma were found in 107 persons who had at least an elevated AFP test. Most elevations of AFP could be attributed to pregnancy. Of the 792 HBsAg-positive males screened, 19 (2.4%) had at least an elevation of AFP, and 9 were found to have primary hepatocellular carcinoma with 6 asymptomatic at the time of the blood test as noted already. Three of these six were detected in a prospective manner, i.e. all had normal AFP 6 to 12 months before their first elevation of AFP. In all six patients, the AFP level increased very rapidly, doubling every 1 to 3 months.

This is by far the most well controlled study indicating that AFP can be used as a screening test for primary hepatocellular carcinoma in a high risk population with hepatitis B virus infection. It also reveals that early stage of surgically resectable tumor can be detected by a simple AFP test.

As illustrated from the reports presented above, should a tumor marker be used as an aid for diagnosis of cancer, it should be able to provide a parameter to differentiate malignant neoplasm from benign disease, and also to identify an early resectable tumor which can be successfully treated. In these respects, AFP certainly is a clinically useful diagnostic marker for primary hepatocellular carcinoma.

In addition to primary hepatocellular carcinoma, AFP in combination with human chorionic gonadotrophin (HCG) can be used effectively for clinical diagnosis of testicular tumors of germ cell origin. Circulating AFP and HCG have been reported to correlate with histology of testicular tumor. It has been well documented that elevated serum AFP is associated with tumors containing endodermal sinus, yolk sac elements and HCG with choriocarcinomatous elements or syncytial giant cells. In fact, these markers are so well studied that recommendations have been made that every patient with a testicular tumor should have a preoperative determination of serum AFP and HCG, then at least twice weekly in the first six weeks after operation (NORGAARD-PEDERSEN 1979). This also may apply when a germ-cell tumor of ovary is suspected. It should be noted, though, that combined AFP and HCG tests still would miss a small portion of patients with germ cell tumors of the testes. On the patients who were found to exhibit measurable and elevated AFP and/or HCG, the levels and rates of change of AFP and HCG in the postoperative follow-up, and during and after chemotherapy, can provide additional information on the course of disease.

Both CEA and AFP are well known biochemical tumor markers and tumor associated antigens. The term antigen is more of an operational use than that of classical immunological terminology. Since, as far as human hosts are concerned, the presence of anti-CEA or anti-AFP antibody has not been unequivocally documented. In recent years with the availability of an enormous amount of monoclonal antibodies generated, many previously unrecognized tumor associated antigens and components have been reported. Some of them have been used in cancer management especially patient monitoring. Only those that have been reported extensively will be presented here.

2.3 CA 19-9 Antigen

CA 19-9 is a monoclonal antibody-defined tumor marker representing a carbohydrate antigenic determinant of glycoprotein and glycolipid substances (DEL VILLANO et al. 1983). Like other tumor associated antigen markers, low concentration of CA 19-9 is found in healthy individuals, but frequently increased in sera from patients with gastrointestinal adenocarcinomas, especially those of the pancreas. Similar to that of CEA, CA 19-9 is detected in fetal, normal and neoplastic gastrointestinal tissues.

Serum CA 19-9 measurement has been shown to be of more clinical value, i.e. sensitivity and specificity, than CEA in pancreatic cancer (STEINBERG et al. 1986). Serum levels of CA 19-9 also has been reported to assist in the differentiation of chronic pancreatitis from pancreas cancer. Although it has been claimed that early localized pancreatic adenocarcinoma can be detected by the measurement of CA 19-9, this interesting and clinical application remains to be documented with further study (GUPTA et al. 1985). The test kit of CA 19-9 is commercially available. So far, no tumor marker has been available that can identify patients with early and resectable pancreatic tumor.

2.4 CA 125 Antigen

CA 125 is an ovarian cancer associated mucin-like antigen as recognized by a murine monoclonal antibody (BAST et al. 1981). Its clinical use in ovarian cancer is by a radioimmunoassay test, and the kit is available commercially. In the initial clinical report (BAST et al. 1983), only 1% of apparently healthy blood donor controls and 6% of benign disease patients were detected to have an elevated serum CA 125 level. Significantly, 82% of patients with ovarian cancer exhibited an elevated level, some with extremely high levels. Serum CA 125 levels appear to correlate with tumor burden and stages of disease. Twenty-nine percent of patients with non-gynecological cancers also exhibited an elevated CA 125 level, especially those with pancreatic cancer (59% of 29 patients).

Like most other tumor markers, serum CA 125 is more useful in monitoring the course of epithelial ovarian cancer. CA 125 has been reported to be effective in monitoring the progression or regression of disease in more than 90% of

ovarian cancer patients. For common epithelial ovarian cancers, serum CA 125 has been reported to be the most valuable marker for disease management, better than CEA and CA 19-9. As discussed previously, AFP and HCG are useful for ovarian germ cell tumor similar to testicular germ cell tumors, while choriocarcinoma may be managed by HCG monitoring.

A murine monoclonal antibody, F36/22, has been shown to identify 100% of epithelial ovarian cancers as examined by tissue immunostain techniques (CROGHAN et al. 1983). Our unpublished data reveal that more than 95% of patients with active epithelial ovarian cancers exhibit an elevated serum F36/22 antigenic components, while patients without evidence of disease have normal range of antigen level. Pregnant women and patients with non-epithelial ovarian cancers, benign ovarian and benign gynecological conditions all have normal blood levels. Preliminary study involving serial specimens also reveals that F36/22 may be an effective marker for monitoring therapy and recurrent disease in epithelial ovarian cancer patients. Neither CA 125 nor F36/22 has been shown to be of clinical value for the early detection of common epithelial ovarian carcinomas.

3 Organ-Site and Cell-Type Specific Antigen

AFP, CEA, CA 19-9 and CA 125 are tumor associated antigens and clinically useful in the sense that their quantitative expression is utilized as an aid to differentiate neoplastic disease from normal or benign conditions. There is another class of substance that is organ-site and cell-type specific and has been used effectively as tumor marker. The best example is a human prostate-specific antigen, commonly abbreviated as PA or PSA (WANG et al. 1979). In fact, PA has been approved by the Food and Drug Administration (USA); the first tumor marker to receive such an approval since CEA was approved in the early 1970s.

Unlike most of tumor associated antigens, PA is present at an almost equal concentration in normal, benign hypertrophic and cancerous human prostatic tissues (WANG et al. 1982). It is not present in other normal or tumor tissues. This tissue specificity is confirmed by both biochemical and immunocytochemical techniques, and by both polyclonal and monoclonal antibody reagents. In the prostate, PA is located in the ductal epithelial cells and not in other cellular components. PA is a glycoprotein of Mr 33,000 with microheterogeneity in carbohydrate contents. The complete amino acid sequence of peptide portion of PA has been elucidated (WATT et al. 1986). Despite the well-characterized physico-chemical properties, the biological function of PA is not known, although proteolytic activities have been shown to be associated with PA (BAN et al. 1984). Test kits for serum PA assay are available commercially, and that manufactured by Hybritech Incorp. has been approved by the FDA for clinical use as an aid in management of patients.

Prostatic acid phosphatase (PAP), a well known tumor marker, has been used for over 50 years to assist in diagnosis and monitoring of prostate cancer

(GUTMAN and GUTMAN 1938). From data available to date, PA is superior to prostatic acid phosphatase; and this is primarily due to the fact that PA is absolutely specific for the prostate. Although serum assay of PA alone cannot be used for early detection of prostate cancer, because of the PA levels of early stage of prostatic cancer patients are often overlapped with those with benign prostatic hypertrophy, its role in monitoring therapy and predicting recurrent disease has been firmly established. Further, combination assay with prostatic acid phosphatase may be of use in early diagnosis of prostate cancer.

Simultaneous serum assays of PA and PAP have detected more patients with prostate cancer than either assay alone (KURIYAMA et al. 1982). Combination assays have revealed a discordance between these two markers, reflecting the expression of these two biochemically and immunologically distinct prostate glycoproteins may represent different aspects on the biology of prostate cancer. Since patients with benign prostatic hypertrophy also exhibited slightly elevated levels of both markers, which can be set as the operation cut-off points for a combination test and thus decrease the false positive rate. Using this approach, data are available supporting that both assays are of additive value and suggesting that diagnosis of early stage of prostate cancer is feasible in certain circumstances.

Single assay of serum PA alone has been shown to be of prognostic and monitoring applications of prostate cancer. Data obtained from large-scale and long-term clinical evaluations have been analyzed and reported (KILLIAN et al. 1985). In patients with regionally confined prostate cancer who had received curative therapies, levels of PA are effective in predicting early recurrence of disease and are of prognostic value in disease-free survival time, irrespective of stage and therapy of patients. A single elevated PA level also has been shown to be of predictive value for recurrence of disease. For patients with early stages (B_2 to D_1) of prostate cancer with regionally confined disease, an association of increasing serum levels of PA to increasing tumor burden and decreasing time to disease recurrence is statistically established. Therapeutic manipulation based upon the PA level may improve the patients' survival in a prospective manner.

For patients with advanced prostate cancer who were receiving treatments, the levels of serum PA were correspondent with clinical courses of patients. Using patient as his own reference, PA levels in general increase as disease progresses and decrease as disease regresses. In addition for those patients with disease progression, a quantitative and significant association between the risk of progression or death and a PA level exceeding a certain elevated value has been statistically established (KILLIAN et al. 1985). Thus, the prognostic reliability of PA is firmly established.

Certainly, should a biochemical tumor marker be clinically useful and practically applicable, it should be better than existing assays for management of patients. In this respect, PA has been compared with other commonly used markers for prostate cancer and shown to be the best marker in its reliability for predicting disease progression (KILLIAN et al. 1986). Acid phosphatase, prostatic acid phosphatase, total alkaline phosphatase and its bone isoenzyme all have been shown previously to be useful in varying degrees for management

of prostate cancer (KILLIAN et al. 1981). Comprehensive statistical analyses on a large series of patients have revealed that either PA or PAP is of value in detection of disease progression in monitoring early stages of prostate cancer. Progression of disease in patients with advanced prostatic cancer can be monitored reliably by PA assay alone.

In summary, as far as serum PA in monitoring of prostate cancer is concerned, it is reasonable to state that what CEA can do for colon-rectum cancer, PA can do for prostate cancer, and may be better. Practical use of PA as an immunohistochemical marker for prostatic neoplasms is detailed in Sect. 8.

4 Steroid Hormone Receptor

An important clinical value of biochemical tumor marker is its use in staging of disease. Although CEA has been reported to be of some value in staging surgically resectable patients in lung cancer (VINCENT et al. 1979), the best marker for such a clinical utility is estrogen receptor. The importance of estrogen receptor protein (estrophilin) analysis in staging of patients with estrogen-related tumors is well known as a guide to therapy and prognosis. Biochemical assays, based upon the interaction between a radioactive estrogen ligand and the receptor protein at the receptor binding site, are commonly used. It requires sufficient amount of fresh tumor tissue and tedious labor-intensive exercise with careful quality control to preserve biologically active binding site (WITTLIFF et al. 1982). False estrogen receptor-poor results is the most common problem, which often deprives a patient from the benefit of hormonal therapy.

Monoclonal antibodies directed against distinct determinants of human estrogen receptor protein have been generated (GREENE et al. 1980), and immunoradiometric or enzyme-linked immunosorbent assays also have been developed (GREENE and JENSEN 1982). The estrogen receptor contents measured by monoclonal antibody-based assay are found to be indistinguishable from those as quantified by conventional biochemical technique, such as sucrose gradient ultracentrifugation.

A test kit of enzyme immunoassay (EIA) for estrogen receptor measurement became available recently commercially (Abbott Laboratories). Two reports evaluating this kit presented somewhat different results. In comparison with the results obtained from classical dextran-coated charcoal method, high estrogen receptor levels were detected by the EIA method in one report (RAAM and VRABEL 1986). However, another report indicated that the EIA method is valid in measuring estrogen receptor contents as compared with those as detected by established radioligand-based assays, dextran-coated charcoal and hydroxylapatite (HOLT and BOLANOS 1986). Estrogen receptor in breast, ovarian and uterine carcinomas can be accurately measured. Regardless of these results, at the present stage of development monoclonal antibody-based immunoassays will be the method of choice in the foreseeable future.

This notion has been fortified by most recent reports involving multicenter evaluation of EIA method in both Europe and the USA (LECLERCQ et al. 1986;

JORDAN et al. 1986). ER-EIA monoclonal immunoassay (Abbott) was evaluated in eleven laboratories. Results suggest that the EIA method is comparable in measuring estrogen receptor contents with conventional steroid binding assays. Routine use of this newly developed immunoassay can be anticipated in the not too distant future.

Theoretically, progesterone receptor can be quantified similarly. As of this date, no such test is yet available.

It cannot be overemphasized the clinical importance of steroid hormone receptors. It also should be noted that quality control assurance of the assay is of utmost importance in obtaining accurate laboratory results. Proficiency-testing specimens are readily available and should be performed. Availability of specific monoclonal antibody for estrogen receptor also provided a useful tool for immunocytochemical assessment of tumor tissues (McCARTY et al. 1986). Potential clinical significance of this approach is to be discussed later in Sect. 8.

5 Potential Markers for Early Cancer Diagnosis

5.1 Thyrocalcitonin

For early detection of cancer, AFP is by far the best candidate as already discussed. There is another potential marker which also has been shown to be effective, i.e. thyrocalcitonin in medullary thyroid carcinoma (COOPER 1976). Calcitonin is a 32 amino acid peptide hormone produced in non-follicular thyroidal C-cells. Physiologically it plays an important role in calcium utilization and bone resorption. It is a hypocalcemic and hypophosphatemic hormone. Data available suggest the presence of a closed-loop feedback system between the gut (gastrin) and thyroid (calcitonin) (COOPER 1976), and gastrin-calcitonin relationship in controlling the level of calcium during intestinal absorption.

The application of calcium or more conveniently of gastrin for a provocative test in early diagnosis of medullary thyroid carcinoma in high risk family members of the patients has been reported. Suspected patients with medullary thyroid carcinoma would respond to the injection of the synthetic gastrin analogue, pentagastrin, to produce a rapid and highly elevated serum level of calcitonin. Subclinical or questionable cases of this C-cell tumor have been identified by this simple manipulation which involves a provocative test of pentagastrin injection and evaluation of blood calcitonin by immunoassay. Pentagastrin injection is more commonly practiced than calcium infusion. Urine calcitonin level also has been suggested to be a reliable screening test (SILVA et al. 1979).

5.2 Fibrinogen Degradation Products

Detection of urinary fibrinogen degradation products could be a simple and potential test for diagnosis of bladder cancer. The association of urinary bladder carcinoma and elevation of urinary fibrinogen degradation products has been

known for some time (WAJSMAN et al. 1975). Recently, a monoclonal antibody-based enzyme linked immunosorbent assay of fibrinogen degradation products was reported (MCCABE et al. 1984). The specificity of the test was reported to be 96% with a false-positive rate of 5%. Although these results are not acceptable for screening use of urinary bladder cancer, it is a reasonable expectation that a better assay could be developed in the near future.

It would be worthwhile to make some comments regarding serological use of biochemical tumor markers at this point. At the present stage of scientific and technical development, the most difficult and yet the most rewarding clinical application is cancer screening for general population or asymptomatic subjects in high-risk populations. Markers of absolute specificity and sensitivity are required. The former is a dream rather than a goal; the latter is an achievable goal, as exemplified by AFP for primary hepatocellular carcinoma. More realistic is the use of biochemical tumor markers in early detection of cancer. Since target patients are those already with signs or symptoms suspected or suggestive of cancer, the requirement of specificity is differentiation of malignant from benign disease, and that of sensitivity is identification of early curable lesion from advanced metastatic tumor (HERBERMAN 1982). AFP, HCG, PA and calcitonin are potential tumor markers of this category.

Another two clinical applications of biochemical markers for cancer are their use in staging of disease, and monitoring of therapeutic efficacy and disease recurrence. Steroid hormone receptors is a good example of the former, and many tumor markers, those discussed here and others published, are proved parameters for the purposes of the latter. Therefore, at present only biochemical markers suitable for these last two clinical applications are mostly in practical use.

6 Enzymes and Isoenzymes

Enzyme has been traditionally used as a biochemical marker for cancer. Abnormality in enzyme activity is a major biochemical characteristic of uncontrolled tumor cell growth. This phenomenon has been used as a laboratory parameter in differentiation of tumor cell from its normal counterpart, and used as a target in developing therapeutic agents against cancer. The use of enzyme as tumor marker is based upon the concept that alteration in the gene expression during malignant transformation can be detected at the level of end product. A qualitative difference in enzyme expression would be an ideal tumor marker. No such cancer specific enzyme has been identified and clinically utilized. Enzymatic imbalance in tumor cells has been used instead for clinical evaluation of cancer.

Biochemical activity of the enzyme is commonly used as a parameter for tumor marker. With recent availability of immunologic reagents, mass of enzyme protein or specific antigenic determinant also has been measured. However, the measurement of an enzyme alone still cannot clinically detect a small tumor mass, since the secreted enzyme is vastly diluted or actively metabolized

in serum, urine or other extracellular fluids. Despite these inherent problems, enzyme and isoenzyme are useful probes as an aid for management of cancer patients. Some recent developments using enzymes and isoenzymes as markers for diagnosis and treatment monitoring are briefly discussed.

6.1 Prostatic Acid Phosphatase

Acid phosphatase of the prostate is the first enzyme marker for cancer. Serum acid phosphatase activity by conventional biochemical assays has been used for over 50 years as a laboratory aid for metastatic prostate cancer (GUTMAN and GUTMAN 1938). Prostatic acid phosphatase (PAP) is one of a few enzyme markers exhibiting relative organ and cell-type specificity. Although acid phosphatase of the prostate is the most well known and investigated enzyme marker for cancer, its biology in prostate or prostate cancer remains totally unknown. Information available recently has shown that PAP is related to hydrolysis of tyrosine-phosphate in phosphoprotein molecules (LI et al. 1984). Its role in oncogene and malignant transformation thus has been speculated, since tyrosinekinase is a well known product of oncogene.

PAP is biochemically a glycoprotein of 100,000 molecular weight, consisting of two dimers and approximately 10% carbohydrate and 90% peptide with microheterogeneity (i.e. multiple isoelectric points) (CHU et al. 1982; LIN et al. 1983a). N-terminal amino acid is lysine and partial sequence is also known (LIN et al. 1983b). Reagents for biochemical and immunological assays are readily available from commercial sources.

It should be noted that PAP is prostate specific rather than prostate-tumor specific. Thus, the assay alone is of limited use in distinguishing early prostate cancer from benign prostatic hypertrophy, although its clinical utilization can be enhanced by other prostate tumor markers. PAP is not suitable as a screening modality for prostate cancer.

Clinically, PAP is useful in identifying metastatic prostate cancer, and in monitoring disease status and therapeutic response of prostate cancer (CHU et al. 1982). There are some recent well-controlled reports describing the role of PAP in monitoring prostate cancer with similar though not identical results. One report found that the PAP level in some patients did decrease to normal range after successful treatment, but the incidence of decrease in the PAP level was less with increasing severity of the disease (COOPER et al. 1983). Serial longitudinal follow-ups of PAP in patients who had been treated with hormonal and chemotherapy are available. The results revealed that PAP assay is a valuable indicator for treatment response and disease progression. One study reported that although serum PAP level decreased in patients responding to hormonal therapy, only in a small number of patients responding to chemotherapy did the serum PAP decrease (ZWEIG and IHDE 1985). These data suggest that PAP is more sensitive in indicating disease progression than disease regression, and that PAP assay in general can be considered to be of value in confirming clinical assessment of patients (KILLIAN et al. 1986).

As discussed already, PAP is a useful tool in detecting disease recurrence in patients with early stage of prostate cancer who have received apparently curative therapy (KILLIAN et al. 1985). It can be used also as a prognosticator for predicting disease-free survival time. Although not as reliable as prostate-specific antigen, PAP still is a reasonably good marker for monitoring disease progression in advanced prostate cancer (KILLIAN et al. 1986). Since prostate-specific antigen is relatively new and may not be available readily, PAP then will be the choice of biochemical marker. When combined with alkaline phosphatase and its bone isoenzyme, the clinical effectiveness of PAP can be greatly enhanced (KILLIAN et al. 1981).

6.2 Neuron-Specific Enolase

Neuron-specific enolase (NSE) is another important enzyme marker of cancer. Enolase is a key enzyme involving the synthesis of ATP in glycolysis. Clinical significance of NSE is based upon its presence only in the cells associated with amine precursor uptake and decarboxylation (APUD) in the neurons of the diffuse neuroendocrine system. This unique feature provides the basis for NSE to serve as an enzyme marker for tumors of APUD or neuroendocrine origin. Highly specific antibody has been used for assay development. It should be emphasized also that NSE is not a tumor specific enzyme.

Small cell carcinoma of the lung was the first tumor shown to be associated with elevated NSE level (CARNEY et al. 1982). Even patients with limited disease (40%) exhibited mildly elevated serum NSE levels. NSE level was related to extent of disease. Virtually all patients with metastases at 3 or more sites were shown to have a highly elevated NSE. Treatment response was shown to be effectively monitored by serial NSE measurements in patients receiving combination chemotherapy. Furthermore, the source of serum NSE was identified to be originating from patients' tumor cells, as documented from continuous cell lines established from patients. These initial data suggest some interesting points. A new enzyme tumor marker has been identified. A simple serum NSE assay can be used effectively in monitoring clinical status of small cell carcinoma of the lung, one of the few cancers that can be treated effectively, and thus may contribute to overall survival of these patients. This is one exciting development in recent years using enzymes as tumor markers.

Serum NSE also has been shown to be of prognostic value in metastatic neuroblastoma. More than 95% of these patients had an elevated NSE. Patients exhibiting highly elevated NSE levels (>100 ng/ml) were generally associated with poor prognosis, especially in infants of less than 1 year old at the time of diagnosis.

Serum NSE, when combined with pancreatic polypeptide and HCG, also could be useful biochemical marker for determining prognosis and monitoring therapy response in pancreatic islet cell tumors and carcinoid tumors of the gastrointestinal tract. NSE is usually elevated in nonfunctioning pancreatic islet cell tumors, but less frequently in glucagonomas and intestinal islet cell tumors.

Additional clinical use of NSE is its diagnostic application in immunohistopathology of these tumors (KLOPPEL et al. 1983).

6.3 Galactosyltransferase

During the past ten years, the potential importance of glycosyltransferases in cancer has been extensively investigated. Data available indicate that their role in tumor metastasis is still largely speculative and that their role as tumor markers has been shown to be of some value. Glycosyltransferases are involved in the transfer and addition of a specific carbohydrate from a donor to a specific protein or glycoprotein acceptor. Galactosyltransferase, sialyltransferase and fucosyltransferase are more investigated than others in clinical oncology. These three enzymes catalyze the addition of galactose, sialic acid and fucose, respectively, to their specific acceptors.

A variety of cancer patients have been shown to exhibit elevated serum total galactosyltransferase activity (SCHWARTZ 1982). Like many other non-specific enzyme markers, more patients with advanced disease have elevated value than patients with early staged disease. Its primary use is in monitoring and prognosis (DAVEY et al. 1986). Also like other enzymes, serum galactosyltransferase activity is the sum of many isoenzymes. One isoenzyme, designated galactosyltransferase II (GT-II), has been identified to be primarily associated with cancer. An initial paper indicated that serum GT-II was elevated in 85/117 patients with colorectal cancer, 18/23 breast cancer, 13/20 lung cancer, 15/18 pancreas cancer and 12/16 gastric cancer, along with some patients in many other cancers, but no elevation was found in melanoma or osteosarcoma (PODOLSKY et al. 1981).

A prospective study on GT-II isoenzyme involving 270 patients in detecting pancreatic cancer and comparing it with other biochemical (CEA, AFP, ferritin, C1q binding and RNAase) and physical diagnostic modalities has been reported (PODOLSKY et al. 1981). GT-II isoenzyme was shown to be the most sensitive (67%) and specific (98%) for differentiating malignant pancreatic cancer from benign disease. GT-II was as sensitive as ultrasound and computerized body tomography were, but was less sensitive than endoscopic retrograde cholangiopancreatography. Combining with GT-II and radiologic or endoscopic test resulted in greater sensitivity than any single test alone. However, combination with other serologic markers indicated above yielded no significant increase in detecting pancreatic cancer. In this prospective study, CA 19-9 was not included since it was not yet available at that time. Serum RNAase detected only 30% of pancreas cancer and in 14% of patients with benign disease. Apparently RNAase was not of any clinical value in pancreatic cancer.

Although a simple ELISA using specific monoclonal antibody to assay GT-II isoenzyme has been reported, no further clinical data to support the initial promising study on pancreatic cancer were available. It appeared that this monoclonal antibody-based assay was more useful for ovarian cancer.

It should be mentioned again at this point that as far as serologic markers and pancreatic cancer are concerned, there is no reliable assay available to date. CA 19-9, DU PAN-2 (METZGAR et al. 1984), and perhaps GT-II, pancreas cancer associated antigen and pancreas specific antigen have been shown to be of value in various degrees (LOOR et al. 1984). Biochemical marker for pancreas cancer represents the most difficult area of diagnostic oncology.

6.4 Sialyltransferase

Serum sialyltransferase activity has been reported to be associated with cancer patients for a long time. Although a significant number of cancer patients exhibited abnormal sialyltransferase activity, a variety of non-neoplastic and benign disorders also were associated with elevated levels (SCHWARTZ 1982). Its clinical value is limited, except perhaps in monitoring disease status and therapy response.

6.5 Fucosyltransferase

At least three different fucosyltransferases have been described. Elevated levels of fucosyltransferase activity have been reported in patients with solid tumors and in AML and non-Hodgkin's lymphoma (SCHWARTZ 1982). An elevated enzyme level generally is associated with proliferative and secretory processes of both normal and neoplastic cells. Therefore, its clinical value in cancer diagnosis is extremely limited.

6.6 Terminal Deoxynucleotidyl Transferase

Most of the enzyme markers so far discussed are identified with solid tumors, very few enzymes have been shown to be associated with nonsolid tumors. Among this very small group of enzymes, terminal deoxynucleotidyl transferase (TdT) is the most extensively studied and is a potential marker for some forms of leukemia (SRIVASTAVA 1982). TdT is a DNA-polymerizing enzyme by catalyzing the addition of deoxynucleotide to the 3'-hydroxyl groups of a primer polydeoxynucleotide without the requirement of a template polynucleotide. TdT was found more than twenty years ago first in calf thymus, and reported to be present in ALL cells and T-ALL cells about ten years later. Antibody specific for TdT without cross-reactivity with α , β or γ DNA polymerase is available. Biochemical assay is still widely used as an analytical tool.

Highly elevated TdT is more commonly found in patients with ALL. Some patients with CML, AML and non-Hodgkin's also exhibited an elevated level. The enzyme level in lymphocytes from normal peripheral blood generally is very low. In CML, TdT positive cells usually increase during lymphoblastic crisis.

Most clinical application of TdT is its use in prognosis for adult patients with leukemia. TdT activity is generally correlated with frequency and duration of remission, and survival in patients with ALL, ANLL and malignant non-Hodgkin's lymphoma. It is of less value for young patients. Combining with different levels of expression by adenosine deaminase, TdT also can be used for subclassification of ALL; e.g. T-ALL, TdT positive and high adenosine deaminase; non-T and non-B ALL, TdT positive and moderate adenosine deaminase; B-ALL, TdT negative and low adenosine deaminase (MERTELSMANN 1982).

6.7 Lysozyme

Lysozyme is another enzyme reported to be of some clinical value in leukemia. Lysozyme is found primarily in monocytes and neutrophils. An elevated level is commonly associated with granulocyte destruction. Elevated serum lysozyme levels are associated with acute granulocytic leukemia, acute myelomonocytic leukemia, and AML. Patients with ALL rarely have an elevated level. Therefore, an elevated lysozyme can be used to exclude the presence of ALL. Also, serial measurement of lysozyme value has been shown to be of value in following the disease course and chemotherapy (SCHWARTZ 1982).

6.8 Other Enzyme and Isoenzyme Markers

There is an extensive list of other enzymes and isoenzymes that have been reported to be associated with cancer and are of some clinical value. Only a few more common markers are briefly mentioned. γ -Glutamyltranspeptidase (γ -GTP) is well known in its association with preneoplastic liver and hepatoma in experimental animals. γ -GTP has been shown in human cancer to be associated predominantly with secondary hepatic metastasis and can be useful in combination with CEA or other assay (CHU and DOUGLASS 1986). Another enzyme marker reported to be useful in detecting hepatic metastasis and primary hepatocellular carcinoma is 5'-nucleotide phosphodiesterase isoenzyme V (CHU 1982). It is an exonuclease and different from 5'-nucleotidase.

Adenosine deaminase, in addition to that already mentioned with TdT, is shown to be a biochemical marker for CML, since an elevated level is found in almost 90% of patients in the accelerated phase. Thymidine kinase isoenzyme I is reported to be a valuable marker for identifying sub-group of adult non-Hodgkin's lymphoma who respond poorly to therapy and require a new therapeutic approach.

Isoenzymes of alkaline phosphatase have been extensively investigated. Several isoenzymes have been identified in cancer patients' sera: Nagao isoenzyme in ovarian cancer, liver isoenzyme in hepatic metastasis and bone isoenzyme in bone metastasis (CHU 1982; KOTTEL and FISHMAN 1982). Most clinical application is in combination with other markers. The combination of liver isoenzyme with CEA is an economical screen method for liver metastasis. The combination of bone isoenzyme with PAP is effective for monitoring advanced prostate cancer (KILLIAN et al. 1981).

Aldolase activity was recently shown to be associated with cancer. There are two primary aldolase isoenzymes A and B. During the process of malignant transformation, the aldolase isoenzyme pattern is changed from B-dominant, liver or adult type, to A-dominant, muscle or fetal type. The aldolase B level in the serum is known to increase in patients with liver cancer and hepatic disease due to its high content in the liver and is released from the damaged hepatic tissue (ASAKA and ALPERT 1983). Whether it would be a better parameter than existing hepatic markers already discussed remains to be seen.

An increase in enzyme activity or protein mass so far has been discussed as a parameter for manifestation of cancer. In contrast to all these observations, the decrease in an enzyme activity, α -L-fucosidase, has been reported to be associated with some ovarian cancer patients (BARLOW et al. 1981). Tumor burden, disease stage, histologic type or grade of differentiation do not appear to be correlated with decreased level of enzyme activity. Familial study suggested that the deficiency of enzyme may be heritable and may be associated with an increased risk of developing ovarian cancer. Further studies certainly are needed to confirm this interesting report.

7 Other Biochemical Markers for Cancer

7.1 Ferritin

Ferritin is a well known protein related to iron storage and metabolism, and is present abundantly in the liver, spleen and bone marrow. Biochemically it exists in isomeric forms and is generally termed as isoferritins. The change in isoferritin molecules is associated with embryological development and pathological/malignant transformation. A carcinofetal form of ferritin has been identified (ALPERT 1982).

Serum ferritin is elevated mostly in patients with cancer related to liver and bone marrow cells. Highly elevated levels are found in patients with AML and other myeloproliferative diseases. Successful chemotherapy results in decrease of serum ferritin level to normal range, as leukemic cells and Hodgkin's lymphomas synthesize ferritin at a faster rate than that of normal lymphocytes. Patients with liver carcinoma and with acute or chronic liver inflammation all exhibit elevated serum ferritin levels. Ferritin is also elevated in patients with teratoblastoma and breast cancer. The major use of serum ferritin level is in evaluating prognosis and therapy response. It should also be noted that ferritin synthesized by tumor cells is different biochemically and immunologically from that of normal tissues.

7.2 Polyamines

The association of polyamines (spermidine, spermine and putrescine) with cancer was first reported about fifteen years ago (RUSSELL 1982). They have been unequivocally established as biochemical markers for normal and malignant cell growth. Although recent investigations have concentrated on basic work related to pharmacology and biochemistry of tumor cells, their use as biochemical tumor markers should not be overlooked. In fact, data available have shown that urinary and plasma levels of spermidine are related to tumor cell loss, and that those of putrescine are associated with both the number of tumor cells in cell cycle and tumor cell loss. These findings provide the basis for

polyamines to serve as effective markers for monitoring cancer patients during therapeutic manipulation. Putrescine can also be useful in determining whether the chemotherapy affects a subsequent phase of cell proliferation.

Measurement of polyamines are commonly performed by tedious and labor-intensive high pressure liquid chromatography. That is one major reason why these markers have not been routinely used. Since polyamines are relatively small molecules in size, reliable and simple immunochemical assays are more difficult to establish than other protein tumor markers.

7.3 Immunoglobulins

Abnormal immunoglobulins are well-known tumor markers. In fact, Bence Jones protein is perhaps the first tumor marker used effectively in clinical diagnosis of cancer patients (BENCE JONES 1847). The abnormal feature of immunoglobulins have been well known for patients with multiple myeloma and Waldenstrom's macroglobulinemia. Monoclonal gammopathies are most commonly observed in cancer patients involving plasma cells where a single malignant clone of precursor cell outgrow other cells and resulting in the production of a homogeneous monoclonal immunoglobulin. Biclinal gammopathies are rare but do occur. Detection of abnormal serum or urine immunoglobulins can be easily accomplished by routine electrophoresis with appropriate and specific antibody reagents that are readily available.

7.4 Peptide Hormones

Peptide hormones ectopically produced by non-endocrine tumors are well-recognized tumor markers. They are produced by tumor cells that are normally not regarded as being engaged in the synthesis and secretion of the hormones. So far only peptide hormones, their precursors or fragments have been documented. In addition, ectopic production of multiple hormones is another common feature.

The hormones commonly found in ectopic tumor production are adrenocorticotrophic hormone and related molecules, parathyroid hormone, gonadotrophin, growth hormones, prolactin, calcitonin, arginine vasopression, vasoactive intestinal peptide, and somatomedin, among others. Production of growth hormone-releasing activity or corticotrophin-releasing activity also has been observed. Oat cell carcinoma of the lung and carcinoid tumors of the liver, stomach, colon and thymus are the most common cancers producing ectopic hormones. Their use as biochemical markers in diagnosis, monitoring and localization to cancer has been reported. Apart from serving as biochemical markers which may be a secondary effect, ectopic hormone production by tumor can provide clues for investigating biologic significance and pathological implication of these tumors (NEVILLE 1982).

7.5 Immune Complexes

Elevated circulating antigen-antibody immune complexes have been used as parameters for a wide variety of malignant diseases, from lung, breast, colon-rectum cancer, thyroid cancer, to malignant melanoma, osteosarcoma, and leukemia/lymphoma. Unfortunately, a variety of non-malignant disorders also exhibit elevated immune complexes. Furthermore, all the assay procedures used for detection and measurement of circulating immune complexes are non-specific, thus diminishing the clinical value of immune complexes (CHU et al. 1983; SALINAS and HANNA 1985).

Theoretically the formation of antigen (cancer)-antibody (host) immune complexes is a result of the patient's natural immunological defense function. Measurement of such parameters should be of great value in clinical oncology, provided that antigen-specific assay is available. To this date, no such assay or methodology is yet available. Therefore, the isolation and characterization of the antigen components of immune complexes should be the focus for cancer immunodiagnosis. This information also is critical for the elucidation of immune complexes in human pathology and the development of new concepts in pathogenesis of immune complex-associated diseases.

8 Immunohistochemical Markers

In addition to serologic assays, biochemical markers can be used in the tissue and cell stain for immunopathologic assessment of tumors. Conventional morphological examination is enhanced by immunohistochemical stain for markers expressed by tumors. Further classification, phenotyping and differential diagnosis of tumor can be achieved at either light or electron microscopic level. Immunoperoxidase stain and immunofluorescence technique are most commonly used. Techniques and other related subjects are described in detail elsewhere in this book. A few biochemical markers that have been described already in this chapter and shown to be of practical application in tumor pathology are discussed.

One of the best examples in the use of biochemical markers in *in vitro* cell and tissue stain is the localization of estrogen receptor protein in breast cancer and other estrogen-sensitive tumors. Estrogen receptor has been traditionally recognized to include two forms, cytosol and nuclear, and that these parameters have been used in elucidation of action mechanism of estrogen and providing the basis for hormonal therapy (WITTLIFF et al. 1982).

Using monoclonal antibodies specific for estrogen receptor protein, recent reports have indicated that estrogen receptor is exclusively localized in nucleus of the cells (KING and GREENE 1984). The presence or absence of nuclear immunostain was significantly associated with the cytosol level of estrogen receptor as determined by conventional radioligand binding assay. These results suggest that previously observed cytosol and nuclear forms of the estrogen receptor may be present only in the nuclear compartment in both steroid-binding and

nonbinding forms. Data available also suggest the unoccupied estrogen receptor recovered in low salt cytosol preparation from tissue homogenate and determined by steroid binding assay represents the portion of estrogen receptor that is loosely bound to nuclear fraction (GREENE and JENSEN 1982).

The availability of monoclonal antibodies specific for estrogen receptor protein provides another tool for estrogen receptor measurement by simple immunohistochemical technique. Comparison of results obtained from immunohistochemical assay for estrogen receptor in breast tumors with those of traditional steroid-binding assay has resulted in highest degree of correlation (KING et al. 1985). A clear and significant association was shown between the presence or absence of nuclear staining for estrogen receptor and the level of cytosolic receptor measured in tumor extracts. All tumor specimens with estrogen-receptor negative immunostain were found to have insignificant levels of cytosolic estrogen receptor. Of clinical interest is that those tumors expressing none to minimum levels (0 to 636 fmol/g) of cytosolic estrogen receptor indeed contain decreased degree of immunostaining in both the cellularity and the percentage of stained tumor cells. These were the tumors from those patients generally presumed to be hormone unresponsive. Specimens with less than 300 fmol/g of cytosolic estrogen receptor show only focal nuclear staining.

The defined technique using estrogen receptor as an immunohistochemical marker also can identify sub-populations of hormone-unresponsive cells in patients, due to the heterogeneity of tumor cells. Additional consideration of therapeutic manipulation thus can be made in these patients. With further refinement, immunohistochemical procedure hopefully will provide valuable information regarding patients' prognosis and treatment responsiveness. Immunohistochemical reagent kit for estrogen receptor is now available commercially.

Detection of early metastasis or micrometastasis, and differential diagnosis of metastatic tumor are of critical importance in management of patients. For these purposes, it is not necessarily required to use tumor-specific markers. Antibody reagents to organ-site or cell-type specific antigen markers, and to tumor associated antigens have been extensively utilized for these purposes. Again, only a few examples using biochemical markers already discussed in this chapter will be illustrated.

Using conventionally prepared formalin-fixed paraffin-embedded tissue sections, PA has been shown to be an effective immunohistochemical marker for detection of secondary metastatic prostate tumors and for differential diagnosis (NADJI et al. 1981). Since PA is prostate specific, it is expressed also in metastatic tumors of prostate origin. With our reagents prepared in-house, we have been able to detect all secondary metastatic prostate tumors examined, including those in the regional lymph node and bone marrow. Similar results have been reported by other investigators. This test is most useful in identification of distant metastasis and in differential diagnosis of tumor involving prostate and adjacent tissues, especially the differentiation of poorly differentiated transitional cell carcinoma of the bladder from prostate carcinoma.

Another clinical application of histochemical markers is on cytopathologic assessment of tumor cells in serous effusions or extracellular fluid. It is well known that one of the most difficult diagnostic problems in tumor cytology

is to differentiate malignant cells from mesothelial cells. A couple of monoclonal antibodies have been shown to be of great value in the use of this very area.

Monoclonal antibody B72.3, recognizing TAG72 antigen, has been shown to stain adenocarcinoma cells in effusion specimens. In one study using large numbers of specimens involving both malignant and benign effusions, greater than 90% of adenocarcinomas were identified without staining any mesothelial cells (SZPAK et al. 1984). Another murine monoclonal antibody F36/22, recognizing a human ductal carcinoma antigen, also exhibits such a quality, especially for identification of ascitic epithelial ovarian carcinomas (CROGHAN et al. 1984).

9 Oncogene Products as Biochemical Cancer Markers

Recent advancement in molecular biology has identified the expression of more than twenty genes that appear to be associated with the conversion of normal cells to neoplastic cells, so called oncogenes (GARRETT 1986). Proteins encoded by some of these oncogenes also have been identified. Monoclonal antibodies directed against specific fragment (synthetic peptide) of oncogene products are available and some have been applied in cancer detection.

Since the presence of the oncogene products, if any, in circulation will be extremely small in amount, so far all reports concerning their expression have been obtained from examination of tissue sections. The oncogene coded protein most extensively studied is *ras* oncogene protein p21, a protein product encoded by the members of the *ras* oncogene, the major neoplastic transformation-inducing genes of human solid tumors.

In one study involving its expression in prostate cancer, all normal prostate and benign prostate tissues were shown to be devoid of p21 expression (VIOLA et al. 1986). In 29 patients with prostate cancer, p21 was detected in 2 of 6 prostate with Gleason Grade I carcinoma, 4 of 6 with Grade II, and all of 17 tumors with higher grades (III to V). Semiquantitatively, the expression of p21 in prostate tumor tissues was correlated strongly with nuclear anaplasia and inversely related to the degree of glandular differentiation. These results appear to suggest that *ras* oncogene p21 is a "prostate tumor-specific" marker, and that increased expression is associated with high grade, presumably more malignant tumors. Certainly, this interesting and provocative finding requires further confirmation with additional studies.

Interesting data on the expression of *ras* oncogene p21 also has been obtained from human breast tissues (OHUCHI et al. 1986). Enhanced expression was found in invasive carcinoma, while decreasing expression was detected in carcinoma in situ, atypical hyperplasia and nonatypical hyperplasia, respectively. Carcinomas from postmenopausal patients were generally associated with higher levels of p21 expression than that from premenopausal patients. No correlation in p21 expression was found with estrogen receptor status. Additionally, normal mammary epithelium in terminal duct lobular units from patients with hyperplasia demonstrated higher level of p21 expression than did epithelium in large ducts. This finding appears to be consistent with the notion that these are the areas preferentially undergoing malignant transformation. Taking all these results together, it would suggest that an enhanced expression of *ras* p21 may be involved in the early malignant development of human breast tumor. This exciting observation again needs to be confirmed.

Without question, the reports concerning oncogene product and cancer diagnosis will be forthcoming at a greater rate in the next few years. The use of oncogene related products in tumor therapy would be another area of clinical interest.

Several oncogene probes for cancer markers including that mentioned above and those for leukemia/lymphoma and others are already available from commercial sources.

10 Biochemical Markers for Carcinogen Testing

Another potential application of biochemical tumor markers is their use in carcinogen testing. In fact, this is an area of investigation that could provide an enormous scientific return. The time-consuming and expensive animal bioassay in rodents is still the best available method and standard technique currently officially acceptable for identifying and evaluating chemical carcinogens. Although a large series of animals are generally required, ambiguous statistical results are often obtained. Therefore, a biochemical marker capable of indicating the cause of malignant transformation or correlating the progress of carcinogenesis by a carcinogen would offer a simple and objective criterion for the identification of chemical carcinogen. Qualitative or quantitative biochemical markers of carcinogenicity will enhance the validity of existing short-term bioassays for chemical carcinogens.

In addition to the determination of maximum tolerated dose, animal bioassay for carcinogens primarily depend upon evaluation of morphologic changes in affected organs following carcinogen exposure. Should a biochemical marker be identified or available to indicate morphologic changes associated with carcinogen exposure, this marker would be of great value in carcinogen testing. Several biochemical markers are effective in indicating such changes.

Serum AFP has been shown to be a very reliable marker for determining exposure to liver carcinogens in animals, especially in rats, regardless of the nature of neoplastic lesions (SELL et al. 1983). Early prolonged elevations of serum AFP or sudden drastic elevations of AFP can be used as conclusive evidence of activity by strong hepatocarcinogens. Some carcinogens may not produce such patterns of serum AFP elevation even after potentiation with choline-deficient diet and may result in a possibly false negative result. In mice that are exposed to chemical hepatocarcinogens or develop spontaneous primary hepatocellular carcinoma, an elevation of serum AFP may not be observed in early stage. If an AFP elevation is produced in animals upon exposure to carcinogens, immunostain also will provide another probe to delineate the cellular mechanisms involved.

Gamma-glutamyltranspeptidase (γ -GTP) is another well known biochemical marker for preneoplastic hepatocyte foci and of hepatocellular carcinoma in animals (RICHARDS 1983). Although its function and biologic significance in normal cells and in malignant transformation is still unclear, γ -GTP has been used increasingly as an enzyme marker for chemical hepatocarcinogens. Simple

histochemical stain allows easy identification of putative preneoplastic hepatocytes. It should be noted, though, that γ -GTP is not a tumor marker per se, it is a marker of cell differentiation, cell aging and reduced cell proliferation rather than of elevated cell proliferation. Furthermore, some rat and human hepatocellular carcinomas and most mouse hepatomas are devoid of γ -GTP expression.

Polyamines are well known for their important role in cell growth, and have been discussed as potential biochemical tumor markers. Ornithine decarboxylase is a critical enzyme involved in metabolism of polyamines, especially in catalyzing the formation of putrescine (diamine) from ornithine, serving the precursor for spermidine (tertiary amine) and spermine (quarternary amine). Induction of ornithine decarboxylase during tumor promotion and in transformed cells as a result of chemical carcinogenesis or other mechanisms has been suggested to be a potential universal marker of neoplastic expression (HADDON and GREENFIELD 1983). This notion represents a basis for its use in carcinogen testing but requires further investigation.

Biochemical markers, such as aryl hydrocarbon hydroxylase, epoxide hydroxylase, DT-diaphorase, and others, have been shown to be of some value as enzyme markers for chemical carcinogenesis.

The use of DNA-carcinogen adducts for possible monitoring exposure to chemical carcinogen is a relatively new and extremely important area of investigation. Considerable characterization of DNA component structurally modified by chemical carcinogen has been reported. Several biochemical and immunologic methods are available which detect and quantify human DNA modified by carcinogens, even to the level of accuracy in defined restriction fragments of DNA molecules modified to less than one carcinogen per DNA molecule (BUSTIN et al. 1983). This new approach represents a great potential research and scientific reward for biochemical markers for cancer.

11 Summary

At this stage of development, there is no tumor specific biochemical marker. Although sensitive and specific procedures are available for measurements of cancer-associated biochemical markers, none has been shown to be of value as a general screen for cancer. Biochemical markers do have potential as screening tools for high-risk populations and symptomatic patients, e.g. α -fetoprotein for primary hepatocellular carcinoma, thyrocalcitonin for medullary thyroid carcinoma, and perhaps fibrinogen degradation products for carcinoma of the urinary bladder. Also, biochemical markers, such as estrogen receptor protein, are clinically useful for staging of breast cancer and other estrogen-sensitive tumors. Most of the biochemical markers available to date are of clinical utility as a means of monitoring the response to treatment and/or of detecting disease recurrence at an early stage. At present, there are only two markers, carcinoembryonic antigen and prostate specific antigen, approved by the Food and Drug Administration. Conventional radioimmune assay and biochemical assay, in-

cluding enzyme-linked immunosorbent assay, are still commonly applied in detection of biochemical markers. Other new approaches or technologies, such as immunohistochemistry, flow cytometry and molecular biology, are being increasingly utilized. Another potential application of biochemical tumor markers is their use in carcinogen testing. Biochemical marker can serve as a simple and objective criterion correlating morphological changes in malignant transformation. Finally, recent advancement in molecular biology has identified oncogene encoded protein as a potential biochemical marker for human cancer.

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Immunohistochemical Methods for the Demonstration of Tumor Markers

H. DENK

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1 Introduction

Immunohistochemistry plays a major role in biological and biomedical research as well as in diagnostic histopathology. In pathology, it has not only contributed significantly to our understanding of basic pathogenetic principles but also to the precision of diagnosis (see TAYLOR and KLEDZIK 1981; DE LELLIS et al. 1979; for further information). An accurate classification of neoplasms is an important prerequisite of the newly developed specific and highly efficient therapeutic approaches in oncology. A major problem in histopathology is the identification and classification of tumors on the basis of light microscopic appearance in routinely stained and processed sections. This task is relatively easily accom-

plished in well differentiated neoplasms with (more or less) close resemblance to the respective non-neoplastic tissue, but may be difficult or even impossible in undifferentiated tumors and in small tissue fragments. In these situations, the evaluation of tumor cell constituents or products (tumor markers) is a valuable supplement for refinement of diagnosis. The family of tumor markers includes various immunoglobulin classes in tumors or atypical proliferative conditions of lymphoreticular origin, hormones, particularly in endocrine tumors, tumor cell constituents, such as membrane and cytoskeletal components, and oncofetal substances (for further information see HEYDERMAN 1983 and this volume).

As a promising tool, immunohistochemistry started with the first description of antibody labeling with a fluorescent dye by COONS et al. (1941), and has been considerably improved with the introduction of enzyme labels and the unlabeled antibody methods (STERNBERGER 1979; VAN NOORDEN and POLAK 1983; DENK and PLATZER 1982; TAYLOR 1980; BOSMAN 1983). Its superior sensitivity and specificity now allows its application to fixed and paraffin-embedded material, the material of choice referred to the surgical pathologist. Numerous modifications of the original procedures have been described with the aim increasing sensitivity, specificity and applicability further. A significant improvement, particularly with regard to specificity, has been achieved by the introduction of the monoclonal antibody technology (for review and further information see DIAMOND and SCHARFF 1982). Moreover, immunochemical analyses of antibodies and antigens (for example, by immunoblotting procedures) allows a more precise definition of antigenic determinants recognized by the antibodies. However, the still expanding range of application of immunohistochemical techniques raises the risk of pitfalls and misinterpretations of results and makes proper evaluation of the methodology, particularly with respect to its specificity, imperative. Moreover in diagnostic pathology, immunohistochemistry should be guided by conventional histopathological evaluation and cannot replace the experience and skill of the pathologist. Diagnostic errors resulting from uncritical use of these costly and time-consuming procedures must be avoided.

The present review will focus on the various methods applied today in diagnostic pathology in general and in oncology, for tumor marker detection in particular.

2 Principles of Immunohistochemistry

Immunohistochemistry allows in situ-identification of antigenic cell or tissue components, including secretion products, on the basis of specific antigen-antibody interactions which are visualized by suitable labels at the light and electron microscopic level. Besides pure immunological methods, depending solely on the specific interaction of antibodies (or antigen) with the tissue-bound antigen (or antibodies), non-immunological fixation of substances in combination with immunological methods (mixed methods), or non-immunological binding alone can be successfully used for visualization of diverse cell and tissue components (for recent review see, for example, VAN NOORDEN and POLAK 1983; TAYLOR

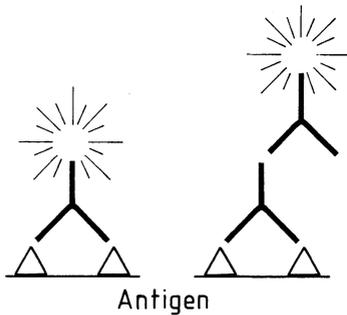


Fig. 1. In direct immunofluorescence (*left*) the fluorochrome-labeled antibody reacts with the tissue bound antigen. In indirect immunofluorescence (*right*) the labeled secondary antibody marks the position of the first antibody bound to the antigen

1983; BOSMAN 1983). Labels used for visualization of binding are either fluorescent dyes (immunofluorescence), enzymes (immunoenzymatic methods), electron dense substances and particles for light and electron microscopic immunocytochemistry (e.g. colloidal gold, ferritin), and other particles (e.g. erythrocytes).

2.1 Immunofluorescence Microscopy

Immunofluorescence microscopy (Fig. 1), first introduced by COONS et al. (1941), is based on labeling of the antibodies with fluorescent dyes (fluorochromes). Several fluorochromes are suitable for antibody labeling, including fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), lissamine rhodamine B 200 sulphonyl chloride (RB 200SC) and Texas Red[®] sulphonyl chloride. An optimal fluorochrome-protein ratio is required for low signal-to-noise ratio, that is the intensity of specific staining in relation to nonspecific background staining. Since conjugation of antibody with label involves a chemical reaction it may to some degree affect antibody structure and immunoreactivity adversely. The disadvantages of immunofluorescence microscopy are the impermanence of staining (although this disadvantage can now largely be overcome by embedding the stained frozen section in special media such as Moviol[®]), the need for special and expensive equipment (fluorescence microscope), the need, in many instances, for frozen or specially treated tissues, and difficulties in the analysis of structural details in frozen material subjected to immunofluorescence staining.

2.2 Enzymes as Labels

Attempts to overcome disadvantages inherent in immunofluorescence techniques resulted in the widespread use of enzymes as markers (Fig. 2) (STERNBERGER 1979; NAKANE and PIERCE 1966; VAN NOORDEN and POLAK 1983). Horseradish peroxidase and alkaline phosphatase, glucose oxidase, and beta-galactosidase are used as marker enzymes which, for visualization, are developed histochemically with various substrates (chromogens) yielding differently coloured end-products (CORDELL et al. 1984; MASON et al. 1983; VAN NOORDEN

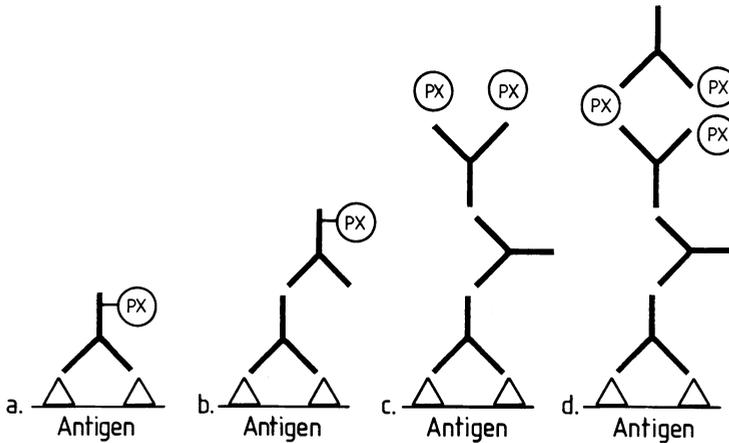


Fig. 2. The coupled enzyme (peroxidase) methods (a, b) resemble the immunofluorescence methods (direct and indirect immunoperoxidase method). In the unlabeled methods the enzyme marker (peroxidase) is fixed to the site of the antigen by an antibody sequence (the final antibody directed to the enzyme). In the antibody bridge method a peroxidase antibody is bound to a primary antibody of the same species by a bridging antibody (c). The enzyme is added as the fourth layer. In the peroxidase-anti-peroxidase (PAP) method a peroxidase-anti-peroxidase PAP complex is bound to the primary antibody by a bridging antibody (d)

and POLAK 1983; for review of recent literature). Glucose oxidase has the advantage that no endogenous enzyme activity exists in mammalian tissues (see CLARK et al. 1982; GOWN et al. 1986 for further references), in contrast to alkaline phosphatase and peroxidase, which has to be blocked in order to avoid false positive results (peroxidase by methanol- H_2O_2 or periodate oxidation; alkaline phosphatase by levamisole, acetic acid, or periodate oxidation). These procedures occasionally result in impairment of antigenicity (for review and references see MASON et al. 1983). A sensitive method involves the combination of glucose oxidase and horseradish peroxidase (KUHLMANN and PESCHKE 1986) which are coimmobilized onto the same cellular sites and act as components of a reaction sequence. The H_2O_2 generated during the glucose oxidase reaction is the substrate of horseradish peroxidase and is utilized for the oxidation of chromogens. This method is superior to conventional immunoperoxidase labeling for the localization of antigens in tissues rich in endogenous peroxidase activity.

The chromogen used most frequently in the peroxidase methods is 3,3'-diaminobenzidine (DAB) in H_2O_2 , yielding a brown color which can be further intensified by treatment with osmium tetroxide or $NiCl_2$, $CoCl_2$ or $CuSO_4$ (ADAMS 1981; HSU and SOBAN 1982; TROJANOWSKI et al. 1983; DE JONG et al. 1985; see also section 6 on sensitivity). Alternative substrates are 4-chloro-1-naphthol (blue color, but not alcohol resistant), 3-amino-9-ethylcarbazol (red color), and p-phenylenediamine-HCl/pyrocatechol (black color) (see MASON et al. 1983; VAN NOORDEN and POLAK 1983). The 1-naphthol-basic dye technique can be used as alternative to DAB in immunoperoxidase techniques (MAURO et al. 1985). The oxidation product of 1-naphthol is capable of binding basic dyes resulting in precipitates with characteristic color, e.g. blue-green with

azur A, toluidine blue, cresyl violet, and Giemsa stain, dark green with methyl-green, blue-violet with crystal violet, red-violet with safranin, etc., which are alcohol-resistant. In alkaline phosphatase immunohistochemistry the enzyme label is revealed with naphthol AS-MX (or naphthol AS-BI) plus Fast Red (red color), hexazotized New Fuchsin (red color) or Fast blue (blue color) (CORDELL et al. 1984; DE JONG et al. 1985). Glucose oxidase activity is detected with a substrate medium containing B-D glucose, nitro blue tetrazolium and phenazine methosulfate (KUHLMANN and AVRAMEAS 1971; GOWN et al. 1986).

Horseradish peroxidase was originally selected as marker enzyme because it could easily be coupled to antibodies and yielded a stable color reaction. However, chemical coupling procedures have several short-comings, particularly by affecting adversely antibody as well as enzyme reactivities (STERNBERGER 1979; VAN NOORDEN and POLAK 1983). Therefore, unconjugated enzyme-antibody methods, such as the peroxidase-antiperoxidase-method (STERNBERGER 1979), have been developed in which the marker enzyme is fixed to the site of the antigen by several steps of antibody binding instead of chemical coupling. The superior sensitivity of these methods allows the detection of antigens, including tumor markers, in routinely fixed and processed material making retrospective studies on a large scale possible. In addition, the excellent structural preservation of fixed material and counterstaining allow a combination of immunohistochemistry with traditional light microscopy and thus facilitate interpretation (TAYLOR 1980; BOSMAN 1983; VAN NOORDEN and POLAK 1983).

2.3 Colloidal Gold and Other Particles as Labels

The stable and rapid binding of colloidal gold particles to proteins, including antibodies, enzymes, protein A, and lectins, without affecting biological activity is exploited in light as well as in electron microscopic immunocytochemistry (including scanning electron microscopy) (Fig. 3). With colloidal gold – coupled probes neither substrates (as in enzyme methods) nor expensive equipment (as in immunofluorescence microscopy) are required for visualization (DE MEY 1983b). Immunogold procedures can be combined with other staining methods, e.g. enzyme cytochemistry, for correlation of morphological with functional criteria (DE WAELE et al. 1983). For visualization of the gold label under the light microscope (red staining) a relatively high local concentration of immunogold reagent is necessary. Sensitivity is considerably increased and, hence, less reagent is required when immunogold staining is combined with silver precipitation (HOLGATE et al. 1983). Metallic gold catalyzes the reduction of silver ions (derived, for example, from silver lactate) to metallic silver in the presence of a reducing agent (e.g. hydroquinone) resulting in an enlarging shell of silver grains around the gold particle which is then easily visible at the light microscopic level (black staining). Several modifications of this method have been designed (SPRINGALL et al. 1984; FRITZ et al. 1986; DANSCHER and NÖRGAARD 1983 for resin-embedded tissue). Advantages are the high sensitivity (see below), the lack of diffusion of the reaction product, low background staining, and easy combination with routine staining procedures, e.g. haematoxylin-eosin staining.

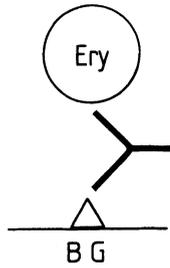
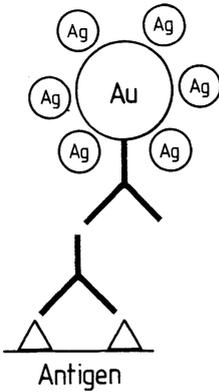


Fig. 4. In the erythrocyte adherence reaction erythrocytes (*Ery*) containing blood group antigens are fixed by a bridging antibody to blood group antigens (*BG*) present in the tissue. The site of the antigen is indicated by erythrocyte adherence

◁ **Fig. 3.** The secondary antibody labeled with colloidal gold binds to the unlabeled primary antibody which reacts with the tissue-bound antigen. The color reaction is intensified by silver precipitation

Particles containing a specific antigenic structure, either artificially coupled to its surface or present in their membranes, e.g. blood group antigens in erythrocytes, can be used as probes by fixation to identical antigens present in tissues or cells by a bridging antibody. An example is the erythrocyte adherence reaction for the demonstration of tissue-bound blood group antigens (Fig. 4; see DENK and PLATZER 1982 for review), originally described by DAVIDSON 1972.

3 Immunohistochemical Methods: Types and Mechanisms

3.1 Direct Labeled Antibody Method

The labeled antibody identifies the tissue-bound antigen by specifically binding to the antigenic sites (Fig. 1, Fig. 2). This method is less sensitive and requires a vast library of labeled antibodies depending on the antigens to be studied.

3.2 Indirect Labeled Antibody Method

An unlabeled (primary) antibody bound to its specific antigen in the tissue is revealed by a labeled secondary antibody raised in another animal species against the primary (Fig. 1, Fig. 2). This method exceeds the direct method in sensitivity. Since primary antibodies are produced in a limited number of animal species, preferentially in rabbits, sheep, goats, pigs, guinea pigs, only relatively few different labeled antibodies are required to detect a diversity of antigens.

3.3 Unlabeled Antibody Enzyme Bridge Method

A bridging antibody fixes an anti-enzyme antibody (derived from the same species as the first antibody) to the unlabeled first antibody directed against

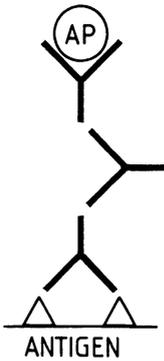


Fig. 5. An alkaline-anti-alkaline phosphatase complex (APAAP) is fixed to the primary antibody by a bridging antibody. When the primary antibody and the alkaline phosphatase antibody are monoclonal (mouse) an anti-mouse IgG is used as bridging antibody

the antigen (STERNBERGER et al. 1970; STERNBERGER 1979; VAN NOORDEN and POLAK 1983). The enzyme is added and immunologically bound in a final step and visualized histochemically (Fig. 2c). Since chemical coupling of antibody and enzyme is avoided maximum reactivity of both is guaranteed. In the three step enzyme-anti-enzyme complex method a preformed antibody-enzyme complex (e.g. peroxidase-antiperoxidase complex, PAP; alkaline phosphatase-antialkaline phosphatase complex, APAAP; CORDELL et al. 1984) is applied (Fig. 2d, Fig. 5). The concentration of enzyme molecules at the antigenic site, and hence sensitivity, can be increased by repeating the sequences (multistep methods). It has been shown that a linear relationship exists between the number of incubation cycles and the amount of enzyme, e.g. peroxidase, bound. Consequently, a high enzyme (e.g. peroxidase)-to-antigen ratio is achieved and the primary antibody can be used highly diluted giving the advantage of low non-specific background staining (LANSDORP et al. 1984). Several variants of the unlabeled antibody-enzyme method have been described, such as the double PAP technique (ORDRONNEAU et al. 1981). Limitations may ensue from dissociation of anti-immunoglobulin from immunoglobulin, dissociation of anti-peroxidase from peroxidase, and impaired accessibility of substrate within large complexes (LANSDORP et al. 1984).

For monoclonal primary antibodies (rat or mouse) monoclonal peroxidase-antiperoxidase (PAP) immunocytochemical reagents have recently been developed (MASON et al. 1982; CUELLO et al. 1984). Since monoclonal antibodies recognize only one specific type of antigenic determinant, monoclonal PAP complexes have lower molecular weights than polyclonal PAP complexes and probably contain only two molecules of horseradish peroxidase per antibody molecule which facilitates penetration (MASON et al. 1982). The use of monoclonal PAP complexes results in very low background staining and intense specific staining (LANSDORP et al. 1984). In "hybrid-hybridoma" immunocytochemistry "bi-specific" monoclonal antibodies which recognize both peroxidase and another antigen are introduced (MILSTEIN and CUELLO 1983).

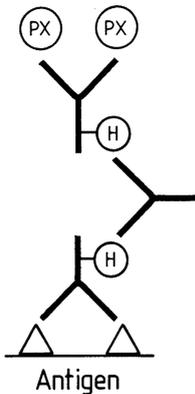


Fig. 6. Uncoupled hapten bridge method. Primary antibodies as well as enzyme antibodies are haptened and bridged by an antibody directed to the hapten

3.4 Hapten-Labeled Antibody Method

Labeling of a primary antibody with a hapten (e.g. arsanilic acid, dinitrophenol, p-amino-benzoylglycine, p-aminobenzoylglutamic acid), which is then recognized by a labeled hapten antibody, is the principle of this reaction. Since large numbers of haptens can be coupled to the antibody its antigenicity towards the second antibody is increased and sensitivity augmented (CAMMISULI and WOFISI 1976; JASANI et al. 1981; MASON et al. 1983). In a modified version of this method an unlabeled anti-hapten antibody (reacting with the haptened primary antibody) is used as the second and a hapten-labeled enzyme antibody or PAP complex as the third layer (Fig. 6).

3.5 Labeled Antigen Method

This method is highly specific and is based on the binding of a labeled antigen to its specific antibody before this antibody reacts with its other binding site with the tissue-bound antigen (MASON and SAMMONS 1979). Enzymes, radioactive isotopes, or gold particles can serve as labels. The necessity of labeling individual antigens is a disadvantage of this method (see VAN NOORDEN and POLAK 1983; BOSMAN 1983 for further information).

3.6 Biotin-Avidin Method

Avidin is an egg white glycoprotein (molecular weight 68,000) with pronounced affinity to the vitamin biotin (see HSU and RAINE 1984 for review). In the labeled avidin-biotin technique (GUESDON et al. 1979; HSU et al. 1981; BOORSMA et al. 1986) biotin-labeled antibodies and enzyme-labeled avidin are applied in sequence. In addition to horseradish peroxidase alkaline phosphatase can be used as marker enzyme. With biotin-labeled secondary antibodies (3-step technique) only few biotinylated antibodies are required to detect a wide range of primary antibodies. However, biotin-labeled primary antibodies (2-step tech-

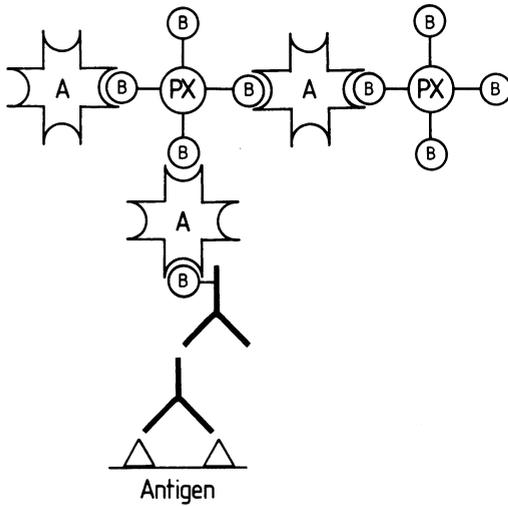


Fig. 7. A biotinylated antibody (either primary or secondary antibody) is visualized by an avidin (*A*)-biotin (*B*)-peroxidase (*PX*)-complex. Since several complexes can bind, several peroxidase molecules are fixed to the place of the tissue bound antigen increasing the sensitivity of the reaction

nique) have the benefit of lower background staining and thus yield a high signal-to-noise ratio, resulting in a distinct staining reaction. In the direct avidin-biotin bridge method avidin bridges the biotin-labeled primary antibody and the biotin-labeled enzyme. In the indirect bridged avidin-biotin technique (HSU et al. 1981) the biotin-labeled secondary antibody and the biotin-labeled enzyme are combined by avidin. The reagents of the different steps are either applied sequentially or a preformed avidin-biotin-peroxidase complex (ABC) is used (Fig. 7). The high sensitivity of the indirect bridged avidin-biotin technique may ensue from the following facts: (1) Biotin is bound to the antibody or the enzyme (peroxidase) through amino groups. Since multiple biotin moieties can be introduced into immunoglobulin or peroxidase, the number of binding sites for avidin is increased; (2) avidin has four active binding sites for biotin, not all of which react with antibody-associated biotin but which do react with biotin molecules coupled to peroxidase leading to amplification of the staining reaction by formation of a large complex containing multiple peroxidase molecules; (3) penetration of the reagents is facilitated by their smaller size (HSU and RAINE 1984). In order to further enhance antibody penetration biotin labeled Fab fragments can be used for light as well as for electron microscopy.

The quality of the ABC reagent also determines sensitivity. An excess of free avidin molecules decreases sensitivity by competition with the avidin-biotin-peroxidase complex for the biotin molecules coupled to the antibody. On the other hand, with biotin-peroxidase in excess the biotin binding sites of the avidin molecules in the complex may become saturated and the complex is then devoid of free biotin-binding sites impeding its reactivity with the biotinylated (secondary) antibody.

Non-specific binding of ABC is observed with high concentrations and can be diminished by appropriate dilutions (for other pitfalls see section 9).

Further possible pitfalls of avidin-based systems may result from the fact that avidin at neutral pH is highly positively charged (isoelectric point around

10) and, therefore, non-specifically attaches to negatively charged molecules, including nucleic acids and phospholipids (SAVAGE 1986).

Avidin can be replaced by streptavidin, which is a protein from *Streptomyces avidinii* and has four high-affinity binding sites for biotin. It can be conjugated with labels without loss of biotin-binding characteristics. Labels used in the streptavidin system include fluorescein, Texas red, enzymes (peroxidase, alkaline phosphatase, β -galactosidase), colloidal gold, and ¹²⁵I. Non-specific binding of streptavidin to negatively charged molecules and to lectins is avoided by its lack of carbohydrate residues and by an isoelectric point around neutral pH (see SAVAGE 1986 for further information). Amplification of the signal can be achieved by biotinylation of the second antibody at multiple sites or by using streptavidin containing several label molecules per molecule.

3.7 Labeled Protein A Method

Protein A is a highly resistant cell wall constituent present in most strains of *Staphylococcus aureus* and consists of a single polypeptide with a molecular weight of 42,000 and an isoelectric point value of 5.1. It shows a high affinity to the Fc region of immunoglobulins, particularly of IgG, which makes it a suitable tool for antibody recognition (see ROTH 1984 for additional information). Differences, however, exist in its binding affinities to IgG subclasses derived from different species (see ROTH 1984 for information). Since it binds strongly to rabbit and guinea pig but rather weakly to goat and sheep immunoglobulins, reagents derived from the former species should be preferred. Protein A can be labeled with several substances, including fluorescein isothiocyanate or colloidal gold particles, and is usable in both light and electron microscopic immunocytochemistry (see also BENDAYAN and DUHR 1986 for modifications).

3.8 Lectins

Lectins are sugar binding proteins or glycoproteins (derived from plants, bacteria, fungi, invertebrates, and vertebrates) which non-covalently bind to specific carbohydrate residues of polysaccharides and glycoproteins, and are able to agglutinate cells containing complementary saccharides (PONDAR 1983; ALROY et al. 1984). Lectins coupled to labels, including fluorescent dyes, enzymes and electron-dense particles, have been used as probes in carbohydrate-directed histochemical studies.

Lectin histochemistry has contributed to our knowledge of the distribution of sugar residues, including blood group-related antigens, on cell surfaces and secretions during maturation, differentiation, but also malignant transformation (for different lectin and sugar specificities see ALROY et al. 1984). Evaluation of the presence, deletion, or distribution of lectin receptors may help to define neoplastic changes and, consequently, the biological behaviour of tumor cells, and may, therefore, contribute to our understanding of pathological processes, in addition to providing diagnostic and prognostic hints.

In addition to direct methods with labeled lectins, lectin binding sites are also revealed by the PAP as well as the ABC method in formalin-fixed and paraffin-embedded material. In the PAP method, the tissue sections are first

incubated with a lectin followed by sequential incubations with lectin antibodies, secondary antibodies, and a corresponding PAP complex. In the ABC method, a biotinylated lectin is revealed by ABC. Controls include blocking of the lectin by preincubation with the specific sugar.

4 Comparison of Different Immunohistochemical Techniques

4.1 Immunofluorescence Versus Immunoenzymatic Techniques

Important factors determining the reliability of immunohistochemistry are the sensitivity of the technique as well as the detection efficiency reflected by the signal-to-noise ratio. A valid comparison of different methods in this respect requires a defined test system (VALNES et al. 1984). VALNES and BRANDTZAEG (1984) concluded from their studies using artificial substrates (human immunoglobulins IgA and IgG incorporated) that only minor differences in sensitivity and efficiency exist between the direct and indirect immunofluorescence, the indirect immunoperoxidase conjugate and the unlabeled PAP methods. According to their experience, masking of antigenicity in formaldehyde-fixed substrates played some role in immunofluorescence but less in immunoenzymatic techniques and could be overcome by raising conjugate concentrations, prolongation of incubation time, and protease pretreatment. However, in the opinion of most authors enzyme bridge, PAP and avidin-biotin methods surpass the direct and indirect methods in sensitivity (e.g. BERGROTH 1983; see also VAN NOORDEN and POLAK 1983).

4.2 PAP Versus ABC Method

By using the ABC method staining intensity is increased in comparison with the PAP technique, thus allowing considerably higher dilution of primary antibodies (HSU et al. 1981). However, other authors (STERNBERGER and STERNBERGER 1986) disagree on the basis of their studies using a new method of quantification by image analysis of optical densities. STERNBERGER and STERNBERGER (1986) obtained stronger staining intensities, and hence sensitivities (signal-to-background ratios), with the PAP than with the ABC method. In their hands, the PAP method was ten times more sensitive than the ABC method within a certain range of antibody dilutions.

4.3 Immunogold-Silver Staining Versus PAP Method

Immunogold-silver staining excels the PAP technique in sensitivity. Increased sensitivity (up to 200 fold) is particularly obtained by the silver precipitation step (see also SPRINGALL et al. 1984), but inclusion of detergent (0.5% Tween 80 or 0.2% Triton X-100) and higher sodium chloride concentrations into the

buffers, higher pH of the immunogold reagent and modifications of the silver development procedures (SPRINGALL et al. 1984) may also contribute to enhancement of staining. Small-sized gold particles adsorbed to affinity-purified antibodies facilitate penetration of the labeled antibodies.

5 Simultaneous Detection of Two or Several Antigens

5.1 Double Immunofluorescence

Differently labeled secondary antibodies (fluorescein isothiocyanate, rhodamine isothiocyanate, Texas Red) are used as a mixture or in sequence to detect primary antibodies derived from different species (indirect method). Primary antibodies conjugated with different fluorochromes can also be used (direct method). Immunofluorescence may also be combined with other methods, e.g. with the immunogold (WANG and LARSSON 1985) or with the PAP method (GAUDINO et al. 1982).

5.2 Immunoenzymatic Techniques

Simultaneous demonstration of multiple antigens by enzyme immunohistochemistry may be achieved by using one type of enzyme with different substrates or different enzymes with or without elution of the preceding sequences (for information and references see WANG and LARSSON 1985; MASON et al. 1983). If one enzyme is used with different substrates for labeling of two different antigens the procedure has to be performed sequentially with the disadvantages of prolongation of the immunohistochemical procedure. With two different enzymes, peroxidase is usually applied in the first sequence. Alkaline phosphatase, as the second enzyme, can be developed with different substrates revealing reaction products with excellent contrast to the DAB reaction product as well as to haematoxylin as counter stain (MASON et al. 1983; VALNES and BRANDTZAEG 1984). For double labeling a combination of avidin-biotin-glucose oxidase in conjunction with avidin-biotin-peroxidase method can be used (GOWN et al. 1986): the avidin-biotin-peroxidase with DAB development is applied first, followed by an elution step (5% acetic acid or 10% oxalic acid) and/or incubation with Triton X-100 (0.1%), or Tween 20 (0.1%), and then by avidin-biotin-glucose oxidase system with nitro blue tetrazolium as chromogen. For maximum staining intensity in paraffin-embedded tissue approximately ten-fold higher concentrations of primary antibodies were required in the avidin-biotin-glucose oxidase than in the avidin-biotin-peroxidase system, but this varied with different antibodies and antigens (GOWN et al. 1986).

In the elution techniques, the antibodies of the first reaction are eluted after color development and the section is then incubated for demonstration of the second antigen using an immunoenzymatic procedure with a differently colored reaction product either by changing the enzyme or the substrate (MASON et al. 1983; WANG and LARSSON 1985). Difficulties of removal of the first sequence antibodies

may arise with antibodies with high binding avidity, and some of the methods efficient in this respect may adversely affect antigenicity (TRAMU et al. 1978; STERNBERGER 1979; see also WANG and LARSSON 1985). Removal of the immunoreagents of the first sequence without affecting the DAB reaction product before application of the second sequence for staining of the second antigen can be achieved by acidification (for further references see STERNBERGER and JOSEPH 1979) or by dimethyl formamide treatment (VANDESANDE and DIERICKX 1975) by which most antigen-antibody complexes are dissociated.

Elution procedures may be avoided by modifications of the techniques, for example by a mixed PAP-immunofluorescence staining sequence (LARSSON et al. 1979). In other non-elution procedures differently labeled primary antibodies, primary antibodies derived from different species, labeled antigen and protein A are used.

According to STERNBERGER and JOSEPH (1979) in the unlabeled antibody enzyme (PAP) method, performed under standard conditions, color mixing did not occur despite the fact that primary antibodies with differing specificities in the first and second sequence were from the same animal species, provided that the first sequence primary antibodies were sufficiently highly concentrated. Apparently, the DAB oxidation product is able to mask antigenic and catalytic sites of the first sequence of immunoreagents thus obviating elution prior to application of the second sequence for double antigen staining. Another possibility is that excess linking antibody in the first sequence blocks all antigenic determinants of the primary antibody and that the PAP complex of the first sequence blocks all free combining sites of the linking antibody.

Another non-elution technique introduced by WANG and LARSSON (1985) is based on the observation that formaldehyde vapour selectively destroys the antigen – combining sites of the second layer anti-IgG antibodies. Under these conditions antibodies of different specificities raised in the same species can be used in sequence for the demonstration of several antigens in indirect immunohistochemical staining. The prerequisite of the suitability of this technique, however, is the stability of the antigens towards formaldehyde fixation. Conditions, which have to be established in pilot studies, include optimal dilutions of primary antibodies and optimal (minimal) time of formaldehyde vapour treatment. Good results have been obtained with several peptide hormones (WANG and LARSSON 1985).

In the hapten sandwich double labeling methods the haptenated primary antibody directed to the first antigen is linked to the haptenated third stage (e.g. PAP) by a antihapten (e.g. to dinitrophenol) bridging antibody. The second antigen is revealed by a second sandwich with another hapten (e.g. p-aminobenzoylglutamic acid or p-aminobenzoylglycine; for further information see MASON et al. 1983).

If alkaline phosphatase is conjugated with (periodate oxidized) horseradish peroxidase a violet reaction product is revealed upon sequential alkaline phosphatase (Naphthol AS-MX/Fast blue) and horseradish peroxidase (3-amino-9-ethylcarbazole) development. This double enzyme conjugate can be coupled either to an antigen (for determination of antibody producing cells) or to antibodies and allows (in combination with pure alkaline phosphatase and horseradish peroxidase immunohistochemistry) the simultaneous detection of three different antigens or antibodies in a single section after a one-step incubation and a two-step standard cytochemical procedure (CLAASSEN et al. 1986).

5.3 Other Techniques

Double staining can also be achieved by sequential application of immunogold reagents, the first staining sequence intensified with silver precipitation reaction (black reaction product) and the second without (red color of colloidal gold) as described by MANIGLEY and ROTH 1985, or by a combination of immunogold-silver staining with the ABC method (SAKO et al. 1986).

6 Sensitivity

Sensitivity can be defined in terms of the smallest amount of antigen to be detected and, therefore, depends greatly on the ratio of specific to nonspecific (background) staining. Sensitivity depends on the quality of the antibody, the chemical structure of the antigenic determinant to be detected, and on the methodology (as described above).

Increasing the incubation time with the different layers can improve specific staining, and, in addition, allows further dilution of the antibodies resulting in decreased background reaction. Repetition of layers, e.g. of the primary antibody layer after washing or of the second layer after application of the PAP complex, can increase the amount of label concentrated at the antigenic site, and thus the signal-to-noise ratio. Combination of immunogold techniques with the PAP method with development of peroxidase (with DAB) and subsequent silver intensification results in further increase in sensitivity (SCOPSI and LARSSON 1985).

Reduction of non-specific background staining can be achieved by using highly specific antibodies, such as affinity-purified or monoclonal antibodies. In immunofluorescence microscopy, background staining may result from binding of unconjugated fluorochrome (e.g., fluorescein isothiocyanate), which can be removed, by Sephadex G-25 chromatography (see VAN NOORDEN and POLAK 1983 for further information). Nonspecific adsorption of antibodies can be prevented by pretreatment of antisera with tissue powders. Nonspecific reactive sites in the tissue section are blocked by normal serum applied prior to the immune reaction. This is, of course, not applicable if protein A is used as a component of the immunohistochemical procedure because of its affinity to the Fc portion of immunoglobulins. Moreover, non-specific binding of antibodies can also be minimized or prevented by detergents such as Triton X-100 or Tween 20 included in the washing buffers (VAN NOORDEN and POLAK 1983; JUHL et al. 1984). Pretreatment of dewaxed formalin-fixed paraffin sections with (2%) Tween 20 and dilution of the additional layer (first and second antibody layer in indirect peroxidase immunohistochemistry) with the detergent (0.05–2%) significantly reduces background staining as evaluated with blood group antigen A in human urothelium (JUHL et al. 1984). Antibody penetration may also be facilitated by detergent action.

Increased sensitivity can also be obtained by changing the chromogen protocol in peroxidase immunohistochemistry (TROJANOWSKI et al. 1983). According

to TROJANOWSKI et al. (1983), with neuro-and glial filaments as antigens DAB in combination with imidazole exceeded all other protocols (amino-ethylcarbazole, 0-tolidine, paraphenylenediamine-pyrocatechol, tetramethylbenzidine, diaminobenzidine, and diaminobenzidine at neutral pH with or without intensification with cobalt chloride and at pH 5.1) in sensitivity. 0-tolidine was the least sensitive chromogen in the hands of these authors although the results may vary with other systems. The positive effect of imidazole added to the DAB incubation medium is apparently due to acceleration of DAB oxidation especially at neutral pH (STRAUS 1982).

Further important determinants of sensitivity are the chemical structure and, particularly, the stability of the antigens in question, especially in fixed and embedded material. Denaturation of antigenic determinants occurs with aldehyde fixatives in addition to masking of antigenic determinants by formation of intermolecular linkages (BRANDTZAEG and ROGNUM 1983, 1984a, b; see VALNES et al. 1984 for further information). Masking may also result from cross-linking with other proteins as shown by VALNES et al. (1984) and this can be prevented by protease pretreatment (see RADASZKIEWICZ and DENK 1982 for further information).

7 Fixation and Embedding Procedures

Fixation and embedding may destroy and/or mask antigenic determinants. On the other hand, however, formaldehyde fixation diminishes the net positive charge of tissues by blocking amino groups and, consequently, inhibits electrostatic (nonspecific) attraction of antibodies. In immunohistochemistry, adequate fixation causes a rapid and total immobilization of the antigens along with a sufficient preservation of antigenicity and maintenance of accessibility of the antigens to the immunochemical reagents. However, excellent tissue preservation and antigenicity are often mutually exclusive and therefore compromises have to be made.

Particularly detrimental to antigenicity may be the denaturing effect of aldehyde fixatives (e.g. formaldehyde) at low antigen concentrations. With IgA and IgG as antigens, BRANDTZAEG and ROGNUM (1983, 1984a, b) found that the concentration of IgG detectable in formaldehyde-fixed specimens had to be 8 times higher than in ethanol-fixed ones. The difference was less pronounced with IgA. In Bouin – and Susa – fixed material more than 150 times higher antigen concentrations were required for the detection of IgG, but only 3–8 times higher for IgA (BRANDTZAEG and ROGNUM 1984a, b) indicating the influence of chemical structure, substrate matrix, and other variables.

Antigenic masking by aldehyde fixatives not only depends on the antigen concentration but also on environmental proteins surrounding the antigen which may prevent antigen – antibody interaction by steric hindrance (see BRANDTZAEG and ROGNUM 1983).

However, even fixatives which cross-link proteins and which are commonly used in pathology, like buffered formaldehyde solution, paraformaldehyde or

Bouin's fixative can yield satisfactory results in immunohistochemistry (RADASZKIEWICZ and DENK 1979; BRANDTZAEG and RÖGNUM 1983, 1984a, b; NATHRATH et al. 1985; HOPWOOD 1985; VAN EWIK et al. 1984; BEROD et al. 1981; CURRAN and GREGORY 1980; MEPHAN 1982; JUDD and BRITTEN 1982). Glutaraldehyde mixed in low concentrations with formaldehyde or paraformaldehyde often gives positive results, but destroys most of the antigenic determinants if applied in higher concentrations (see RADASZKIEWICZ and DENK 1982 for references and further information). Alternative fixatives are dimethylsuperimide (reacting specifically with the ϵ -amino-groups of lysine) and periodate-lysine-paraformaldehyde (MCLEAN and NAKANE 1979; see also RADASZKIEWICZ and DENK 1982) which acts by oxidation of membrane carbohydrates to aldehydes (by periodate) and cross-linking via the amino groups of lysine. This fixation procedure, therefore, stabilizes carbohydrate moieties with preservation of antigenic sites (MCLEAN and NAKANE 1979) and has been found to be suitable for a variety of immunohistochemical reactions, including those performed with monoclonal antibodies on paraffin-embedded material (BRENES et al. 1986; HANCOCK et al. 1982). Fixation time is usually not critical, but prolonged fixation (several days) may adversely affect antigenicity (BRENES et al. 1986 for further information).

Alteration of antigenicity may be prevented by using dehydrating substances for fixation instead of aldehyde fixatives, such as ethanol, methanol, ethanol-acetic acid, chloroform or ethyl ether which act by protein precipitation. Ethanol, however, does not immobilize all protein antigens equally well in order to resist prolonged incubation and washing procedures during immunohistochemical staining (BRANDTZAEG and RÖGNUM 1983).

During routine paraffin embedding procedures temperature-dependent destruction of antigenic determinants may occur which can be prevented by using paraffins with lower melting points instead of the conventional paraffin. The paraffin embedding procedure designed by SAINTE-MARIE (1962) after alcohol fixation and dehydration at low temperature (4° C) leads to excellent antigen preservation but is less practicable in routine pathology. An expanded range of antigens can also be detected in paraffin-embedded tissue after freeze-drying (JUDD and BRITTEN 1982; STEIN et al. 1984) or acetone fixation (JUDD and BRITTEN 1982; TANAKA et al. 1984). Several antigens can be demonstrated in plastic-embedded tissue sections after previous fixation in (1%) paraformaldehyde and digestion with trypsin (BECKSTEAD 1985) or in undecalcified methylmethacrylate – embedded formalin-fixed bone biopsies (SCHRÖDER and DELLING 1986).

In conclusion, therefore, it does not seem to be possible to develop a fixative of general applicability but the techniques should be modified depending on the antigens, the antibodies, and the methods to be applied.

Immunohistochemistry is complicated if material requiring decalcification has to be processed (MATHEWS and MASSON 1984). MATHEWS and MASSON (1984) demonstrated that treatment of tissues with neutral EDTA, formic, or acetic acid after formalin fixation combined with trypsin treatment did not adversely affect immunoreactivity of a variety of antigens, including immunoglobulins, factor VIII-related antigen, lysozyme and keratin. However, with decalcifying agents containing mineral acids unreliable results were obtained. Under these

conditions, trypsin concentration and duration of treatment had to be reduced in most cases after extended periods of decalcification, although with some decalcifying agents the tissue was refractory to trypsin action (for detailed information consult MATHEWS and MASSON 1984).

MULLINK et al. (1985) regard fixation in a mercuric chloride – formaldehyde mixture followed by decalcification in acetic acid-formaldehyde-saline as the best procedure for antigen preservation (including CEA and lysozyme) and protection of morphological details in paraffin-embedded human bone marrow biopsies. Trypsinization was usually not needed except for the demonstration of factor VIII-related antigen in megakaryocytes. To date it appears that not all markers specific for a certain cell type (say, lymphocyte subsets) can be detected under these circumstances. Frozen sections are often required.

8 Proteases and Other Pretreatments

Specific immunostaining of several (but not all) antigens can be enhanced by pretreatment of dewaxed sections with proteases, such as pronase and trypsin (see DENK et al. 1977; RADASZKIEWICZ and DENK 1982). Among the various enzymes tested, including pronase, papain, pepsin, trypsin, hyaluronidase (for demonstration of fibronectin; HOLUND and CLEMMENSEN 1982; see also DENK et al. 1977; PINKUS et al. 1985; CURRAN and GREGORY 1978; HUANG et al. 1976; MEPHAM et al. 1979) trypsin seems to be the most suitable in the hands of most authors since its action is easily controlled and structural details of the section are better preserved. Trypsin hydrolyzes arginine-lysine bonds. Attention to the type of trypsin as well as to incubation times is essential and the results may vary accordingly (see PINKUS et al. 1985; HAJDU 1983). The mechanism of the beneficial effect of protease pretreatment, however, is not fully elucidated. According to HUANG et al. (1976) certain immunoreactive sites are unmasked, due eventually to release of protein cross-links, and more antigenic sites become available to interaction with the antibody (see also RADASZKIEWICZ and DENK 1982 for further information). However, individual treatment is needed and optimal regimens should be assessed in pilot studies for different fixation conditions and antigens. Common fixatives, such as formalin-mercury, Bouin and Carnoy, formalin-acetic acid, fix immunoglobulins at least in such a way that antigenicity is preserved and demonstrable without protease pretreatment. However, in tissues fixed with neutral (buffered) formalin, the demonstration of certain antigens (immunoglobulins) requires pretreatment with proteolytic enzymes (MEPHAM 1982). A number of fixatives were also tested, particularly with respect to their effects on immunoglobulins, by CURRAN and GREGORY (1980). Sections of tonsil fixed with buffered (4%) formaldehyde revealed positive immunostaining (PAP method) only after trypsinization, whereas enzyme pretreatment was not required with tissues fixed with Bouin's or Carnoy's fluid, with solutions containing mercuric chloride, and particularly with formol-saline containing (2–10%) acetic acid, as well as with a combination of acetic acid (10%) – formol-saline and formol sublimate (CURRAN and GREGORY 1980).

9 Specificity and Pitfalls

The specificity of the immunohistochemical staining reaction has to be thoroughly established and should be evaluated with respect to antibody and method specificity (see VAN LEEUWEN 1986).

Affinity purification of antibodies on Sepharose-4B-CNBr columns to which the antigen is coupled or on gels containing antigens separated by SDS-polyacrylamide gel electrophoresis is a practical method to reveal monospecific antibodies (see DE MEY 1983 for review). Considerable progress regarding specificity has been made by the development of the hybridoma technology for production of monoclonal antibodies. Moreover, this technique creates the potential for obtaining large amounts of antibodies against a specific antigenic determinant even if impure antigens are used for immunization, and is thus important in oncology, for example to detect tumor-specific antigens (see DIAMOND and SCHARFF 1982; BHAN 1984). Disadvantages are the often poor avidity and stability of monoclonal antibodies and the possibility of cross-reactivity (DIAMOND and SCHARFF 1982).

The identification and characterization of the antigen recognized by the antibodies is another basis for specific immunohistochemistry. This can be achieved by immunoprecipitation, immunoblotting procedures and radioimmunoassay (VAN NOORDEN and POLLAK 1983; DE MEY 1983 for review). Method specificity can be checked by absorption of primary antibodies with their respective antigens or omission of one of the reagents of the staining sequences.

False positive results may be caused by peroxidase-like activity in haeme (in erythrocytes) which is blocked by methanol/H₂O₂. Endogenous peroxidase (catalase in peroxisomes and cytochrome c in mitochondria) is capable of reacting with DAB-H₂O₂ but is inhibited by methanol, nitroferricyanide or phenylhydrazine, as well as (10%) methanol and (3%) H₂O₂. Free reactive groups in the tissue may lead to non-specific binding of IgG (particularly after aldehyde fixation) and this is prevented by pretreatment with normal serum (not recognized by the immunoreagents), borohydride, serum albumin, or buffers containing lysine, glycine, or TRIS. Charged groups within proteins can be responsible for ionic interactions with antibodies which can be minimized with buffers with increased ionic strength (e.g. 0.5 M NaCl). Moreover, incubation of tissue sections at pH 8.6 also minimizes the influence of ionic charges since IgG has an isoelectric point at 8.6. Hydrophobic interactions of antibodies and labels with hydrophobic tissue components can be prevented by addition of Triton X-100 to the media. Immunoglobulin aggregates (eliminated by dilution or high speed centrifugation) may adhere nonspecifically to hydrophobic tissue components (or embedding media). Natural and contaminating antibodies (due to impurities of the antigen preparation used for immunization) should also be considered as source of false-positive results. In frozen sections, unwanted non-specific background staining by the ABC method may occur in liver, pancreas and kidney, possibly due to the presence of biotin or related substances reacting with avidin. This non-specific binding can be abolished by pretreatment of the section with avidin followed by biotin (HSU and RAINE 1984). Non-specific avidin binding can also be induced by the presence of endogenous lectins (for

example, in nerve fibers) which react with mannose-containing glycoproteins, such as avidin. This unwanted reactivity can be blocked by pretreatment with alpha-methyl-D-mannoside (HSU and RAINE 1984). The reaction of mast cell granules with ABC in frozen as well as paraffin sections can be prevented by using the ABC solution at pH 9.4 (BUSSOLATI and GUGLIOTTA 1983).

False negative results may be due to modification of antigenic sites by fixation, dehydration and embedding procedures. Antigen-antibody interaction may be hampered by impaired antibody penetration, which is then facilitated by non-ionic detergents (Triton X-100, 0.1–0.5%). Steric hindrance exerted either by embedding medium or associated proteins may also impede interaction of antibodies with the antigen. In the unlabeled antibody-enzyme method, a high concentration of antigen within the tissue section and insufficient dilution of the primary antibodies may prevent bridge formation by the second antibody (see VAN LEEUWEN 1986 for references).

10 Outlook

Immunohistochemistry has contributed considerably to our understanding of basic biological processes but also to refinement of histopathologic evaluation. It plays a particularly valuable role in tumor histopathology, provided strict controls and critical interpretation are used. The immunohistochemical demonstration of a given substance depends on several factors, including quantity and quality of the antigen to be determined, antibody reactivity and the reliability and efficiency of the immunohistochemical procedure employed. Commercial institutions now play a major role in provision of reagents as well as in specificity testing. This should, however, not obscure the fact that valuable information in histopathology can only be obtained by those who understand the basic principles and pitfalls of the reactions and who are experienced in histopathological diagnosis.

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Epithelial Tumor Markers: Cytokeratins and Tissue Polypeptide Antigen (TPA)

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1 Introduction

The concept of morphological tumor markers is derived from the fact that tumor cells express a specific, albeit limited, program of differentiation that usually represents at least a part of the differentiation program of the putative cell of origin. Epithelial tumor markers, which are discussed in this and the following contributions, should facilitate the identification and characterization of poorly differentiated and/or metastatic epithelial tumors in tissue sections, even though the morphological appearance of such neoplasms may be uncharacteristic.

The epithelial cell system is highly complex. Thus, the process of epithelial differentiation does not simply involve a single cell type but rather a variety of cell forms of different histogenetic origin that differ widely with respect to their structure and function. However, normal epithelial cells have several morphological features in common, most notable the presence of desmosomes (maculae adhaerentes) and tonofilaments. Malignant epithelial tumors may lose these features to varying degrees, resulting in the absence of a recognizable epithelial morphology in certain anaplastic epithelium-derived tumors, even at the electron microscope level (GHADIALY 1980). Recently, in diagnostic histo-

pathology, molecular components that are specifically expressed in epithelial cells have acquired great importance as epithelial tumor markers or, more correctly, epithelial differentiation markers. Such markers have two main applications, i.e., in distinguishing epithelial from nonepithelial tumors, and in distinguishing different types of epithelial tumors. The present chapter is concerned with cytokeratins, a family of cytoskeletal proteins that have been convincingly demonstrated to be very reliable epithelial markers. Moreover, there is an increasing body of evidence indicating that the individual proteins of this family can be used for the subtyping of different epithelia and different epithelial tumors.

2 Cytokeratin as a General Marker of Epithelia

2.1 Cytokeratin-type Intermediate Filaments

Intermediate-sized filaments (intermediate filaments) are a ubiquitous cytoplasmic filament system (filament diameter, about 10 nm) and are part of the cytoskeleton. These filaments can be divided into five classes (cytokeratin, vimentin, desmin, glial filaments, and neurofilaments) on the basis of their protein composition, which is differentiation specific (for reviews see LAZARIDES 1980; FRANKE et al. 1982; OSBORN and WEBER 1983).

Cytokeratin filaments are expressed in epithelial cells. At the electron microscope level, they are indistinguishable from other types of intermediate filaments, being arranged in loose bundles or, in stratified squamous epithelia, occurring as tonofilaments that form densely packed bundles (tonofibrils). A typical feature of cytokeratin filaments is their association with the cytoplasmic plaques of desmosomes. In certain exceptional cases, they may be present in the form of paranuclear globular aggregates, also referred to as fibrous bodies (RACADOT et al. 1964; see Section 2.6).

2.2 Distribution of Cytokeratin Filaments in Normal Tissues

In 1978, using immunofluorescence microscopy, it was found that antibodies raised against prekeratin (α -keratin) obtained from epidermis react with filament arrays in cultured cells of diverse origin, including simple epithelia of the mammary gland and kidneys as well as in a number of epithelial tissues, even though keratinization is completely absent in these cell types (FRANKE et al. 1978a, b; SUN and GREEN 1978). Since then α -keratin related proteins have been detected in all types of epithelial cells (FRANKE et al. 1979a, 1981a-d; SUN et al. 1979) and have been collectively termed cytokeratins or keratins. As these polypeptides are related to each other (see Section 3.1), they have certain antigenic determinants in common. Therefore, certain polyclonal (FRANKE et al. 1981a) and monoclonal (Table 1) antibodies are capable of detecting the cytokeratin

Table 1. Broad-spectrum cytokeratin antibodies

Antibody	Cytokeratin polypeptides recognized	References
K _G 8.13	CKs nos. 1, 5, 6, 7, 8, 18	GIGI et al. 1982
AE 3	CKs nos. 1–8	TSENG et al. 1982; COOPER et al. 1985
Clone 80	Basic CKs (including CKs nos. 1 and 5)	VAN MUIJEN et al. 1984
LP 34	Several CKs	LANE et al. 1985
lu-5	Most CKs	VON OVERBECK et al. 1985; FRANKE WW, unpublished
KL-1 ^a	Epidermal CKs (55–57 KD polypeptide group) ^b	VIAC et al. 1983

^a Basal layer of stratified squamous epithelia negative

^b No biochemical data for non-epidermal CKs available

filaments present in all epithelial cells; these antibodies can be regarded as being broad-spectrum cytokeratin antibodies (Table 1).

In normal tissues, the cytokeratin filaments recognized by such broad-spectrum cytokeratin antibodies have been found (usually arranged in fibrillar cytoplasmic arrays) in all cell types that, according to the classical definition, are considered to be epithelial cells. Thus, cytokeratin filaments are not only expressed in the epidermis – the classical tissue of keratin research – but also in all types of noncornifying stratified squamous epithelia, pseudostratified epithelia, myoepithelial cells, and even simple epithelia (FRANKE et al. 1978a, 1979a, 1980, 1981a; SUN et al. 1979; ALTMANNBERGER et al. 1981b; OSBORN and WEBER 1983), including epithelia of mesodermal origin, e.g., renal tubular cells (BACHMANN et al. 1983; RAMAEKERS et al. 1983c; HOLTHÖFER et al. 1984), epithelial cells of the upper male and female genital tract (CZERNOBILSKY et al. 1985; ACHTSTÄTTER et al. 1985), and mesothelial cells lacking any connection with other epithelial surfaces of the body. Cytokeratin filaments are also present in most endocrine cells (HOEFLER et al. 1984a; RAMAEKERS et al. 1983c; MIETTINEN et al. 1984a, 1985a–c; VAN MUIJEN et al. 1984a), including cells of the dispersed neuroendocrine system (HOEFLER and DENK 1984) such as Merkel cells of the skin (MOLL et al. 1984), which thus are also true epithelial cells in spite of their simultaneous expression of neural features.

Numerous studies have shown that nonepithelial cells lack cytokeratin filaments. These cytokeratin-negative cells include the various types of mesenchymal cells, myogenic cells, neuronal cells, glial cells, and melanocytes which, in turn, are characterized by the presence of other types of intermediate filaments (for a review see OSBORN and WEBER 1983). Certain epithelioid tissues, e.g., the so-called lens epithelium (RAMAEKERS et al. 1980), the pigment epithelium of the retina (at least in chicken), Sertoli cells (FRANKE et al. 1979b; for the detection of occasional cytokeratin-positive cells in human seminiferous tubules, see MIETTINEN et al. 1985d), glomerular podocytes (BACHMANN et al. 1983; RAMAEKERS et al. 1983c; HOLTHÖFER et al. 1984), and vascular endothelium (FRANKE et al. 1979c) are also cytokeratin negative, whereas they do express vimentin filaments. These cell types cannot, however, be regarded as being truly epithelial because they contain neither desmosomes nor tonofilaments. Immunohistochemical staining of certain muscle cells for cytokeratins has been reported by HUITFELDT and BRANDTZAEG (1985).

Thus, cytokeratin (as recognized by broad-spectrum antibodies) can be regarded as being a general, a specific, and, perhaps, the best marker of epithelial differentiation.

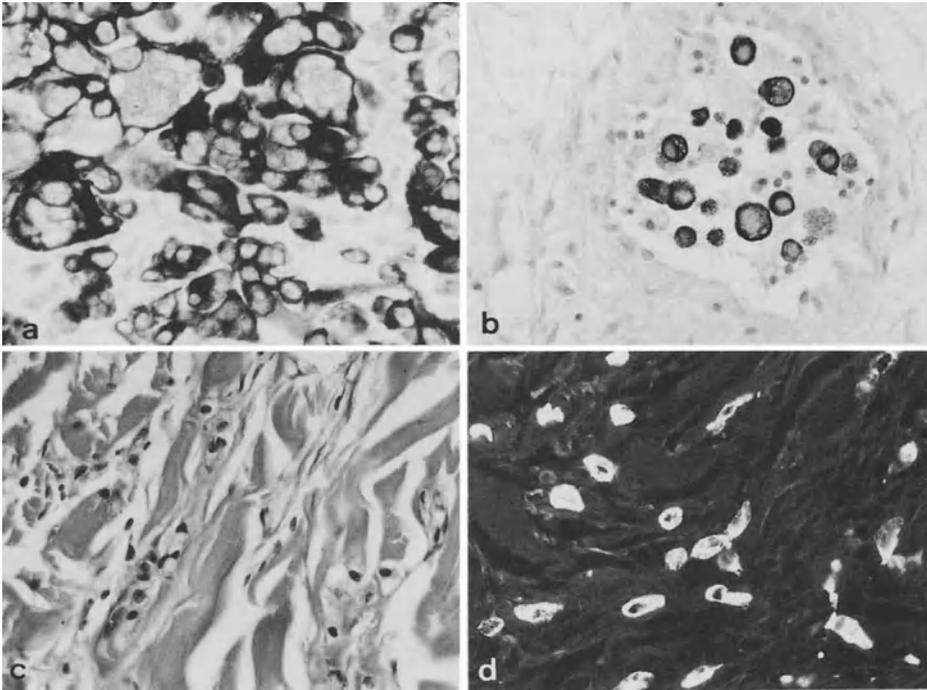


Fig. 1 a–d. Immunocytochemical detection of cytokeratin in epithelial tumors, using broad-spectrum cytokeratin antibodies. **a** Anaplastic carcinoma, peritoneal metastasis (primary tumor probably in ovary), strongly positive with cytokeratin antibody AE1/AE3 (paraffin section; ABC-peroxidase). **b** Lymphangiosis carcinomatosa derived from an adenocarcinoma of the stomach, showing strong staining of individual tumor cells in a lymphatic vessel close to a lymph node with cytokeratin antibody AE1/AE3 (paraffin section; ABC-peroxidase). **c, d** Disparately growing tumor cells in a skin metastasis of a breast carcinoma, which are hard to recognize in HE-staining (**c**), are clearly identified by the cytokeratin antibody (**d**; antibody AE1/AE3; paraffin section; immunofluorescence microscopy). **a, b** $\times 240$; **c, d** $\times 190$

2.3 Expression of Cytokeratin Filaments in Malignant Tumors

From the observation that cultured carcinoma cells such as HeLa cells (a cell line derived from a cervical adenocarcinoma) contain intermediate filaments of the cytokeratin type (sometimes together with vimentin filaments), FRANKE et al. (1978a, 1979d) proposed the use of intermediate-filament antibodies for demonstrating whether a given tumor is of epithelial origin. Using available broad-spectrum cytokeratin antibodies, it has since been found that malignant epithelial tumors, including their metastases, consistently maintain the expression of cytokeratin-type intermediate filaments (Fig. 1 a; BANNASCH et al. 1980; BATTIFORA et al. 1980; LÖNING et al. 1980; SCHLEGEL et al. 1980; ALTMANN-SBERGER et al. 1981 a; CASELITZ et al. 1981; GABBIANI et al. 1981; SIEINSKI et al. 1981). Numerous recent studies have further confirmed and demonstrated that the expression of cytokeratin filaments is a constant feature of all carcinomas,

Table 2. Cytokeratin-positive tumors

Tumor type	References
Squamous cell carcinomas (skin, upper digestive and respiratory tract, lung, cervix uteri)	1
Nasopharyngeal carcinomas (including lymphoepitheliomas)	2
Thymomas	3
Transitional cell carcinomas	1
Adenocarcinomas (lung, breast, gastrointestinal tract, female genital tract, prostate)	1
Choroid plexus carcinomas	4
Renal cell carcinomas ^a	5
Adenomatoid tumors	6
Malignant mesotheliomas (epithelial, fibrous)	7
Chordomas	8
Adamantinomas	9
Synovial sarcomas	10
Epithelioid sarcomas	11
Thyroid carcinomas	12
Adrenal cortical carcinomas ^b	13
Epithelial neuroendocrine tumors (Merkel cell carcinomas, carcinoid tumors, islet cell tumors, medullary carcinomas of thyroid, small cell carcinomas of lung)	14
Embryonal carcinomas, teratocarcinomas, choriocarcinomas, endodermal sinus tumors	15
Undifferentiated/anaplastic carcinomas	1, 16

^a Rare cases cytokeratin-negative but vimentin-positive

^b 13 of 25 cases were cytokeratin-positive

References: 1 BATTIFORA et al. 1980; SCHLEGEL et al. 1980; BANNASCH et al. 1980; GABBIANI et al. 1981; ALTMANNBERGER et al. 1981a, 1982; SIENSKI et al. 1981; CASELITZ et al. 1981; ESPINOZA and AZAR 1982; NAGLE et al. 1983; SABA et al. 1983; RAMAEKERS et al. 1983b, c; MAKIN et al. 1984; PINKUS et al. 1986; 2 MIETTINEN et al. 1982b; MADRI and BARWICK 1982; 3 BATTIFORA et al. 1980; LOENING et al. 1981; 4 MIETTINEN et al. 1986; 5 HOLTHÖFER et al. 1983; HERMAN et al. 1983; 6 BARWICK and MADRI 1982; SAID et al. 1982; 7 CORSON and PINKUS 1982; HOLDEN and CHURG 1984; LA ROCCA and RHEINWALD 1984; CHURG 1985; BLOBEL et al. 1985b; 8 MIETTINEN et al. 1983a; RAJU et al. 1983; 9 ROSAI and PINKUS 1982; 10 MIETTINEN et al. 1982a, 1983b; see also pp. 155–178; 11 CHASE et al. 1984; see also pp. 155–178; 12 MIETTINEN et al. 1984a; 13 MIETTINEN et al. 1985c; 14 For references, see Section 2.6; 15 KAHN et al. 1983; BATTIFORA et al. 1984; MIETTINEN et al. 1985d; RAMAEKERS et al. 1985a; 16 LAUDER et al. 1984; KAHN et al. 1984

irrespective of their degree of differentiation (for reviews, see OSBORN and WEBER 1983; RAMAEKERS et al. 1983c; MIETTINEN et al. 1984b; ERLANDSON 1984; CORSON 1986). The various types of malignant tumors which have been found to be cytokeratin positive are listed in Table 2. In contrast to other epithelial markers such as epithelial membrane antigen (EMA; PINKUS and KURTIN 1985; PINKUS et al. 1986), cytokeratin filaments are usually uniformly distributed among carcinoma cells. In view of the steadily accumulating data concerning the heterogeneity of tumor cell populations (FIDLER et al. 1978; NICOLSON 1984), the degree of stability of cytokeratin expression in tumors is remarkably high. Therefore, cytokeratin is also a reliable marker for undifferentiated and anaplastic carcinomas (Fig. 1a), for disparately growing infiltrating carcinoma cells (Fig. 1c, d), for metastasizing single carcinoma cells in lymph nodes and the

bone marrow (Fig. 1b), and for isolated carcinoma cells in suspension, e.g., in effusions and other body fluids.

Tumors containing spindle cells of a sarcomatoid appearance, e.g., mesotheliomas of the fibrous type, monophasic synovial sarcomas, and some carcinomas of the kidneys, skin (CORSON 1986), and other sites, are cytokeratin positive (Table 2) and are thus of an epithelial nature (for the co-expression of vimentin, see Section 2.5); this is in good agreement with the presence of epithelial features such as desmosomes in these tumors, as revealed by ultrastructural investigations (GHADIALLY 1980; ERLANDSON 1981). These findings are of particular importance for differential diagnosis, for they make it possible to distinguish such tumors from true sarcomas. Cytokeratin is also present in the epithelial subgroup of neuroendocrine tumors, sometimes occurring simultaneously with neurofilaments (see Section 2.6).

In certain rare tumors, the expression of cytokeratin appears to be confined to a subpopulation of cells. This has been reported for adrenal cortical carcinomas, some of which may also exhibit the complete absence of cytokeratin immunoreactivity (MIETTINEN et al. 1985b), suggesting that the extent of epithelial differentiation in these tumors is very low and variable. Certain tumors consisting mainly of vimentin-positive tumor cells, including granulosa cell tumors of the ovaries (CZERNOBILSKY et al. 1987), skeletal and extraskeletal Ewing's sarcomas (MOLL et al. 1987), and seminomas (MIETTINEN et al. 1985d; RAMAEKERS et al. 1985a; DENK et al. 1986) contain sparsely distributed, scattered cytokeratin-positive tumor cells. These tumors are also characterized by the presence of variable numbers of desmoplakin-positive true desmosomes (for the distribution of desmoplakin in human tumors, see MOLL et al. 1986a).

Nonepithelial tumors, including sarcomas, malignant fibrous histiocytomas, lymphomas, malignant melanomas, Schwannomas, gliomas, neuroblastomas, and pheochromocytomas are negative for cytokeratin proteins, but express other types of intermediate filaments (for references, see OSBORN and WEBER 1983; RAMAEKERS et al. 1983c; MIETTINEN et al. 1984b; CORSON 1986). With respect to diagnosis, this is especially important when nonepithelial tumors exhibit a solid or epithelioid growth pattern as is the case in, for example, some sarcomas, lymphomas, and melanomas that only express vimentin filaments.

2.4 Detection of Cytokeratin Filaments in Formalin-fixed, Paraffin-embedded Tissue

The reliable immunocytochemical detection of cytokeratin filaments is possible in frozen tissue, as well as in tissue fixed with alcohol (ALTMANNBERGER et al. 1981b) or Methacarn (GOWN and VOGEL 1984) and embedded in paraffin.

When applied to formalin-fixed, paraffin-embedded material, many cytokeratin antibodies yield false-negative or misleadingly irregular results. Unfortunately, in many cases, only routinely fixed material may be available. This has prompted the search for conditions which allow the detection of cytokeratins in such material (see also pp. 47-69). The broad-spectrum cytokeratin antibodies that are reactive with formalin-fixed, paraffin-embedded tissue are listed in Table 3; among these, the antibody lu-5 appears to have the broadest spectrum of reactivity. Limited proteolytic digestion of sections is essential before incubation with these antibodies. The use of pepsin (MIETTINEN et al. 1983a; our own findings), trypsin (PINKUS et al. 1985; BATTIFORA and SILVA 1986), and pronase (GOWN and VOGEL 1985; our own data) has produced good or, at least, fairly reliable results (Fig. 1). However, even when such procedures are applied, negative results obtained in formalin-fixed material should be interpreted with caution, since small amounts of cytokeratin filaments may escape detection, or the antigens may be damaged during tissue processing.

Table 3. Monoclonal cytokeratin antibodies with broad or extended spectrum reactive on formalin-fixed, paraffin-embedded tissue

Antibody	Cell type spectrum	References
lu-5	Broad	VON OVERBECK et al. 1985
AE1	Extended (secretory simple epithelia ^a and suprabasal layers of epidermis negative)	TSENG et al. 1982; SPAGNOLO et al. 1985; PINKUS et al. 1986
KL-1	Extended (basal layer of stratified squamous epithelia negative)	VIAC et al. 1983
PKK 1	Extended (epidermis negative)	MIETTINEN et al. 1985d; VIRTANEN et al. 1985
CAM 5.2	Extended (most hepatocytes and all stratified squamous epithelia negative)	MAKIN et al. 1984

^a Hepatocytes, pancreatic acinar cells

2.5 Co-expression of Cytokeratin and Vimentin Filaments

In epithelial tumors, cytokeratin filaments are usually the only intermediate-filament class present; however, as has been described for cultured epithelial and carcinomatous cells (FRANKE et al. 1978a, 1979d, e, 1981b; VIRTANEN et al. 1981), certain types of epithelial tumors also express (in the majority of cases) vimentin filaments (Table 4; Fig. 2). This phenomenon was first observed in cells of pleomorphic adenomas of the parotid gland (CASELITZ et al. 1981; KREPLER et al. 1982). The co-expression of cytokeratin and vimentin filaments is particularly striking in cells exhibiting a clearly epithelial structure and tumors derived therefrom, e.g., certain cells of the endometrial glands and adenocarcinomas of the endometrium (MCNUTT et al. 1985), and the follicle epithelium (B. DOCKHORN-DWORNICZAK and W.W. FRANKE, personal communication) and follicular and papillary carcinomas of the thyroid gland (MIETTINEN et al. 1984a). In malignant mesotheliomas, the co-expression of cytokeratin and vimentin occurs most consistently in fibrous tumors and areas consisting of spindle-shaped, fibroblast-like tumor cells, but may also be observed in epithelial-like structures of these tumors (BLOBEL et al. 1985b). Here again, tumors can be seen to maintain a feature of the corresponding normal cells, i.e., pleural mesothelium cells (LARocca et al. 1984). Normal granulosa cells of the ovary (CZERNOBILSKY et al. 1985) as well as granulosa cell tumors (CZERNOBILSKY et al. 1987) co-express both intermediate-filament types. Whereas most renal cell carcinomas co-express cytokeratin and vimentin (Fig. 2; HERMAN et al. 1983; HOLT-HÖFER et al. 1983; WALDHERR and SCHWECHHEIMER 1985; PITZ et al. 1987) the normal adult kidney tubules exclusively express cytokeratin filaments, thus suggesting that, in these tumors, vimentin expression is switched on during tumorigenesis. A rare renal cell carcinoma subtype, the chromophobe cell renal carcinoma (THOENES et al. 1985), as well as renal oncocytomas can be distinguished

Table 4. Co-expression of cytokeratin and vimentin filaments in malignant tumors

Tumor type	References
Adenoid-cystic carcinomas of salivary gland	1
Carcinomas of thyroid gland (follicular, papillary, anaplastic)	2
Adenocarcinomas of lung ^a	3, 4
Large cell carcinomas of lung ^a	4
Renal cell carcinomas (except chromophobe type)	5
Nephroblastomas ^b	6
Malignant rhabdoid tumors of kidney	7
Adenocarcinomas of endometrium	8, 9
Carcinomas of ovary ^a	8
Granulosa cell tumors of ovary ^c	10
Adenocarcinomas of prostate	11
Malignant mesotheliomas	12
Epithelioid sarcomas	13
Chordomas	14

^a Only a minority of tumors revealed vimentin in addition to cytokeratins

^b Co-expression of cytokeratins and vimentin was found in blastema cells

^c These tumors express predominantly vimentin filaments, as well as desmoplakin

References: 1 CASELITZ et al. 1984; 2 MIETTINEN et al. 1984a; DROESE et al. 1984; SCHRÖDER et al. 1986; 3 JASANI et al. 1985; 4 UPTON et al. 1986; 5 HERMAN et al. 1983; HOLTHÖFER et al. 1983; PITZ et al. 1987; 6 ALTMANNBERGER et al. 1984; DENK et al. 1985; 7 VOGEL et al. 1984; 8 McNUTT et al. 1985; 9 DABBS et al. 1986; 10 CZERNOBILSKY et al. 1987; 11 WERNERT et al. 1986; 12 LA ROCCA and RHEINWALD 1984; CHURG 1985; BLOBEL et al. 1985b; 13 MIETTINEN and DAMJANOV 1985; see also pp. 155–178; 14 MIETTINEN et al. 1983; GOWN and VOGEL 1985; COINDRE et al. 1986; ABENOZA and SIBLEY 1986

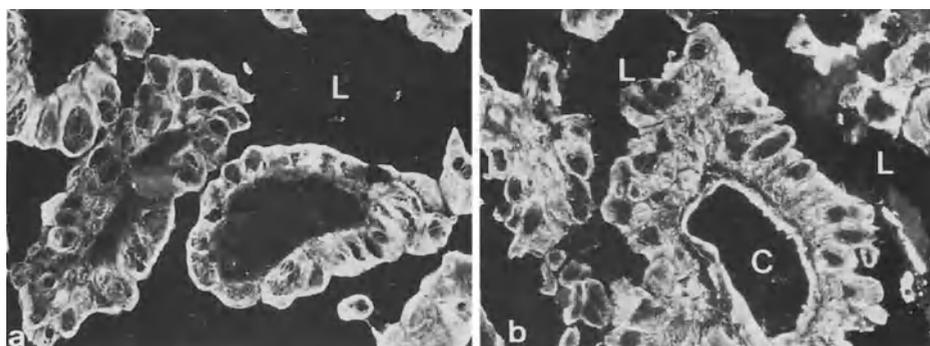


Fig. 2. Co-expression of cytokeratin filaments (**a**; monoclonal antibody against CK no. 18; frozen sections; immunofluorescence microscopy) and vimentin (**b**; antibody VIM-9) by the tumor cells of a renal cell carcinoma (eosinophilic-granular; tubulo-papillary). Note that the vimentin antibody (**b**) also stains the endothelium of a capillary (C). L, lumen. $\times 260$

from the common types of renal cell tumors due to their exclusive expression of cytokeratin filaments (PITZ et al. 1987).

It is of interest that many of the tumors that exhibit the co-expression of vimentin and cytokeratin are derived from cells of mesodermal origin and, therefore, must be histogenetically related to (vimentin-expressing) mesenchymal

cells. It is also important to note that the distribution of the expression of cytokeratin filaments in most of these tumor types is uniform, underlining their true epithelial character, regardless of the co-expression of vimentin. Clearly, testing for the presence or absence of cytokeratin-vimentin co-expression is of value for differential diagnosis, as this feature appears to be restricted to certain carcinoma types (Table 4; see also GOWN and VOGEL 1985; McNUTT et al. 1985).

2.6 Co-expression of Cytokeratin and Neurofilaments

Recently, another type of intermediate-filament co-expression has been reported, i.e., the co-expression of cytokeratin and neurofilaments. This phenomenon appears to be a fairly characteristic feature of certain types of epithelial neuroendocrine tumors (Table 5).

The co-expression of cytokeratins (of the simple-epithelial type; see Section 3) and neurofilaments is most consistently found in neuroendocrine (Merkel cell) carcinomas of the skin (Fig. 3). In a series of 12 patients with such carcinomas, both intermediate-filament types were found in frozen tumor samples of all of the patients (MOLL et al. 1986b; see also HOEFLER et al. 1984b, 1985). In formalin-fixed samples of such tumors, neurofilaments have been detected with less frequency (HOEFLER et al. 1984b; SIBLEY and DAHL 1985). In such tumors, some of the filaments are arranged in fibrous bodies (see Section 2.1), a feature which is of diagnostic significance. Other types of neuroendocrine tumors, including carcinoid tumors of the bronchus and medullary carcinomas of the thyroid gland, also exhibit combined cytokeratin and neurofilament expression in some cases (Table 5); moreover, medullary carcinomas sometimes express vimentin filaments too. In contrast to earlier reports (LEHTO et al. 1983), several recent studies have shown that all small cell carcinomas of the lung contain cytokeratin intermediate filaments, and that most, if not all, of them

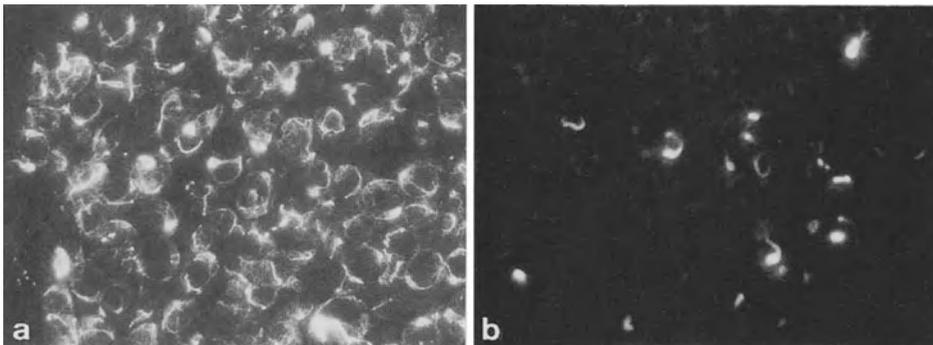


Fig. 3. Co-expression of cytokeratin (**a**; antibody PKK1; frozen sections; immunofluorescence microscopy) and neurofilaments (**b**; antibody 2F11) in a Merkel cell carcinoma of the skin. Note uniform positivity for cytokeratin (**a**), whereas neurofilaments are expressed only by some tumor cells, and are mostly arranged in globular aggregates (**b**). $\times 380$

Table 5. Expression of cytokeratin and neurofilaments in epithelial neuroendocrine tumors

Tumor type	Cytokeratin	Neurofilament	References
Neuroendocrine (Merkel cell) carcinomas of skin	+	+	1
Carcinoid tumors of bronchus	+	+/-	2
Parathyroid adenomas	+	+/-	3
Islet cell tumors of pancreas	+	+/-	4, 5
Medullary carcinomas of thyroid gland ^a	+	+/-	6
Carcinoid tumors of small intestine	+	-/+	4, 7
Small cell carcinomas of lung	+	-	8

^a These tumors may, in addition, express vimentin

References: 1 HOEFLER et al. 1984b; GOULD et al. 1985; SIBLEY and DAHL 1985; MOLL et al. 1986b; 2 LEHTO et al. 1985; BLOBEL et al. 1985a; 3 MIETTINEN et al. 1985a; 4 MIETTINEN et al. 1985b; 5 MOLL et al. 1986a; 6 DROESE et al. 1984; SCHRÖDER et al. 1986; 7 R. MOLL, unpublished results; 8 See text for references

are exclusively cytokeratin positive (VAN MUIJEN et al. 1984b; BLOBEL et al. 1985a; BROERS et al. 1985b; GATTER et al. 1985; MOSS et al. 1986). However, some 'variant cell lines' cultured from small cell carcinomas of the lung have been reported to be neurofilament positive and apparently lack cytokeratin filaments (BROERS et al. 1985a). It remains to be seen whether any in situ tumors have this phenotype (for the finding of neurofilaments in one case of a poorly differentiated squamous cell carcinoma of the lung, see VAN MUIJEN et al. 1984b).

Since normal islet cells of the pancreas (MIETTINEN et al. 1985b) and Merkel cells of the skin (MOLL et al. 1984) are exclusively cytokeratin positive, the corresponding tumors appear to acquire neurofilament expression during tumorigenesis. Obviously, the expression of neurofilaments is an optional part of the neuroendocrine differentiation program, which also involves the expression of other neuronal markers, such as neuron-specific enolase (NSE) and neurosecretory granules containing chromogranin and neuropeptide hormones. With regard to diagnosis, the co-expression of cytokeratin and neurofilaments can be used, for example, to distinguish a Merkel cell tumor from a malignant lymphoma or from a metastasis of a small cell carcinoma of the lung (MOLL et al. 1986b; BATTIFORA and SILVA 1986).

2.7 Co-expression of Cytokeratin and Glial Filaments

This rare type of co-expression has been observed in some normal myoepithelial cells of the parotid gland as well as in some cells present in pleomorphic adenomas (ACHTSTÄTTER et al. 1986), the latter of which also contain vimentin filaments. Cytokeratin, glial, and vimentin filaments may also be present together in choroid plexus carcinomas (MIETTINEN et al. 1986), but it has yet to be shown that they co-exist in individual tumors cells.

3 Diversity and Cell-Type Specificity of Cytokeratin Polypeptides

3.1 The Cytokeratin Family of Proteins

In contrast to all other classes of intermediate filaments, cytokeratin filaments are made up of a highly complex multigene family of proteins. In studies of cytokeratin polypeptides in human tissues, it has been shown that even epidermal keratinocytes contain several cytokeratin polypeptides (FUCHS and GREEN 1978, 1979; SUN and GREEN 1978). Nineteen distinct cytokeratin (CK) polypeptides have been found in the various types of human epithelial cells (CKs nos. 1–19) ranging in apparent molecular weight from 40,000 to 68,000 (Table 6; WINTER et al. 1980; DORAN et al. 1980; FRANKE et al. 1981c; MOLL et al. 1982; TSENG et al. 1982; WU et al. 1982; for reviews, see SUN et al. 1984; QUINLAN et al. 1985; COOPER et al. 1985; for the occurrence of a distinct set of eight keratin polypeptides in hair-forming cells, which are not discussed here, see HEID et al. 1986).

These polypeptides, all of which contain a central α -helical rod piece with an apparent molecular weight of 38,000, are related to each other to varying degrees with respect to their amino acid sequences (QUINLAN et al. 1985; FUCHS et al. 1985; MAGIN et al. 1986). According to their sequence relationships, they can be subdivided into a basic (B) subfamily (type II) comprising CKs nos. 1–8, and an acidic (A) subfamily (type I) comprising CKs nos. 9–19 (Table 6; FUCHS et al. 1981; MOLL et al. 1982; SCHILLER et al. 1982; TSENG et al. 1982; HANUKOGLU and FUCHS 1983; KIM et al. 1983; WEBER and GEISLER 1984; HATZFELD and FRANKE 1985; STEINERT et al. 1985; COOPER et al. 1985). At least one basic and one acidic polypeptide are present in each filament, forming heterotypic tetramer complexes (B_2A_2) that represent the actual subunits of the cytokeratin intermediate filaments (for a review, see QUINLAN et al. 1985). Accordingly, the cytokeratin polypeptides within a given cell are always expressed in pairs and in a coordinated fashion, i.e., a basic (type II) cytokeratin is always co-expressed with an acidic (type I) partner molecule.

With respect to the use of cytokeratins as differentiation markers, it is of particular importance that cytokeratin pairs are expressed in a differentiation-specific manner; thus, cytokeratin expression is correlated with the various epithelial cell types, so that different cytokeratin polypeptide patterns are observed in the various types of epithelia (Table 6).

3.2 Cytokeratin Polypeptide Patterns in Normal Epithelia

The cytokeratins can be roughly divided, according to their expression spectrum, into stratified-epithelium and simple-epithelium-type components. The *stratified-epithelium-type cytokeratins* comprise the higher-molecular-weight polypeptides of each subfamily, i.e., CKs nos. 1–6 of the basic group, and CKs nos. 9–17 of the acidic subfamily. Of these, CK no. 5, which is co-expressed with CK no. 14 and/or CK no. 17, is found in most stratified squamous epithelia as well as (most probably) in the basal cells of pseudostratified epithelia and in myoepithelial cells (Table 6; MOLL et al. 1982, 1983a, b; TSENG et al. 1982; NELSON and SUN 1983; BLOBEL et al. 1984; COOPER et al. 1985; NAGLE et al. 1985b; MOLL and FRANKE 1986); therefore, the cytokeratin pairs, 5/14 and

Table 6. Cytokeratin polypeptide patterns of normal epithelia and epithelial tumors, obtained using two-dimensional gel electrophoresis (MOLL et al. 1982; MOLL and FRANKE 1986). For each tumor, a typical and representative example is shown. *Large dot*, high relative proportion; *small dot*, low relative proportion of the respective polypeptide

Cytokeratin No. ^a Molecular weight ($\times 10^{-3}$)	Stratified-epithelium type										Simple-epithelium type					
	BASIC					10	ACIDIC					BASIC		ACIDIC		
	1	3	4	5	6	11	12	13	14	15	16	17	7	8	18	19
1. Normal epithelia																
Stratified squamous epithelia																
Epidermis	•			•	•	•										
Non-cornifying strat. squam epithelium			•	•	•											•
Corneal epithelium		•		•	•											
Hair follicle outer root sheath				•	•											•
Simple epithelia																
Secretory epithelial cells ^b													•	•	•	•
Intestinal epithelium													•	•	•	•
Ductal epithelium ^c												•	•	•	•	•
Urothelium			•	•				•				•	•	•	•	•
Mammary gland ducts				•				•				•	•	•	•	•
Respiratory epithelium				•	•							•	•	•	•	•
2. Epithelial tumors																
Squamous cell carcinomas																
Skin	•			•	•	•			•	•	•					
Tongue				•	•	•			•	•	•					•
Lung				•	•	•			•	•	•					•
Cervix (moderately differentiated)				•	•	•			•	•	•			•	•	•
Cervix (poorly differentiated)				•	•	•			•	•	•			•	•	•
Hepatocellular carcinoma													•	•	•	•
Adenocarcinomas																
Colon													•	•	•	•
Pancreas													•	•	•	•
Endometrium ^d				•									•	•	•	•
Breast													•	•	•	•
Lung													•	•	•	•
Pleural mesothelioma (epithelial)				•									•	•	•	•
Small cell carcinoma of lung													•	•	•	•
Merkel cell carcinoma of skin													•	•	•	•
Transitional cell carcinoma (GII)				•									•	•	•	•

^a The cytokeratin polypeptides no. 2 (molecular weight 65,500; expressed in epidermis) and no. 9 (molecular weight 64,000; expressed in palmar and plantar epidermis) are not included in this table

^b E.g., hepatocytes, pancreatic acinar cells. Proximal and distal tubular cells of the kidney show the same pattern

^c E.g., bile ducts, pancreatic ducts, renal collecting ducts

^d CK no. 5 present in this special case but not in all cases

5/17, can be regarded as being the principal cytokeratin components of stratified epithelia. The remaining cytokeratin components of such epithelia are more selectively expressed, their presence being correlated with special forms of stratified squamous differentiation, and as shown by immunocytochemical study (see Table 7), they are usually expressed in more differentiated, suprabasal cells. These components include CKs nos. 1, 2, 9, 10, and 11 (typical of epidermis), the CK pair 3/12 (specific for corneal epithelium), and the CK pair 4/13 (typically expressed in noncornifying stratified squamous epithelia; Table 6; MOLL

Table 7. Selective single-polypeptide-specific cytokeratin antibodies

	Antibody	References
1. Antibodies against CK no. 10/11	K _K 8.60 K 92	HUSZAR et al. 1986 PULFORD et al. 1985; GATTER et al. 1982, 1984
2. Antibodies against CK no. 9	Guinea pig antibodies	KNAPP et al. 1986
3. Antibodies against CK no. 3	A E 5	SCHERMER et al. 1986; COOPER et al. 1985
4. Antibodies against CK no. 4	6 B 10	VAN MUIJEN et al. 1986
5. Antibodies against CK no. 13	1 C 7 2 D 7	VAN MUIJEN et al. 1986 VAN MUIJEN et al. 1986
6. Antibodies against CK no. 8	Troma-1	BRULET et al. 1980; TÖLLE et al. 1985
7. Antibodies against CK no. 18	CK 1-4 RGE 53 LE 61 PKK 3	DEBUS et al. 1982 RAMAEKERS et al. 1983a LANE et al. 1982, 1985 VIRTANEN et al. 1985
8. Antibodies against CK no. 7	CK 7	TÖLLE et al. 1985
9. Antibodies against CK no. 19	A 53/B-A2 BA 16, BA 17 LP 2 K K _S 4.62	KARSTEN et al. 1985 BARTEK et al. 1985 LANE et al. 1985 GIGI-LEITNER et al. 1986

et al. 1982; TSENG et al. 1982; for reviews, see COOPER et al. 1985; QUINLAN et al. 1985).

The *simple-epithelium-type cytokeratins* comprise the lower-molecular-weight polypeptides of each subfamily, i.e., CKs nos. 7 and 8 of the basic group, and CKs nos. 18 and 19 of the acidic subfamily (Table 6). These can also be divided into principal components (the CK pair 8/18) that are expressed in all simple epithelia, and more specific components; the latter include CK no. 19, which is found in ductal as well as in intestinal epithelium, and CK no. 7, which exhibits a much more restricted distribution (Table 6; MOLL et al. 1982; TSENG et al. 1982).

In complex epithelia such as the mammary gland epithelium (consisting of luminal and myoepithelial cells) and the respiratory epithelium, the different cell types present often express different cytokeratin polypeptides, resulting in complex patterns when pieces of whole epithelium are analyzed biochemically (Table 6; MOLL et al. 1982). Usually, the luminal cells of these complex epithelia express simple-epithelial-type components, while the basal or myoepithelial cells most likely contain stratified-epithelial-type components (mainly the CK pairs 5/14 or 5/17). Certain cytokeratins are heterogeneously distributed even within a particular epithelial cell layer, e.g., CKs nos. 4 and 13, which are expressed in single cells or in small groups of cells in the glandular epithelium of the prostate gland (VAN MUIJEN et al. 1986).

3.3 Selective Cytokeratin Antibodies

The biochemical diversity of cytokeratin polypeptides is reflected by the bewildering complexity and number of cytokeratin antibodies with different specificities.

ties that have been and are being developed. Selective cytokeratin antibodies are able to resolve expression differences within single cells, and the application of such antibodies is essential when cytokeratin typing is used for tumor diagnosis (Section 3.4). These antibodies, most of which are monoclonal antibodies, have been characterized to varying extents using immunocytochemical screening and biochemical/immunochemical tests. Selective cytokeratin antibodies can be divided into two main classes:

1. Group-specific Antibodies. These recognize two or more different cytokeratin polypeptides but, immunocytochemically, exhibit selectivity for certain types of epithelia.

Antibodies with a relatively extensive cell-type spectrum (being unreactive only with certain secretory epithelia such as hepatocytes and pancreatic acinar cells) fall into this class, e.g., some polyclonal antibodies against keratins from bovine hoof and muzzle epidermis (FRANKE et al. 1978 b, 1979a) and human stratum corneum (SUN et al. 1979), as well as the monoclonal antibodies 34 β E12 (GOWN and VOGEL 1982, 1984, 1985), clone 77 (VAN MUIJEN et al. 1984a), PKK2 (VIRTANEN et al. 1985), EKH4 (recognizing CK no. 14 and other cytokeratins; ETO et al. 1985), AE1 (recognizing many cytokeratins of the acidic subfamily; TSENG et al. 1982; COOPER et al. 1985), KA4 (NAGLE et al. 1985a, b), and LICR-LON-29b (KNIGHT et al. 1985). Other monoclonal antibodies decorate stratified squamous epithelia and myoepithelial/basal cells, e.g., KA1 (NAGLE et al. 1985b), CK B1 (CASELITZ et al. 1986a, b), and LICR-LON-16a (KNIGHT et al. 1985).

Monoclonal antibodies that selectively or preferentially stain stratified squamous epithelia include clone 78 (VAN MUIJEN et al. 1984a), 34 β B4 (GOWN and VOGEL 1984), ZK 61 and ZK 99 (LANG et al. 1986), and K₈.12 (directed against CKs nos. 13 and 16; HUSZAR et al. 1986). Some monoclonal antibodies mainly react with the epidermis, e.g., K20 (GATTER et al. 1982, 1984; PULFORD et al. 1985), AE2 (against CKs nos. 1, 2, and 10; TSENG et al. 1982; COOPER et al. 1985), RKSE 60 (RAMAEKERS et al. 1985b), and KA5 (NAGLE et al. 1985a, b); of these antibodies, the last three do not decorate basal epidermal cells. Other antibodies react preferentially with simple epithelia and recognize at least CKs nos. 8, 18, and 19, e.g., PKK1 (HOLTHÖFER et al. 1983; MIETTINEN et al. 1985a; VIRTANEN et al. 1985).

Only for some of these group-specific antibodies has the exact spectrum of cytokeratin polypeptides that they recognize been determined.

2. Polypeptide-specific Antibodies. These are reactive with only a single cytokeratin polypeptide. At present, antibodies are available for at least nine of the cytokeratin polypeptides (Table 7). Antibodies of this class have been most important for elucidating the correlation between the pattern of cytokeratin expression and cellular differentiation. The most frequently used antibodies of this type are those directed against CK no. 18, which recognize simple epithelia and distinguish them from stratified squamous epithelia. The application of these antibodies for the subtyping of carcinomas (see Fig. 4) will be discussed in Section 3.4.

A negative immunocytochemical reaction with selective cytokeratin antibodies does not necessarily indicate the absence of the respective cytokeratin polypeptide(s), since the antigenic determinants may be masked (see also COOPER et al. 1985). Most polypeptide-specific cytokeratin antibodies do not react with formalin-fixed, paraffin-embedded tissue.

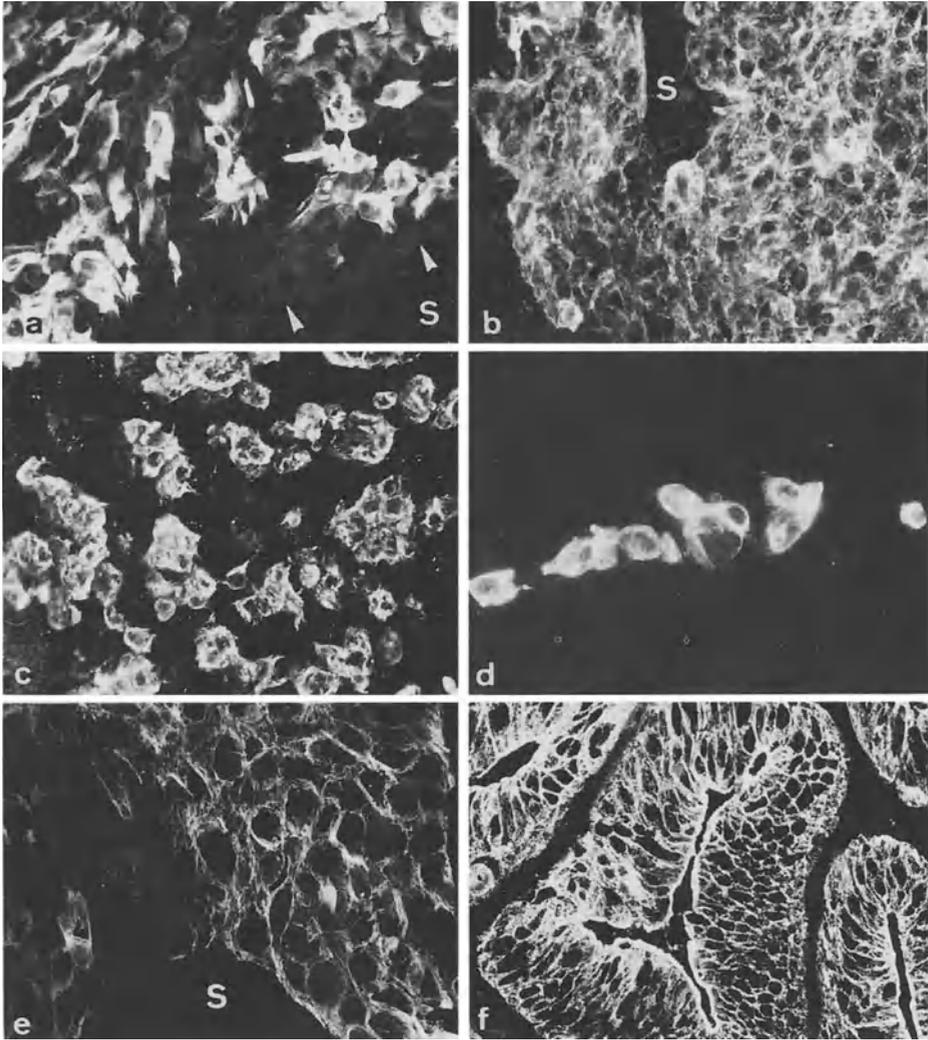


Fig. 4a-f. Detection of individual cytokeratin polypeptides in carcinomas using selective cytokeratin antibodies (frozen sections; immunofluorescence microscopy). **a** In a cornifying squamous cell carcinoma of the skin, antibody K_κ8.60 against CKs nos. 10/11 stains a prominent proportion of tumor cells with central location within the tumor cell nodule (*arrowheads* denote the tumor-stroma(*S*)-interface). **b** Poorly differentiated squamous cell carcinoma of the palatine tonsil positive for antibody 1C7 against CK no. 13. **c, d** Lymphnode metastasis of a transitional cell carcinoma of the urinary bladder, uniformly positive with an antibody (K_λ18.18) against CK no. 18 (**c**), and heterogeneously positive with antibody 1C7 against CK no. 13; the latter polypeptide is characteristic of transitional cell (and squamous cell) carcinomas. **e** Non-cornifying squamous cell carcinoma of the lung showing heterogeneous staining with antibody CK-2 against the simple-epithelial-type CK no. 18. **f** Adenocarcinoma of the colon, uniformly positive with antibody CK-2 against CK no. 18. *S*, stroma. **a, b, d, f** × 240, **c** × 190, **e** × 300

3.4 Differential Expression of Cytokeratin Polypeptides in Carcinomas

The finding of the cell-type specificity of cytokeratin polypeptide patterns has prompted the question as to whether analysis of the individual cytokeratin polypeptides present in carcinomas and their metastases may reveal the type of carcinoma and the epithelium of origin, as has been suggested by FRANKE et al. (1981c). Table 6 shows the typical cytokeratin polypeptide patterns of various types of epithelial tumors as determined using gel electrophoresis (MOLL et al. 1982, 1983a; QUINLAN et al. 1985; MOLL and FRANKE 1986). Complementary immunocytochemical data have been obtained by several investigators using selective cytokeratin antibodies.

Squamous cell carcinomas of various localizations are primarily characterized by the predominance of stratified-epithelium-type cytokeratins, a feature which distinguishes them from nonsquamous cell carcinomas (MOLL et al. 1982, 1983a, b; NELSON et al. 1984). *Squamous cell carcinomas of the skin* usually additionally express small amounts of the epidermis-typical CK pair 1 and 10/11, indicating a limited amount of terminal epidermal differentiation; antibodies directed against these cytokeratins stain a variable proportion of the cells of such tumors (Fig. 4a; LOENING et al. 1980; VIAC et al. 1982; GATTER et al. 1982; THOMAS et al. 1984; HUSZAR et al. 1986). CK no. 1 has not been detected in *squamous cell carcinomas of the oral cavity and esophagus*, which exhibit a relatively primitive, predominantly stratified-epithelium-type pattern (Table 6; Fig. 4b; MOLL et al. 1982, 1983a; NELSON et al. 1984; FUCHS et al. 1984); rather, they may contain small amounts of the simple-epithelium-type CK no. 19, which is also found in normal stratified squamous epithelia of the mucosae in the basal cell layer (BARTEK et al. 1986; FRANKE et al. 1986).

In contrast, *squamous cell carcinomas of the lung and cervix* are characterized by very complex cytokeratin patterns, in which stratified-epithelium-type cytokeratins are predominant, but simple-epithelium-type cytokeratins are also conspicuous (Table 6; MOLL et al. 1983b; BLOBEL et al. 1984; FUCHS et al. 1984; MOLL and FRANKE 1986; see also SAID et al. 1983; BANKS-SCHLEGEL et al. 1984). Accordingly, these tumors are positive, albeit heterogeneously (i.e., only in some cells), for antibodies against the simple-epithelium-type CKs nos. 8 and 18 (Fig. 4e; MOLL et al. 1983b; RAMAEKERS et al. 1983a, 1985b; DEBUS et al. 1984; GATTER et al. 1985). RAMAEKERS et al. (1985b) found that more than 90% of the squamous cell carcinomas of the lung that they examined were positively stained with an antibody against CK no. 18. Interestingly, squamous cell carcinomas of both lung and cervix usually arise (via squamous metaplasia) from simple or pseudostratified epithelia. It should be noted that the degree of differentiation may modify the cytokeratin pattern of carcinomas to a certain, although usually limited, extent (Table 6). As to whether differences in the cytokeratin pattern of squamous cell carcinomas of different origin is of any histodiagnostic value is a question that must remain open until larger series of patients have been investigated.

Carcinomas of the gastrointestinal tract exhibit a strikingly high level of conservation of cytokeratin polypeptide expression (MOLL et al. 1982, 1983a; MOLL and FRANKE 1986). Thus, *hepatocellular carcinomas* express the same

primitive CK pattern (nos. 8:18) as normal hepatocytes (Table 6; DENK et al. 1982; MOLL et al. 1982). The absence of CKs nos. 7 and 19, which has recently also been demonstrated at the immunocytochemical level using polypeptide-specific antibodies (OSBORN et al. 1986), could probably be used to distinguish such tumors from various gastrointestinal adenocarcinomas, including cholangiocellular carcinomas (OSBORN et al. 1986; MOLL and FRANKE 1986). Hepatocellular carcinomas have also been found to be negative for the relatively-broad-spectrum antibodies AE1 (SPAGNOLO et al. 1985) and 34 β E12 (GOWN and VOGEL 1985), this being in accordance with the fact that these tumors express only CKs nos. 8 and 18.

Adenocarcinomas of the colon also exhibit a very characteristic cytokeratin pattern that is identical to that of normal enterocytes, i.e., the presence of CKs nos. 8, 18, and 19, but with CK no. 7 being consistently absent (Table 6; MOLL et al. 1982). The absence of CK no. 7 is especially important for their differential diagnosis with respect to *adenocarcinomas of the pancreas* and *cholangiocellular carcinomas*, which both consistently express CK no. 7 (MOLL et al. 1982, 1983a; OSBORN et al. 1986). This CK can now be specifically detected using immunocytochemical procedures (OSBORN et al. 1986).

Adenocarcinomas of the endometrium and *ovary* often express the full complement of simple-epithelium-type CKs (nos. 7, 8, 18, and 19), but some lack CK no. 7 (Table 6; MOLL et al. 1983b; MOLL and FRANKE 1986; for the co-expression of vimentin, see Section 2.5). The finding of small amounts of the stratified-epithelium-type CK no. 5 in an endometrial adenocarcinoma (MOLL and FRANKE 1986) may indicate a correlation with squamous metaplastic processes. In endometrial adenocarcinomas with squamous metaplasia, antibodies against epidermal cytokeratins stain squamous foci much more strongly than glandular areas (BONAZZI DEL POGGETTO et al. 1983; WARHOL et al. 1984).

Most *invasive ductal and lobular carcinomas of the breast* express all of the simple-epithelium cytokeratins, i.e., nos. 7, 8, 18, and 19, as revealed in biochemical (Table 6; MOLL and FRANKE 1986) and immunocytochemical studies (ALTMANNBERGER et al. 1986) and are thus related to the luminal rather than the myoepithelial cells of normal breast tissue. Most ductal carcinomas of the breast are strongly positive for the antibody 34 β E12 (GOWN and VOGEL 1985). However, in another study, two well-differentiated breast carcinomas differed from all other types of adenocarcinomas tested due to their positive reaction with antibody clone 78, which is relatively specific for stratified squamous epithelial cells (VAN MUIJEN et al. 1984a). Interestingly, in one study, 1 of the 12 ductal carcinomas tested exhibited a positive reaction with antibody CK B1, which is specific for basal (squamous)/myoepithelial cells (ALTMANNBERGER et al. 1986). Similar observations have been made by NAGLE et al. (1986). In biochemical investigations, certain rare breast carcinomas have been found to contain small amounts of stratified-epithelium-type CKs (MOLL et al. 1982; MOLL and FRANKE 1986). Thus, there appears to be a minor subtype of ductal carcinoma of the breast that expresses some cytokeratins typical of stratified epithelia (and myoepithelia) but whose morphology is not detectably different from that of the common breast carcinoma types (ALTMANNBERGER et al. 1986). Intraepidermal Paget cells of mammary and extramammary *Paget's disease* express

simple-epithelium-type cytokeratins (MOLL and MOLL 1985; KARINIEMI et al. 1985; NAGLE et al. 1985a) like breast carcinoma and secretory sweat gland cells and thus can easily be distinguished, using antibodies against such cytokeratins, from surrounding epidermal keratinocytes, as well as from normal and malignant melanocytes.

Interesting differences in cytokeratin polypeptide expression have also been found in *thyroid gland carcinomas*, some of which also express vimentin (Section 2.5). It has been found that, whereas all types of such carcinomas, including follicular carcinomas, exhibit staining for simple-epithelium-type cytokeratins, only papillary carcinomas are positive for antibodies against epidermal cytokeratins (MIETTINEN et al. 1984). The additional cytokeratin polypeptides of papillary carcinomas responsible for this immunoreactivity have yet to be determined.

Renal cell carcinomas exclusively contain simple-epithelium-type cytokeratins in varying patterns, in correlation with the histological tumor type (PITZ et al. 1987). While clear cell carcinomas express predominantly CKs nos. 8/18, eosinophilic-granular and basophilic (small cell cuboidal) carcinomas often also reveal CKs nos. 7 and 19 (for the simultaneous expression of vimentin, see Section 2.5). Chromophobe cell carcinomas typically contain CKs nos. 8 and 18 and, in some cases, no. 7 (PITZ et al. 1987).

Adenocarcinomas of the lung exhibit all four simple-epithelium-type cytokeratins, but they appear to lack stratified-epithelium-type cytokeratins (BLOBEL et al. 1984). Histologically, these tumors may be difficult to distinguish from malignant *pleural mesotheliomas* of the epithelial type. Attempts have been made to distinguish between these tumors on the basis of their cytokeratin expression (for a recent review, see CORSON 1986). Epithelial mesotheliomas have been found to react positively with antibodies against epidermal (callus) keratins, while pulmonary adenocarcinomas are negative or react only weakly with such antibodies (SCHLEGEL et al. 1980; CORSON and PINKUS 1982). This is explained by the results of a recent biochemical study, in which certain stratified-epithelium-type cytokeratins, notably CK no. 5, were present in all five epithelial mesotheliomas tested and in four of the five biphasic pleural mesotheliomas examined (Table 6; BLOBEL et al. 1985b; see also WALTZ et al. 1983). Therefore, the use of antibodies specific for stratified-epithelium-type cytokeratins may prove to be very helpful in tackling this difficult differential-diagnostic problem. The expression of vimentin in the various types of mesotheliomas has been discussed above (Section 2.5).

Small cell carcinomas of the lung, which are characterized by the presence of certain neuroendocrine features such as neurosecretory granules, express cytokeratins (for references, see Section 2.6) of the simple-epithelium-type, of which only CKs nos. 8 and 18 are present in sizeable amounts, these being arranged in very delicate fibrils in the oat cell type of these tumors (Table 6; BLOBEL et al. 1985a; MOLL and FRANKE 1986). Therefore, formalin-fixed tissue sections of such tumors are not usually stained by antibodies against epidermal keratins (GUSTERSON et al. 1982). *Neuroendocrine (Merkel cell) carcinomas of the skin*, whose histology closely resembles that of metastatic small cell carcinomas, can be distinguished from the latter by their fairly consistent (although sometimes rather weak) expression of neurofilaments (see Section 2.6). Merkel

cell carcinomas also express simple-epithelium-type cytokeratins (nos. 8, 18 and, sometimes, 19; MOLL and FRANKE 1985; HOEFLER et al. 1985; MOLL et al. 1986). In addition, a cytoskeletal protein with an apparent molecular weight of 46,000 (IT protein) has been found in Merkel cell carcinomas but appears to be absent in other types of neuroendocrine carcinomas, including small cell carcinomas of the lung (MOLL and FRANKE 1985). This protein, which appears to be related to cytokeratins, is also a characteristic feature of intestinal epithelium and colonic adenocarcinomas (MOLL and FRANKE 1985; MOLL and FRANKE, unpublished results) and would appear to be useful for the differential diagnosis of these tumors.

The normal urothelium exhibits a unique and complex cytokeratin pattern (Table 6; MOLL et al. 1983a; ACHTSTÄTTER et al. 1985). Some antibodies against CK no. 18 only stain the superficial (umbrella) cells of normal urothelium and of low-grade papillary carcinomas, whereas they stain all cell layers in high-grade papillary carcinomas and invasive *transitional cell carcinomas* (RAMAEKERS et al. 1985b). Biochemically, many transitional cell carcinomas show cytokeratin patterns resembling that found in normal urothelium, including the expression of CK no. 13 (Table 6; ACHTSTÄTTER et al. 1985; MOLL and FRANKE 1986; see also Fig. 4c, d) but some grade-III tumors appear to have lost some of these polypeptides.

All of these data clearly show that there are profound differences in the patterns of cytokeratin polypeptide expression in different types of carcinomas. In our own studies, we have not found significant differences between the cytokeratin patterns of primary tumors and their metastases (MOLL and FRANKE 1986). It has to be borne in mind that some cytokeratin polypeptides may be heterogeneously expressed in the cells of a particular tumor, and that the degree of differentiation may also influence the pattern of cytokeratin expression. The expression of cytokeratins can also be influenced by experimental conditions, e.g., alterations in the concentration of vitamin A modify the expression of certain cytokeratin polypeptides, whereas others remain constant (FUCHS and GREEN 1981; ECKERT and GREEN 1984). It seems likely that the stability of expression of some cytokeratins is higher than that of others. In spite of this (limited) variability, it is evident from the data discussed here that many carcinoma types exhibit characteristic cytokeratin polypeptide phenotypes. Many of these differences can be detected using available polypeptide-specific antibodies, and it appears that, for a number of problems of differential diagnosis which cannot be satisfactorily resolved by morphological procedures alone, cytokeratin typing will prove to be very useful diagnostic adjunct.

4 Tissue Polypeptide Antigen (TPA) and Its Relationship to Cytokeratins

Tissue polypeptide antigen (TPA), which was originally prepared from the insoluble tissue residue of pooled carcinomas, was one of the first serum tumor markers (BJÖRKLUND and BJÖRKLUND 1957). The serum TPA level is often



Fig. 5. Immunofluorescence microscopy of cultured cells of the human mammary carcinoma-derived cell line MCF-7, using rabbit antibodies against TPA (WEBER et al. 1984). Note the decoration of a fibrillar cytoplasmic network as is also seen with antibodies against cytokeratins. $\times 600$ (courtesy of Dr. M. OSBORN, Göttingen)

elevated in patients with carcinomas and appears to be correlated with the proliferative activity of carcinomas (BJÖRKLUND 1980). TPA has therefore been regarded as being a marker of proliferation (BJÖRKLUND 1980).

TPA can be localized in normal and malignant cells using immunohistochemical techniques. In cultured epithelial cells, antibodies against TPA stain a fibrillar cytoplasmic network exhibiting a perinuclear concentration (Fig. 5; KIRSCH et al. 1983; WEBER et al. 1984), which is very reminiscent of the pattern of cytokeratin filament staining (FRANKE et al. 1979 d). At immunoelectron microscopy, antibodies against TPA can be seen to bind to cytokeratin filaments (ZIMMER et al. 1985). In a detailed immunohistochemical study, NATHRATH et al. (1985) detected TPA in most types of epithelial cells, there being only a few exceptions, e.g., the epidermis, hepatocytes, and suprabasal layers of noncornifying stratified squamous epithelia (see also LOENING et al. 1983). This cell-type distribution of TPA is almost identical with that of CK no. 19 (BARTEK et al. 1986; FRANKE et al. 1986).

Using immunohistochemical techniques, TPA has also been detected in carcinomas of the breast (BJÖRKLUND et al. 1982), in salivary gland tumors (SEIFERT and CASELITZ 1983; CASELITZ et al. 1983), squamous cell carcinomas of the esophagus, and various gastrointestinal carcinomas (BEHAM et al. 1986), exhibiting intracellular staining patterns similar to those of cytokeratins (BEHAM et al. 1986).

Biochemically, TPA purified according to the procedures of BJÖRKLUND and co-workers (for a review, see BJÖRKLUND 1980) consists of several polypeptides with a main component (TPA:B₁) having an apparent molecular weight of 43,000. Recently, in immunoblotting experiments, an antibody against TPA was found to bind specifically to the simple-epithelium-type CK polypeptides nos. 8, 18, and 19 (WEBER et al. 1984). Similar observations have been reported by BEHAM et al. (1986). When the amino acid sequences determined for fragments of TPA subunits (REDELIUS et al.

1980; LÜNING et al. 1980; LÜNING and NILSSON 1983) are compared with the recently established amino acid sequences of simple-epithelium-type cytokeratins, a peptide fragment from the TPA subunit C reveals identity with respect to 48 out of 58 amino acids with a sequence in the α -helical rod domain of bovine CK no. 8 (MAGIN et al. 1986). Furthermore, complete identity with respect to amino acid sequence has now been shown for the TPA subunits C and E1 and human CK no. 8 (LEUBE et al. 1986).

The relationship between TPA and cytokeratins thus appears to be somewhat clarified. TPA is probably a mixture of proteolytic fragments containing the relatively stable α -helical rod domains of simple-epithelium-type cytokeratins. These fragments are probably released into the serum during necrosis and the lysis of carcinoma cells. Therefore, the TPA serum level is only indirectly correlated with proliferation, and TPA should be regarded as being a broad-spectrum epithelial tumor marker. These findings in no way compromise the clinical usefulness of TPA assays in tumor management, but together with our increasing knowledge of the molecular structure of cytokeratins, may prompt the search for more specific probes for the serological analysis of fragments of the various defined cytokeratin and intermediate filament polypeptides that might provide information concerning the nature of particular tumors.

5 Conclusion

In this article, the molecular and cell-biological features of cytokeratins as well as the various areas of their application in histopathological diagnosis have been discussed. It is evident that the expression of cytokeratins is closely correlated with epithelial differentiation and remaining stable in malignant tumors. Although of value, other epithelial markers such as epithelial membrane antigen (EMA) exhibit expression patterns in tumors that are less reliably correlated with the epithelial phenotype (PINKUS et al. 1986). In addition to the detectable pattern of cytokeratins being useful for the establishment of the epithelial nature of a primary or metastatic tumor, other intermediate-filament types co-expressed with cytokeratins may be highly relevant for diagnosis. Furthermore, cytokeratin subtyping, which is now feasible using immunohistochemical procedures, makes it possible to resolve a spectrum of different cytokeratin phenotypes, thus allowing carcinomas to be precisely classified into subgroups according to their type and origin.

The information obtained by the analysis of cytokeratin and other intermediate-filament proteins is, in principle, independent of morphological features. While it is true that the majority of tumors can be diagnosed using conventional morphological techniques, these markers are very valuable diagnostic adjuncts in problem cases exhibiting an equivocal morphological appearance. The correlation between the expression of the intermediate-filament proteins and previously known morphological data concerning normal histology, normal development, and tumor pathology is strikingly good. Thus, the examination of the expression of intermediate filaments provides a complement to established morphological procedures. It should be emphasized that all histological tumor markers have to be applied critically, and that morphological investigation re-

tains a decisive role. However, in problem cases, the necessity of intermediate-filament and cytokeratin analysis for diagnosis is indisputable.

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Epithelial Tumor Markers: Oncofetal Antigens (Carcinoembryonic Antigen, Alpha Fetoprotein) and Epithelial Membrane Antigen

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1 Introduction

Oncofetal antigens are substances that are produced in high quantities during fetal development as well as during evolution of neoplasms in adult life. The best known oncofetal antigens are CEA and AFP. Their discovery marks the beginning of the still continuing search for tumor antigens which may serve as markers for tumor diagnosis and surveillance. Compared with CEA and

AFP, the epithelial membrane antigen (EMA) is of minor significance, but has gained general recognition as an immunocytochemical marker for epithelial tissues. This article places its emphasis on reviewing the usefulness of CEA, AFP and EMA as tissue markers for malignancies.

2 Carcinoembryonic Antigen (CEA)

2.1 General Properties

CEA is distinguished by 3 properties: (1) It shows an unusually high degree of molecular size heterogeneity due to a variable carbohydrate content; (2) it belongs to a family of closely related glycoproteins displaying a wide tissue distribution; and (3) CEA as well as the CEA-related substances reveal an extraordinarily strong immunogenicity. This makes CEA difficult to define solely on the basis of one analytical method. As long as the genes for CEA have not been cloned and sequenced, a combination of methods, particularly including immunological techniques using polyclonal as well as monoclonal antibodies, are necessary to define the CEA molecule and the structure of the other members of the complex CEA family (for detailed reviews see BURTIN and GOLD 1978; VON KLEIST 1983; ROGERS 1983; SHIVELY and BEATTY 1985).

2.2 Chemistry

Perchloric acid extraction of liver metastases of colon adenocarcinomas is the initial procedure most commonly used to obtain CEA (HAMMARSTRÖM 1985). Further purification steps include ion exchange chromatography, gelfiltration, Con A-Sepharose affinity chromatography and immunosorbent separation (SHIVELEY and TODD 1980). The purity then has to be controlled by SDS-PAGE and immunodiffusion resulting in an about 90% precipitation of the ¹²⁵I-labelled CEA preparation with specific polyclonal and some monoclonal anti-CEA antibodies. CEA prepared by this technique proved to be a large glycoprotein with beta-electrophoretic mobility and a molecular weight of approximately 180,000 daltons consisting of a single polypeptide chain of 829 residues, and about 40 N-acetylglucosamine-asparagine linked oligosaccharide chains (CHANDRASEKARAN et al. 1983). Although only the N-terminal sequence of the intact molecule and of several tryptic fragments have been determined, it seems that the composition of the peptide chain is remarkably constant. The carbohydrate moiety, in contrast, which comprises about 50–60% of molecule shows a considerable microheterogeneity. These carbohydrate chains are predominantly of the tetra-antennary type bearing many terminal sialic acid residues and some fucose residues. In addition, some CEA molecules may express blood group specificity in their carbohydrate side chains, especially for the blood group precursor (H) antigen (ROGERS 1983).

2.3 CEA-related Antigens

So far at least six well established (Table 1) and several putative CEA-related substances have been identified (MATSUOKA et al. 1982; SHIVELY and BEATTY 1985; NEUMAIER et al. 1985). These antigens differ from CEA with respect to molecular weight and/or immunoreactivity. The first of the CEA related antigens was reported by VON KLEIST et al. (1972) and MACH and PUSZTAZSERI (1972) and named nonspecific cross-reacting antigen (NCA) and normal glycoprotein (NGP), respectively. Subsequently a number of other substances with different molecular weight, different carbohydrate content and variations in the N-terminal sequence of the amino acids were described, which now constitute the CEA family.

Table 1. CEA and related antigens^a

Molecular weight (KD)	Terms and synonyms	Carbohydrate content (%)
175-180	CEA (carcinoembryonic antigen)	50-60
150-170	NCA 160 (nonspecific cross-reacting antigen)	45-50
	NCA 2	
	NFA 2 (normal faecal antigen)	
	MA (meconium antigen)	
128	CRA-128 (colorectal antigen)	
	CEA-low	
100	Meconium-antigen 100	
90-110	NCA 95	20-30
	TEX (tumor-extracted antigen)	
80-85	BGP 1 (biliary glycoprotein)	-40
55-65	NCA 55	-30
	NCA 1	
	NGP (normal glycoprotein)	
	CEX	
	CCEA 2	
20-30	NFA 1	10-20

^a from ROGERS (1983); BUCHEGGER et al. (1984); SHIVELY and BEATTY (1985); NEUMAIER et al. (1985)

At present, the exact molecular relationship of NCA to CEA is unclear, and it is far beyond the scope of this review to discuss the issue in detail (for reviews see WAGENER and BREUER 1982; ROGERS 1983; SHIVELY and BEATTY 1985). However, it should be mentioned here that on the basis of the most recent works two models for the structure of CEA and NCA have been proposed (GRUNERT et al. 1985). In the first model it is suggested that all NCA and CEA molecules share an unique polypeptide chain, but differ widely in the degree of their glycolysation. The second model suggests the existence of closely related repeat units in the polypeptide chain and explains the molecular heterogeneity of CEA and NCA by the different frequency of the occurrence of the repeats in the respective molecules. Evidence in support of the second model has been recently provided by gene cloning studies which demonstrated the existence of two very closely related repeats in CEA (ZIMMERMANN et al. 1987).

2.4 Immunology

CEA and the other members of the CEA family are highly immunogenic in all species commonly used for immunization. The majority of the polyclonal antisera raised against CEA react with antigenic determinants both on CEA and CEA related substances. Similar reactivity patterns have become known for many monoclonal antibodies which are currently available. This demonstrates that the CEA related substances have several epitopes in common with CEA and that specificity of a monoclonal antibody for CEA can only be determined by exhaustively investigating all of the potentially cross-reacting antigens (PRIMUS et al. 1983; WAGENER et al. 1983a; GRUNERT et al. 1985; MURARO et al. 1985).

HEDIN et al. (1982) generated eight monoclonal antibodies recognizing six determinants on native CEA, two of the epitopes probably occurring twice in CEA. Two antibodies also reacted with NCA. Recently, the same authors extended the number of epitopes to nine, six of which were also found on NCA 160, NCA 95 and/or NCA 55 (HAMMARSTRÖM 1985). KUROKI et al. (1984) reported 8 different epitopes, while MURARO et al. (1985) found at least 5 epitopes. Using a set of 18 monoclonal antibodies HAGGERTY et al. (1986) defined 9 epitopes when only the reaction with the intact molecule was considered. Twelve epitopes were found when data on binding to NCA, denaturated CEA and CEA fragments were taken into account as well. Seven binding sites

appeared to be CEA specific. Since not all of the monoclonal antibodies recognized epitopes in native CEA, it was suggested that some epitopes may also be carbohydrate in nature.

Human anti-CEA autoantibodies were detected in the sera of some cancer patients. These antibodies were also capable of reacting with the CEA molecule in tissue sections (ENDO et al. 1985).

2.5 Immunocytochemical Spectrum

The fact that CEA belongs to a family of closely related glycoproteins which have several epitopes in common but show a different tissue distribution necessitates the use of specific and well defined antibodies when CEA is localized by immunostaining at the cellular level. Thus it has been shown that with a commercial, unabsorbed polyclonal antiserum against CEA, which contained antibodies to NCA, 93% of breast carcinomas, 85% of mastopathic lesions and 66% of fibroadenomas stained positively, while, after absorption with normal tissues, the same antiserum labelled only 42% of the carcinomas and none of the mastopathic lesions or fibroadenomas (NAP et al. 1984). Another point which has to be considered when studying the immunocytochemical distribution of CEA is the influence exerted by fixation procedures on the antigenicity of CEA. TSUTSUMI et al. (1984) demonstrated that noncancerous formalin fixed paraffin-embedded tissues generally had a lessened stainability with (polyclonal and monoclonal) antisera against CEA and NCA when compared with the reactivity pattern on frozen sections. On sections from formalin-fixed, paraffin-embedded pancreatic duct type carcinomas monoclonal antibodies specific for CEA gave a less intense staining reaction than on frozen sections (TSUTSUMI et al. 1984; BÄTGE et al. 1985). No significant differences between the rate of CEA positivity in frozen and paraffin sections, however, were noted in gastric carcinomas (HEIDL et al. 1986).

2.5.1 Fetal Tissues

Among the fetal tissues the gastrointestinal tract is the major source of CEA (GOLD and FREEDMAN 1965; WAGENER et al. 1983b). Here it can be detected consistently from the 16th week of gestation onwards, with the greatest intensity in the colon (KAUF and BORCHARD 1985). The fetal pancreas, in contrast to the gut, reveals non immunostaining for CEA (TSUTSUMI et al. 1984).

2.5.2 Normal Adult Tissues

Recent studies based on sensitive staining techniques and using polyclonal antisera absorbed with perchloric extracts of lung (or spleen) and normal colonic mucosa, or monoclonal antibodies specific for CEA demonstrated CEA in the superficial part of normal colonic epithelium both in patients with and without carcinomas (WAGENER et al. 1978; PRIMUS et al. 1983; TSUTSUMI et al. 1984; YACHI et al. 1984) (Fig. 1). The failure of other authors to demonstrate CEA in normal colonic mucosa (ISAACSON and LEVANN 1976; GOLDENBERG et al. 1978; WILEY et al. 1981) may be explained by different specificities and sensitivi-

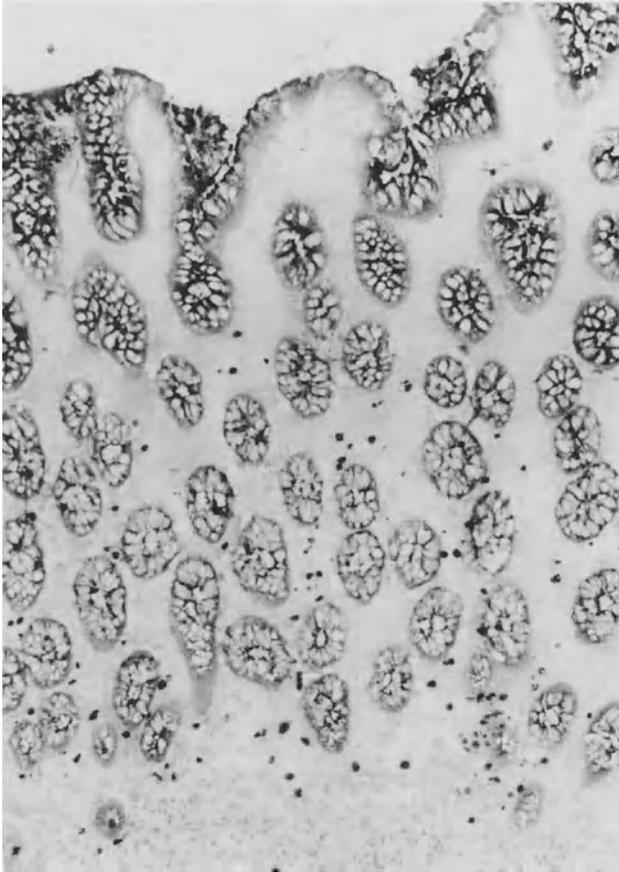


Fig. 1. Frozen section from normal colonic mucosa stained with monoclonal antibody C1P83 specific for CEA. Positive labelling of epithelial cell surfaces. $\times 125$

ties of the various CEA antisera and immunocytochemical methods used. Conflicting results were also reported for the stomach and small intestine in adults. While BURTIN (1985) found no CEA at both sites, TSUTSUMI et al. (1984) applying a monoclonal antibody specific to CEA, observed CEA positivity along the luminal rims of the antral foveolar glands, and ISAACSON and JUDD (1978) using a polyclonal CEA antiserum with minimal anti-NCA activity reported on CEA positivity of the small intestine on the surface of the villi, along the lining of the crypt lumens and within goblet cells. The tissues that are normally free of CEA include those of lung, liver, pancreas, kidney, spleen, prostate and endocrine glands (Fig. 2).

2.5.3 Adult Tissues with Nonneoplastic Changes

Some epithelial tissues which are usually negative for CEA may become positive when undergoing metaplastic or hypoplastic changes. Thus gastric intestinal

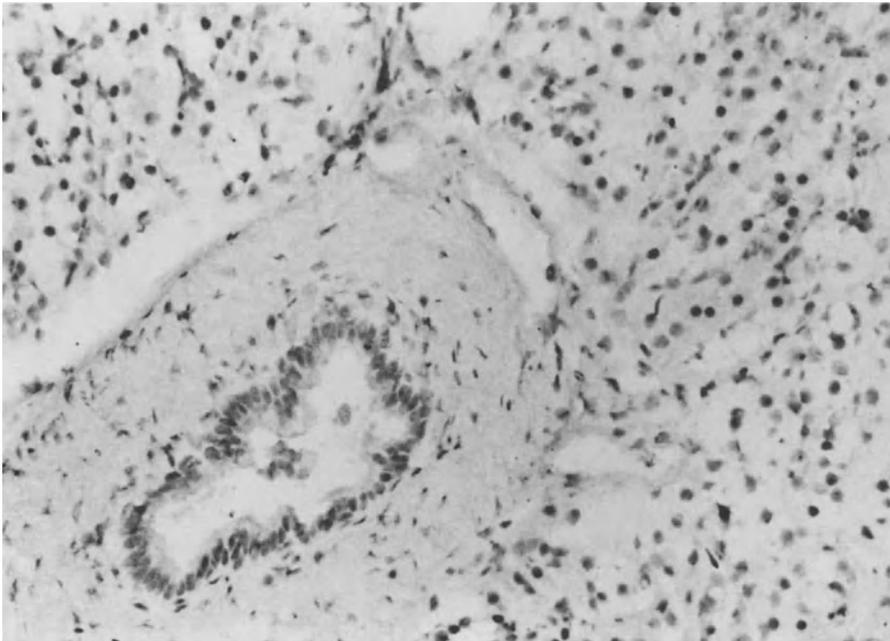


Fig. 2. Frozen section from normal adult pancreas, stained with monoclonal antibody C1P83 specific for CEA. No staining of the duct epithelium (counterstaining with hematoxylin). $\times 250$

metaplasia was described to be positive for CEA (BURTIN et al. 1973, 1977; DENK et al. 1973; WURSTER and RAPP 1979; YACHI et al. 1984). Occasionally CEA positivity was also observed in pancreatic ducts showing mucinous hypertrophy and papillary hyperplasia (BÄTGE et al. 1986; KLÖPPEL et al. 1987). In these lesions CEA positivity appeared to be discrete and was confined to the apical border of the cells (Fig. 3). ALLUM et al. (1986) observed a similar staining pattern in six of ten specimens of chronic pancreatitis. No CEA immunoreactivity could be identified in the hyperplastic prostate (PURNELL et al. 1984).

2.5.4 *Benign Neoplastic Lesions*

Originally it was thought that adenomas of the colon were CEA negative. Using frozen sections and more sensitive immunocytochemical methods it has been shown that all epithelial lesions of the colon, irrespective of benign or malignant, stain for CEA (BURTIN et al. 1972; STENGER et al. 1979). In the pancreas benign lesions such as serous cystadenoma or solid-cystic (papillary-cystic) tumor are consistently CEA negative, while in mucinous cystic tumors with their known malignant potential, those cells showing the greatest degree of atypia may stain for CEA (BÄTGE et al. 1986). Fibroadenomas of the breast and other benign lesions were found to be virtually or totally CEA negative (WALKER 1980; VON KLEIST et al. 1982; MANSOUR et al. 1983; NAP et al. 1984). In pleomorphic adenomas of salivary glands, 11% were positively stained with a monoclonal antibody to CEA (SUMITOMO et al. 1987). In adnexal tumors of the skin, CEA was only detected in sweat gland neoplasms (PENNEYS et al. 1982).

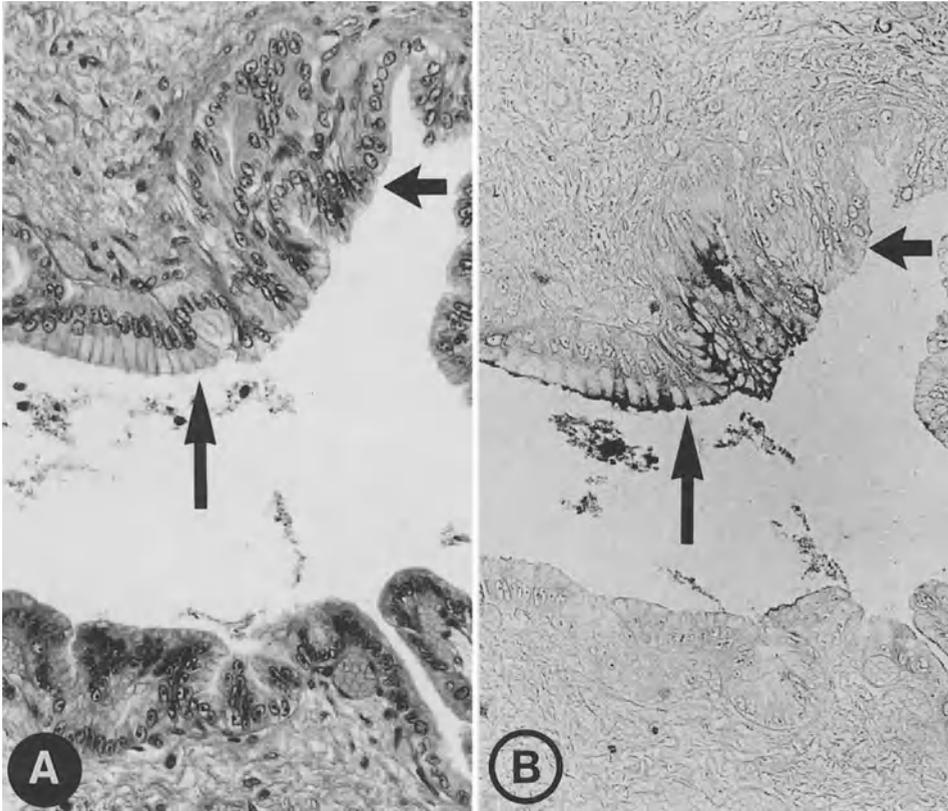


Fig. 3. Consecutive formalin-fixed, paraffin-embedded sections from normal pancreas, stained with Hematoxylin and Eosin (**A**) and monoclonal antibody BMA 130c recognizing CEA (**B**). Apical labeling of papillary hyperplasia of duct epithelium (*long arrows*). Normal duct cells remain unstained (*short arrows*). $\times 250$

2.5.5 Malignant Neoplastic Lesions

Common features for tumors that produce CEA are that they are of epithelial origin and belong predominantly to the variety of mucus producing adenocarcinomas. This association has become particularly evident when well absorbed polyclonal antisera or monoclonal antibodies specific to CEA were used. Consequently the vast majority of the adenocarcinomas arising in the gastrointestinal and biliary tract, the pancreas, lung, breast, salivary glands, ovary and endometrium belong to the CEA positive category. A noteworthy exception from this rule is the well known CEA production observed in the C-cell carcinoma of the thyroid (DELELLIS et al. 1978) and in squamous and some small cell carcinomas of the lung (WACHNER et al. 1984). In general, CEA expression is stronger in well than in poorly differentiated tumors. In well differentiated gland forming adenocarcinomas with mucus production most of the CEA is localized to the

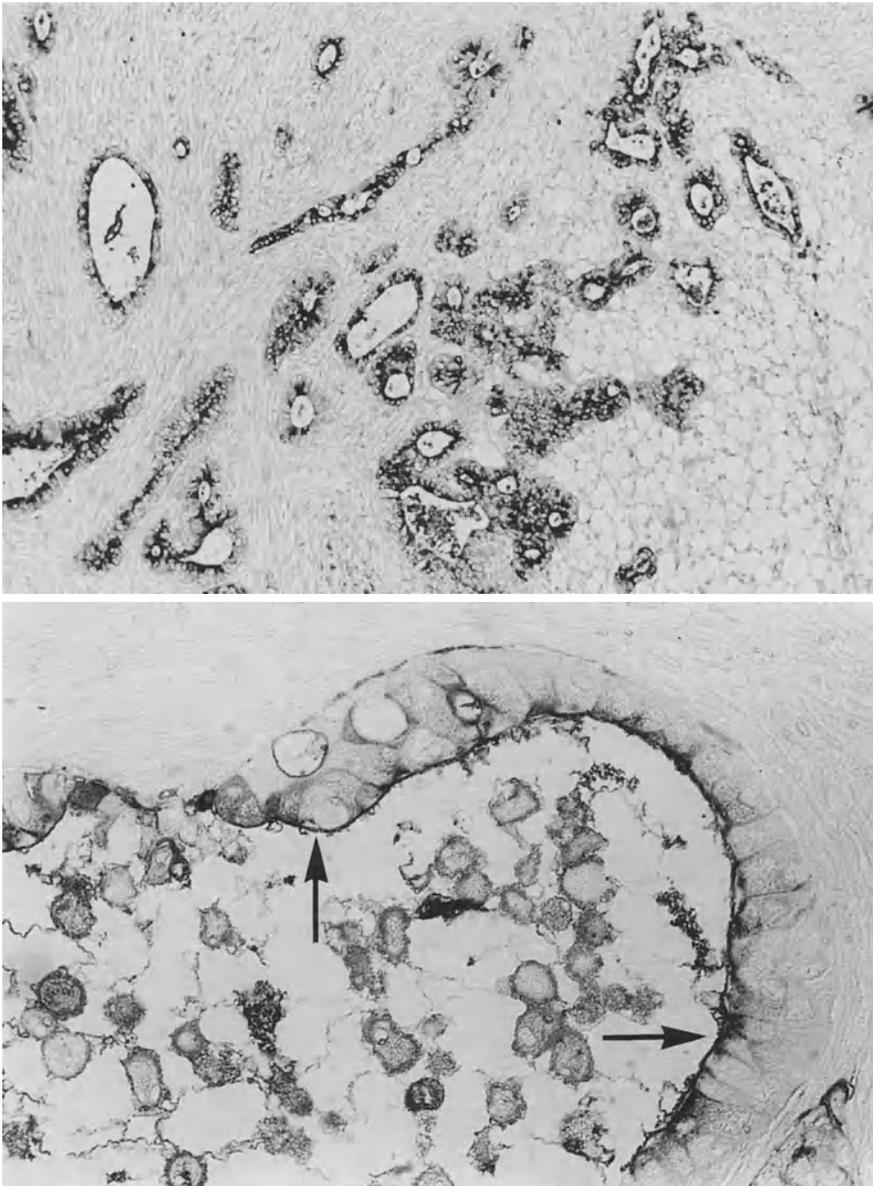


Fig. 4. Formalin-fixed, paraffin embedded sections from well differentiated ductal adenocarcinoma of pancreas, stained with monoclonal antibody BMA130b recognizing CEA and NCA 95. Mainly apical labelling of neoplastic epithelial cells (*arrows*). $\times 125$ and 250

apical cell pole, the luminal border of the epithelium and the luminal content (DENK et al. 1973; STENGER et al. 1979; O'BRIEN et al. 1981; NIELSEN and TEGLBJAERG 1982; BÄTGE et al. 1986) (Fig. 4). Less well differentiated and poorly differentiated adenocarcinomas including signet ring cell carcinomas, where some or most of the mucus is retained within the cell, predominantly show

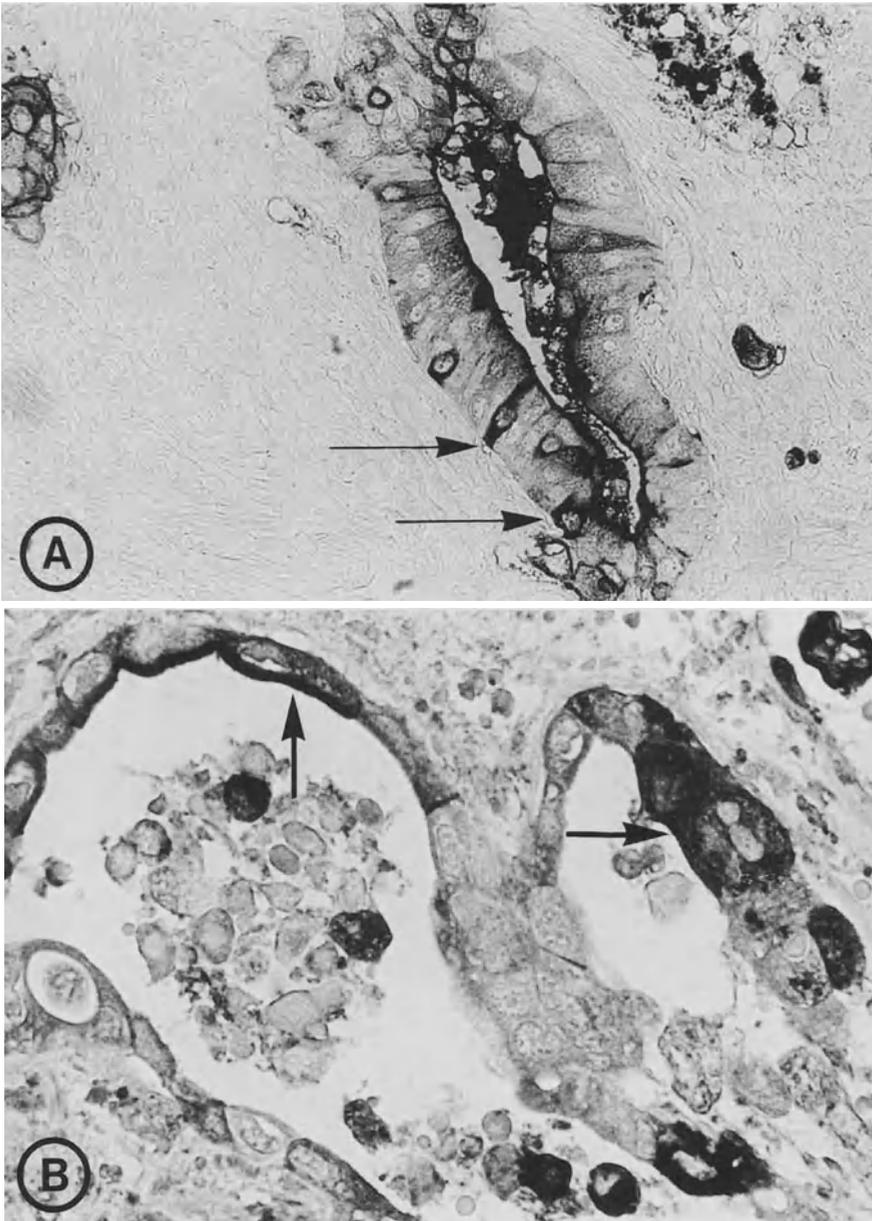


Fig. 5. Formalin-fixed, paraffin-embedded sections from two pancreatic ductal carcinomas of moderate (A) and poor differentiation (B), stained with BMA130b (CEA and NCA 95). Apical and strongly intracytoplasmic labelling (*arrows*). A \times 250, B 640

intracytoplasmic immunostaining for CEA (BURTIN et al. 1973; DENK et al. 1972 and 1973; NIELSEN and TEGLBJAERG 1982; HEIDL et al. 1986) (Fig. 5). CEA is usually absent from anaplastic carcinomas unless there are foci of glandular differentiation where CEA may be positive.

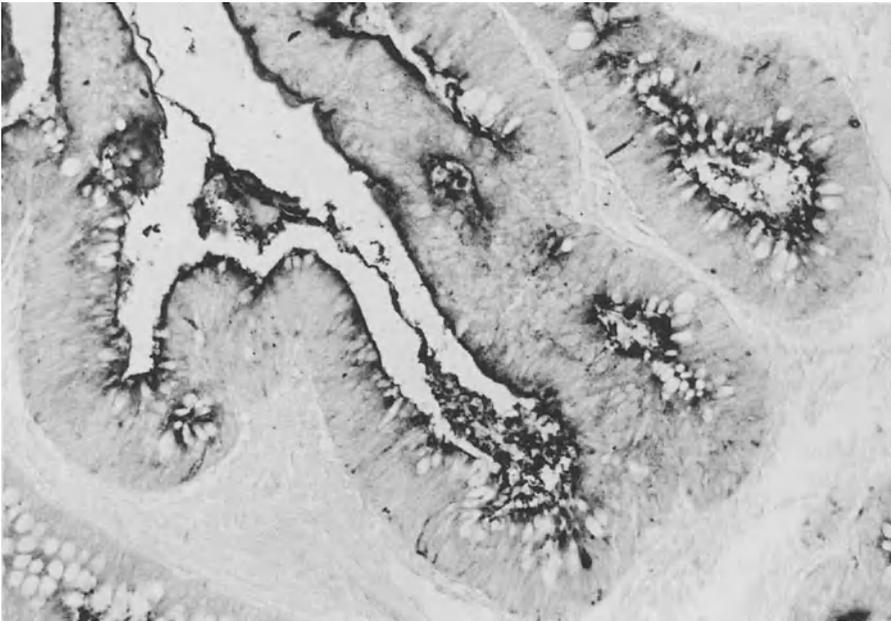


Fig. 6. Formalin-fixed, paraffin-embedded section from colon carcinoma, stained with BMA130c (CEA specific). Positive labelling is apparent at the apical brush border of tumor cells and within lumina. $\times 250$

As the majority of *colon carcinomas* belong to the group of differentiated mucus producing adenocarcinomas they are all CEA positive (ISAACSON and LEVANN 1976; GOLDENBERG et al. 1978; MURARO et al. 1985; JOTHY et al. 1986) (Fig. 6). The same is true for *stomach carcinomas* of the intestinal or diffuse type (HEIDL et al. 1986). In about one third of gastric carcinomas CEA expression was reported to be less intense in lymph node metastases than in the primary. This behaviour appeared to correlate with a worse prognosis (WITTEKIND et al. 1986). The rare adenocarcinomas of the *small intestine*, if well differentiated, are all positive for CEA.

In the *pancreas*, monoclonal antibodies directed against epitopes either present on CEA as well as NCA 95 or on CEA only, were found to discriminate clearly between duct type carcinomas and nonduct type carcinomas such as acinar cell carcinomas and endocrine tumors (BÄTGE et al. 1986). Formalin-fixed, paraffin-embedded material of pancreatic ductal adenocarcinomas disclosed CEA expression in about 60–77% of the cases (BÄTGE et al. 1986; ALLUM et al. 1986). Using frozen sections of duct type adenocarcinomas all tumors reacted positively (KLÖPPEL, unpublished observation).

Earlier examinations on CEA expression in *hepatocellular carcinomas* reported positivity rates of 30% (THUNG et al. 1979; NAKANUMA et al. 1981; IMOTO et al. 1985). Recently however it was found that CEA was consistently absent from hepatocellular carcinomas if these tumors were stained with a monospecific CEA antiserum (KOELMA et al. 1986). This result has been confirmed in an own series of hepatocellular carcinomas which were tested using monoclonal

Table 2. Tumor markers in liver neoplasms^a (Positivity given in percentage)

Neoplasm	Marker					
	AFP	CEA	α -HCG	AAT	KER	VIM
HCC (<i>n</i> =63)	24	0	2	6	95	11
CCC (<i>n</i> =18)	6	72	0	11	100	17
FNH (<i>n</i> =7)	0	0	0	0	100	0
HCA (<i>n</i> =4)	0	0	0	0	100	0

HCC, Hepatocellular carcinoma; CCC, cholangiocellular carcinoma; FNH, focal nodular hyperplasia; HCA, hepatocellular adenoma; KER, Keratin (MAb KL-1); VIM, Vimentin (MAb V9); CEA, Mab specific for CEA (BMA 130 c)

^a Klöppel, Brumm, Schultz, Morohoshi (unpublished observations)

antibodies specific for CEA. In this study, none of 63 hepatocellular carcinomas and 13/18 *cholangiocellular carcinomas* expressed CEA (Table 2).

In *salivary gland tumors*, CEA is mainly expressed in carcinomas with glandular differentiation, but may also be observed in squamous cell carcinomas (CASELITZ et al. 1981a, b; SEIFERT and CASELITZ 1983). The latter carcinomas, however, appeared to be either weakly positive or negative for CEA when tested with NCA absorbed polyclonal antiserum or a monoclonal antibody to CEA (TSUKITANI et al. 1985; SUMITOMO et al. 1987). CEA is positive in *sweat-gland carcinomas* but not in adnexal neoplasms derived from other structures of the skin (sebaceous glands, pilar structures) (PENNEYS et al. 1982).

In *breast carcinomas*, the rate of CEA positivity ranges between 40–90% (SHOUSA and LYSSIOTIS 1978; VON KLEIST et al. 1982; WAHREN et al. 1978; WALKER 1980; NAP et al. 1984; BÖCKER et al. 1985). Using an absorbed polyclonal CEA antiserum BÖCKER et al. (1985) showed that 51% of the breast carcinomas contained more than 5 percent CEA-positive cells. In addition, CEA positivity was found to be correlated with the histologic tumor type, invasive ductal carcinomas being positive more frequently than invasive lobular, tubular and cribriform carcinomas. SHOUSA et al. (1979) suggested a relation between CEA positivity and prognosis. However, such a correlation could not be confirmed by other authors (NAP et al. 1984; VAN DER LINDEN et al. 1985). CEA immunoreactivity could also not be correlated with oestrogen receptor status (BÖCKER et al. 1985; VAN DER LINDEN et al. 1985) and other established prognostic parameters such as lymph node status, tumor type, histologic grade, mitotic activity and others (VAN DER LINDEN et al. 1985).

Virtually all *adenocarcinomas of the lung* express CEA (WACHNER et al. 1984; KIMURA et al. 1985). A high rate of positivity, however, was also found in the other types of lung cancer, especially in squamous cell carcinomas. Here the reactivity appeared to be restricted to the epidermal cell nests. PASCAL et al. (1977) therefore discussed a cross-reactivity of CEA with keratin. This assumption was not confirmed by WACHNER et al. (1984) since preincubation of the sections with a polyclonal anti-keratin serum did not abolish the staining intensity for CEA. CEA staining was weak or absent from *mesotheliomas* (CORSON and PINKUS 1982).

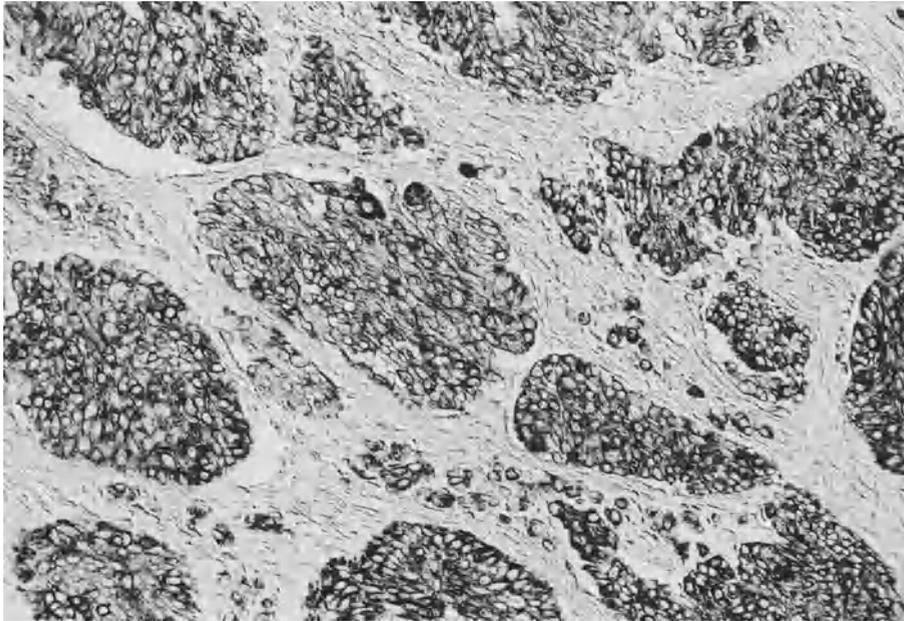


Fig. 7. Formalin-fixed, paraffin-embedded section from medullary thyroid carcinoma, stained with BMA 130 c (CEA specific). Intense intracytoplasmic labelling of all tumor cells. $\times 160$

Ovarian carcinomas, with the exception of the serous, germ cell and anaplastic tumors, and *adenocarcinomas of the uterus* often express CEA (FENOGLIO et al. 1981; CHARPIN et al. 1982). This is also seen in testicular teratomas containing glandular elements (WITTEKIND 1985).

CEA was not found in *renal cell carcinomas* and *melanomas* when tested with a monoclonal antibody to CEA (BÄTGE et al. 1986). In *prostate carcinomas*, only one out of 38 carcinomas stained with a polyclonal CEA antiserum absorbed for NCA (PURNELL et al. 1984). Other authors also using an absorbed polyclonal CEA antiserum reported positive CEA values in up to 65% of prostate carcinomas (VOGEL and HELPAP 1986). The same authors also obtained high CEA scores (about 70%) in *bladder carcinomas* (VOGEL et al. 1984).

Neuroendocrine tumors, except the medullary thyroid carcinomas, proved to be CEA negative (BÄTGE et al. 1986) but may contain some NCA (BISHOPRIC and ORDONEZ 1986). *Medullary carcinomas of the thyroid* express CEA in about 80% of the cases (SCHRÖDER et al. 1986; DELELLIS et al. 1978). The cellular distribution of CEA is very similar to that of calcitonin (Fig. 7). C-cell hyperplasia, the precursor lesion of the hereditary C-cell carcinoma (WOLFE et al. 1980), was reported to remain unstained with CEA antisera as do normal C-cells (DELELLIS et al. 1978). Recent studies, however, revealed CEA positivity in those C-cell hyperplasias which are already accompanied by infiltrating C-cell carcinomas (SCHRÖDER and KLÖPPEL 1987). C-Cell hyperplasias in the absence of an overt tumor proved to be CEA negative (SCHRÖDER and KLÖPPEL 1987). *Nonmedullary thyroid carcinomas* are negative for CEA but may contain NCA (SCHRÖDER and KLÖPPEL 1987).

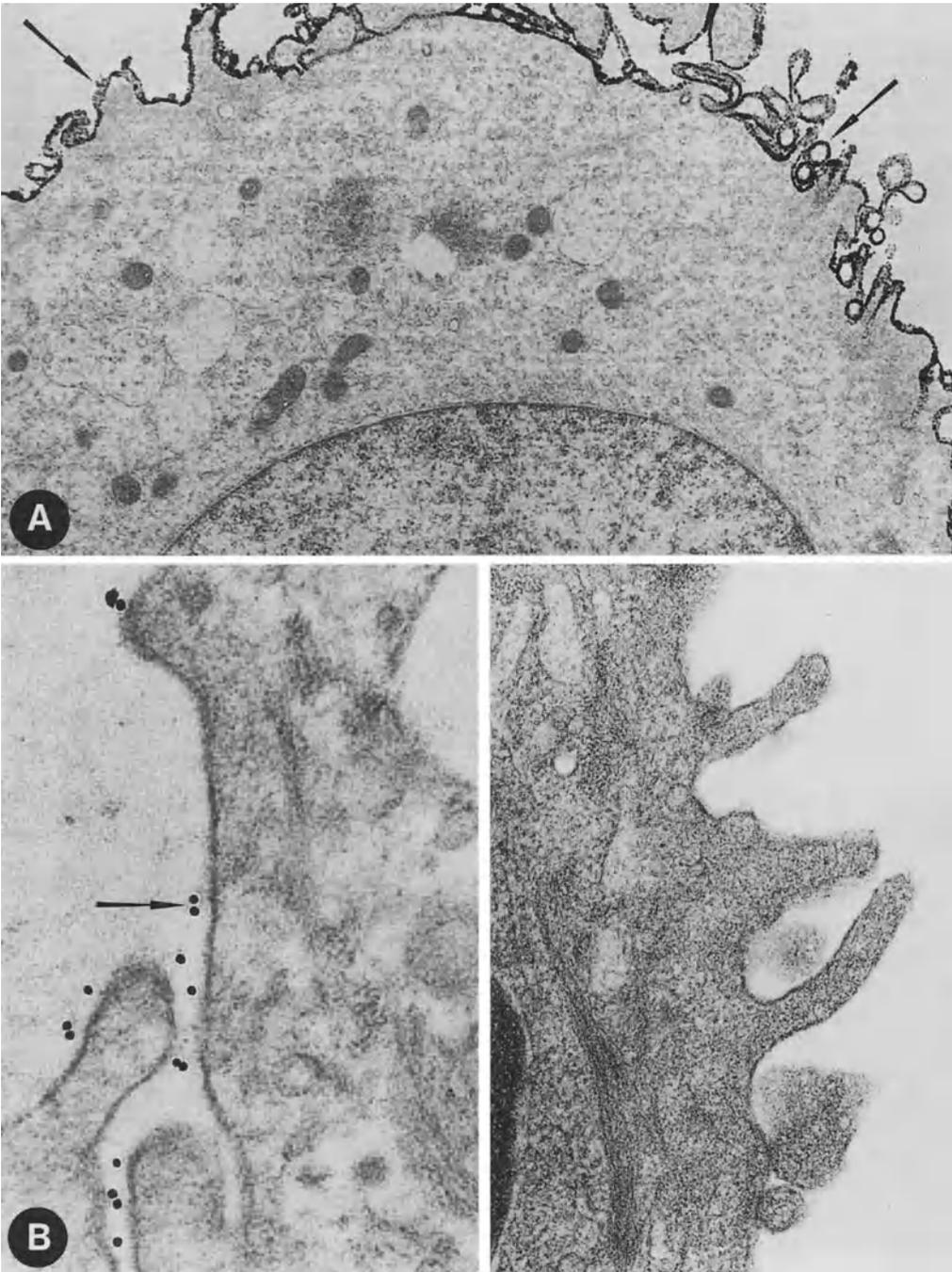


Fig. 8. Electron micrographs of cultured cells of pancreatic ductal adenocarcinoma immunostained with monoclonal antibody C1N3 recognizing epitopes on CEA and NCA. **A** Pre-embedding immunoperoxidase technique: Intense labelling of the surface of an ASPC cell (*arrows*), $\times 6290$. **B** Pre-embedding immunogold technique: Reaction products (*arrow*) on the surface of a QGP1 cell (*left*). Control (*right*). $\times 17000$

Apart from the well known cell lines such as LoVo, QGP-1, WIDR, the COLO series and others (SHIVELY and BEATTY 1985), an increasing number of CEA producing cell lines have been established from colonic carcinomas (SHI et al. 1983), pancreatic ductal carcinomas and other gut carcinomas in various laboratories during recent years. Immunostaining of cultured adenocarcinoma cells locates CEA primarily on the cell membrane and by electron immunocytochemistry CEA can be labelled on the surface of these cells (HERBERMAN et al. 1975; LOHSE, KLÖPPEL, KALTHOFF, SCHMIEGEL, unpublished observations) (Fig. 8).

2.5.6 Ultrastructural Localization

By ultrastructural immunocytochemistry CEA was localized primarily to the apical brush border and the glycocalyx (WOLF et al. 1984). Thus CEA might be anchored into the cell membrane. In addition, it was found in some mucus granules, in small vesicles and within the Golgi apparatus (HUITRIC et al. 1976; AHNEN et al. 1982; HAYNES et al. 1985). These findings suggest that CEA is packaged within the Golgi apparatus and transported from there to the cell surface by small vesicles. Whether mucus granules are also involved in the mechanisms of CEA transportation was doubted in a recent study which demonstrated CEA in small vesicles but not in mucus droplets (SCHULZ et al. 1986).

2.6 Tissue Distribution of CEA-Related Antigens

NCA 160 (NCA 2), NCA 95, NCA 55 (NCA) and BGP 1 show a strong cross-reactivity with CEA. Because of their wide tissue distribution and high tissue concentrations these CEA related antigens account for most of the immunoreactivity observed in normal or neoplastic tissues when unabsorbed or only partly absorbed polyclonal CEA antisera, or monoclonal antibodies detecting epitopes on both CEA and NCA are applied.

The normal tissue source of NCA 160 (NCA 2) is fetal and adult gastrointestinal mucosa (BURTIN et al. 1977). It is absent from monocytes and granulocytes (BURTIN et al. 1973). In contrast, NCA 95 (Fig. 9) and NCA 55 are present in granulocytes (BUCHEGGER et al. 1984). In addition, NCA 55 is found in bronchiolar and alveolar epithelium of normal lung as well as in pancreatic epithelium. BGP I is localized to the surfaces of hepatocytes lining biliary canaliculi and associated with the bile itself (SVENBERG 1976).

Staining of gastric and colonic carcinomas with antibodies specific either to CEA or NCA 160 (NCA 2) resulted in a similar incidence and intensity of positivity (BURTIN et al. 1977). A much higher incidence of immunoreactivity and a stronger staining intensity was observed in breast carcinomas or pancreatic carcinomas when labelled with antibodies recognizing CEA and CEA related substances, notably NCA 55 and NCA 95 (NAP et al. 1984; BÄTGE et al. 1986) (Fig. 10).

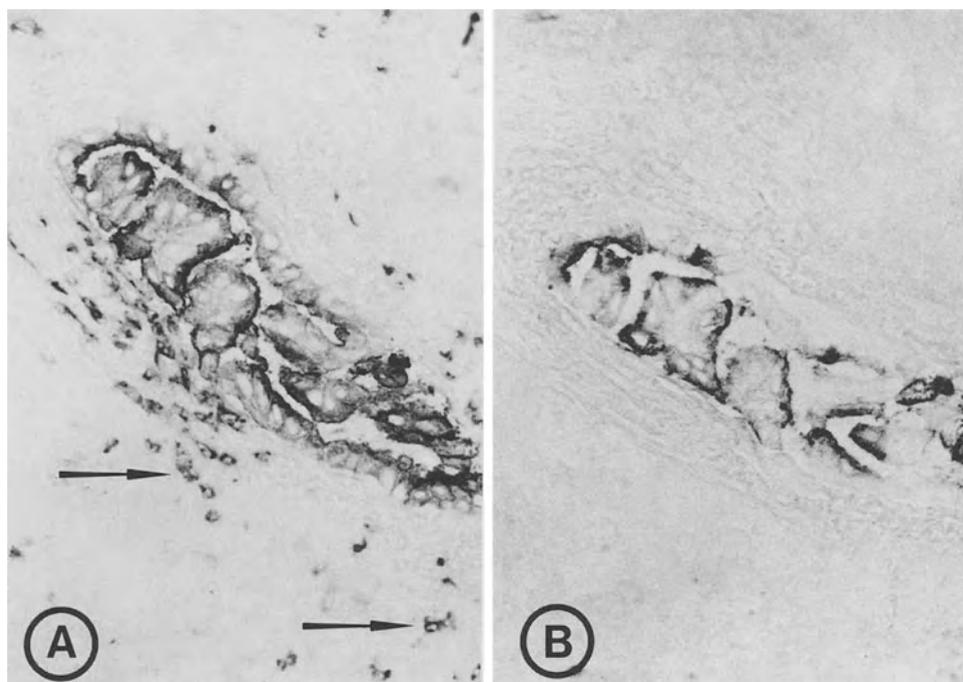


Fig. 9. Frozen section from pancreas with chronic pancreatitis, stained with BMA 130b recognizing epitopes on CEA and NCA 95 (A), and monoclonal antibody C1P83 specific for CEA (B). BMA 130b stains papillary duct hyperplasia as well as monocytes (*arrows*), while C1P83 labels papillary hyperplasia only. $\times 250$

2.7 Circulating CEA Levels and CEA Production

In the absence of inflammatory diseases involving epithelial tissues, elevated serum CEA levels usually indicate carcinomas originating from the gastrointestinal tract, the pancreas, the biliary system, the breast or the lung (NEVILLE and COOPER 1976). Complete removal of such a tumor correlates well with a decline of the elevated serum CEA level to the normal range. Correspondingly, tumor recurrence is usually marked by rising CEA values (STAAB et al. 1985; FLETCHER 1986). There is also a good correlation of serum CEA levels with regression or progression of tumor growth during chemotherapy (AHLEMANN et al. 1980; STAAB et al. 1980). In colorectal carcinomas the circulating CEA concentrations were found to be related to stage of disease (for review see FLETCHER 1986) and rising titres of CEA appeared most frequently in association with hepatic metastases (MACKAY et al. 1974). These observations suggest that the serum CEA levels reflect the general extent of the disease. However, the fact that serum CEA levels show great variability from one patient to another during the same stage of the disease (KALSER et al. 1978) indicates that the relationship between CEA level and tumor extent is rather complex. This assumption is further underlined by studies conducted in nude mice bearing trans-

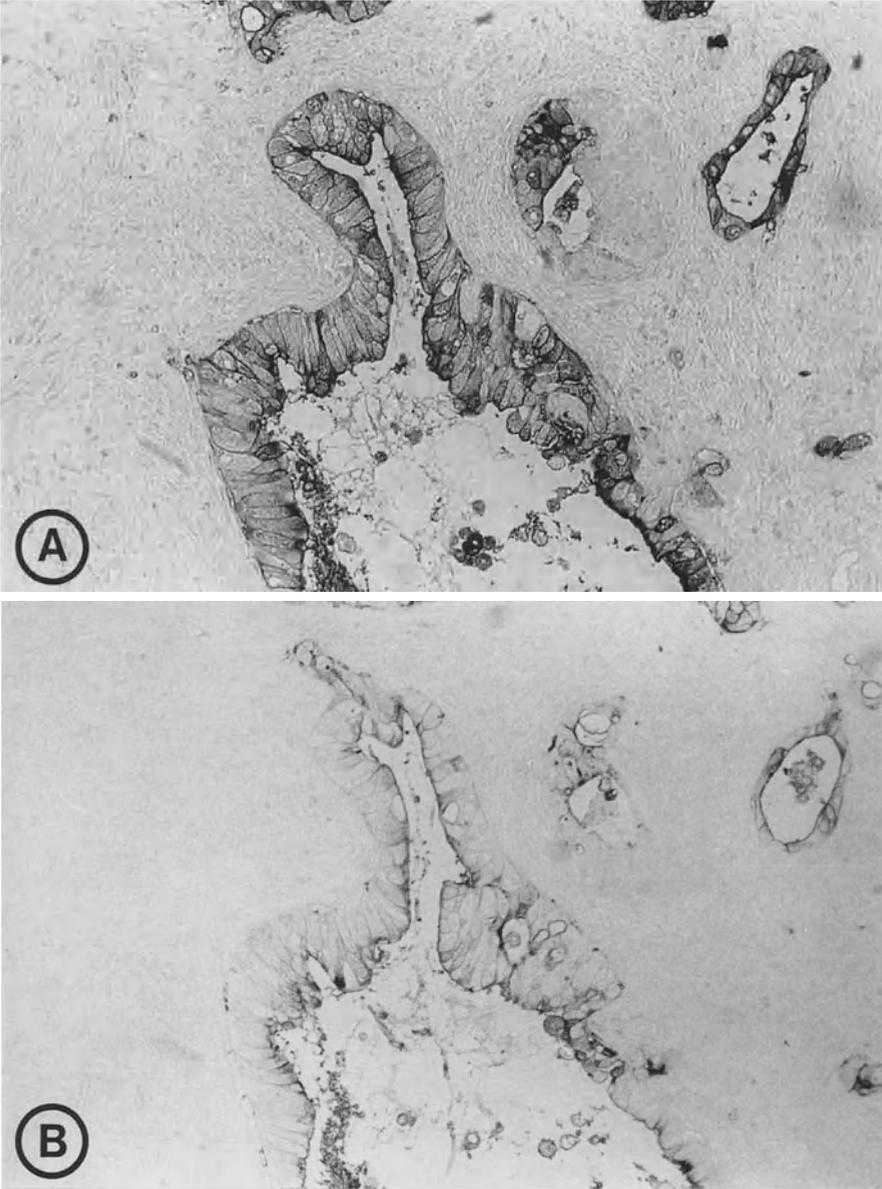


Fig. 10. Consecutive formalin-fixed, paraffin embedded sections from a ductal adenocarcinoma of the pancreas, stained with BMA130b (CEA and NCA 95) (A) and monoclonal antibody C1P83 (CEA) (B). Note the difference in staining intensity. $\times 250$

planted colorectal carcinomas. While, in general, the elevation of the CEA level was found to be proportional to the increase in tumor size and tumor CEA content (STAAB and ANDERER 1982; FIEBIG and VON KLEIST 1983), considerable variability was noted between individual values, making a strict correlation

often impossible (STRAGAND et al. 1980; LEWES and KEEP 1981). It is therefore most probable that serum CEA levels are influenced by a number of different factors. Basically these factors can be divided in two categories: One is related to the variable CEA production and secretion by the tumor, and the other to alterations in the CEA clearance and excretion by the liver (ZAMCHECK and MARTIN 1981). Factors belonging to the first group are (1) the degree of tumor differentiation (though some investigators reported a lack of correlation between tumor grade and CEA level (BIVINS et al. 1975; KALSER et al. 1978), the apparent inability of some carcinomas to release CEA into the extracellular space (VON KLEIST et al. 1982), the preferable secretion of CEA in glandular lumina without getting access to the circulation (BÖCKER et al. 1985), and the degree of necrosis and vascularisation within a tumor (WAGENER et al. 1981 b). The most important variables included in the second category are liver diseases such as acute and chronic hepatitis as well as obstructive jaundice resulting in an impaired capability of the liver to adequately clear the circulation of CEA and excrete it (ZAMCHECK and MARTIN 1981).

2.8 Function

The exact role of CEA during fetal development as well as the regulation of its pronounced expression and synthesis upon malignant transformation of normal cells are still obscure. It is of interest, however, that CEA induces the release of a suppressor factor from normal human lymphocytes (MEDOFF et al. 1984). Alpha-interferon treatment of human breast and colon tumor cells resulted in the enhanced expression of CEA on the cell surface (GREINER et al. 1984). The expression of CEA was found to be inversely related to the detection of histocompatibility antigens (HIRAI et al. 1980), and this heterogeneous expression of HLA-DR antigen and CEA in colonic carcinomas appeared to be associated with DNA-profile variations (ROGNUM et al. 1983). In a recent study it was observed that CEA stimulated the growth of thymic epithelial cells cultured in vitro (SAVINO et al. 1985).

2.9 Future Aspects

One of the most interesting issues with regard to CEA is whether there are CEA molecules which might be specifically associated with certain tumors. This question may be solved by molecular cloning studies which should render it possible to analyze the expression of these genes in normal and malignant tissues. In addition, gene transfer studies may aid in elucidating the biological function of CEA. Finally, such studies should also have implications for the development of new and more specific antibodies to CEA species which may further improve radioimmunodetection of tumors and possibly also tumor therapy.

3 Alpha Fetoprotein (AFP)

3.1 General Properties

AFP, like CEA, is an oncofetal glycoprotein synthesized in significant quantities in the liver, the yolk sac and the gastrointestinal tract of the fetus of many species (GITLIN and BOESMAN 1967). This protein which was first observed by BERGSTRAND and CZAR (1956) in the human fetus, appears as a normal constituent in fetal serum and amniotic fluid, reaching peak concentrations in the blood at about the 12th to 15th week of gestation. After birth AFP almost completely disappears from the blood. Its reappearance is usually associated with the development of hepatocellular carcinomas or yolk sac tumors but may also be seen as a result of hepatocellular damage and regeneration (SELL et al. 1974; KUHLMANN 1981; TUCZEK et al. 1981; BLOOMER et al. 1985a, b).

As a tumor associated antigen it was first described in mice bearing a transplantable hepatoma (ABELEV et al. 1963). Subsequently it was also found to be elevated in sera of patients with hepatocellular carcinoma and testicular germ cell tumors (ABELEV 1974; NEVILLE and COOPER 1976). During pregnancy raised AFP levels in amniotic fluid were observed to be associated with defects of the neural tube and are considered useful for antenatal detection of anencephaly and spina bifida (BUAMAH et al. 1981).

3.2 Chemistry

AFP represents a group of closely-related molecular variants of an acidic glycoprotein with a molecular weight of approximately 70000 and an α 1-globulin electrophoretic mobility (SMITH and KELLEHER 1980). It consists of a single polypeptide chain showing sequence homology with albumin (RUOSHLATI and TERRY 1976) and contains about 3–5% carbohydrate. The AFP of yolk sac tumors differs from that of hepatomas in that it has an additional carbohydrate residue at the epitope which in the hepatoma associated AFP bind Concanavalin A (TSUCHIDA et al. 1984; BUAMAH et al. 1984). The different content of neuraminoacid distinguishes embryonal AFP from hepatoma-AFP (PURVES et al. 1971). Recently a fucosylated variant of AFP was found to be useful for differentiating between benign liver diseases and hepatocellular carcinoma (AOYAGI et al. 1985). For the generation of antibodies, AFP isolated and purified from pools of amniotic fluid is used (KUHLMANN 1975; BELLET et al. 1984).

3.3 Immunocytochemical Spectrum

3.3.1 Fetal Tissues

The fetal liver expresses AFP in single or grouped hepatocytes, preferably accumulating around liver sinus (ENGELHARDT et al. 1971; KUHLMANN 1975; NAYAK et al. 1974; PURTILO and YUNIS 1971; NAYAK and MITAL 1977). The serum concentration was found to be directly proportional to the number of hepatocytes staining for AFP. Ultrastructurally, AFP was localized to the endoplasmic reticulum but not the Golgi region. This suggests that secretion of AFP may

be achieved without the involvement of the Golgi apparatus (PEYROL et al. 1977).

3.3.2 Adult Tissue

In the normal adult hepatocyte AFP remains undetectable by immunocytochemical means because of the low quantity of AFP production. In phases of liver cell regeneration as observed after hepatitis, in liver cirrhosis or experimentally after partial hepatectomy or CCl₄ intoxication, AFP production is slightly enhanced (KUHLMANN 1981; NEVILLE and COOPER 1976; HARADA et al. 1980; RONCALLI et al. 1986). This increase in CEA levels is correlated with growth and cell multiplication of hepatocytes (SELL et al. 1974). Immunocytochemically, AFP staining hepatocytes occur shortly after damage to the liver cells and disappear after restoration of the tissue (KUHLMANN 1981). In chronic liver disease such as alcoholic hepatitis and secondary biliary cirrhosis, AFP positive liver cells were occasionally observed (KOELMA et al. 1986).

3.3.3 Neoplastic Tissues

Although 80–90% of the patients with *hepatocellular carcinomas* have elevated serum AFP levels, immunocytochemical demonstration of AFP is only achieved in 20 to maximally 50% of these tumors (CHU et al. 1974; THUNG et al. 1979; ESPINOZA et al. 1984; IMOTO et al. 1985) (Table 2). AFP expression in the tumors is very patchy and more likely to be found in poorly differentiated than in well differentiated hepatomas (ABELEV 1974; KOELMA et al. 1986; KLOEPEL, BRUMM, SCHULTZE and MOROHOSHI, unpublished observations) (Fig. 11). Pronase digestion does not enhance the score of AFP positive hepatocellular carcinomas (KOELMA et al. 1986). In mice bearing xenotransplanted hepatocellular carcinomas serum AFP levels reflected the degree of tumor growth in the host (HIROHASHI et al. 1979). Experimentally, AFP positive tumors frequently develop in rats treated with different carcinogens (KUHLMANN 1981).

Hepatoblastoma, the liver neoplasm of young children, is nearly always AFP positive (SCHMIDT et al. 1985), while *fibrolamellar hepatocellular carcinoma*, a distinct variant of hepatocellular carcinoma occurring in adolescents and young adults, is frequently AFP negative (CABALLERO et al. 1985; SCHMIDT et al. 1985). *Cholangiocellular carcinomas* invariably behave AFP negative and this is also true for *liver cell adenoma* (Table 2). *Focal nodular hyperplasia* (FNH) was found to be AFP negative in our series (Table 2), while an AFP positive case was observed by KOELMA et al. (1986).

Approximately two-thirds of *testicular teratomas* are associated with elevated serum AFP levels (NEVILLE and COOPER 1976). The increased serum AFP levels correlate with the presence of *endodermal sinus (yolk sac) tumor* elements within these neoplasms. This is due to the fact that AFP is produced and localized in the cells of visceral endoderm lining the endodermal sinuses and in the various-sized intra- and extracytoplasmic PAS-positive hyaline globules in the tumor (ENGELHARDT et al. 1983; ITOH et al. 1974; NORGAARD-PEDERSON et al.

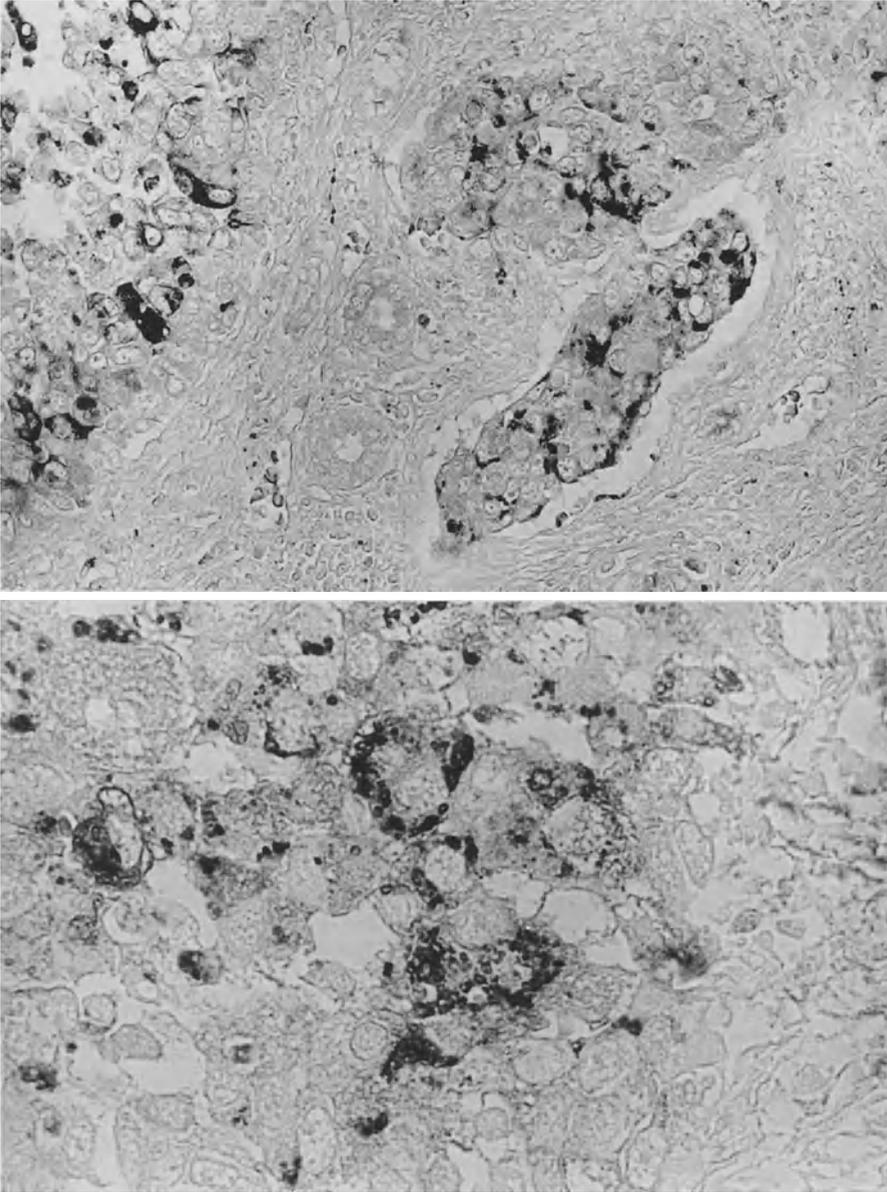


Fig. 11. Formalin-fixed, paraffin-embedded sections from poorly differentiated hepatocellular carcinoma, stained with polyclonal AFP antiserum. Granular intracytoplasmic AFP positivity. $\times 120$ and 640

1974; TALERMAN 1980; BOSMAN et al. 1980; HORN et al. 1980; WITTEKIND 1985). Germ cell tumors located in the ovary and extragonadal sites containing homologous structures also produce AFP in high quantities. Pure *embryonal carcinomas* are associated either with normal or slightly elevated AFP levels and may

show some AFP-positive cells (WAGENER et al. 1981a). AFP production was also reported in some rare germ cell tumors with borderline histology between seminoma and embryonal carcinoma (HOFSTÄDTER 1986; WALT et al. 1986). Pure *seminomas*, *teratomas* or *choriocarcinomas* are AFP negative (JACOBSEN et al. 1981).

Neoplasms not arising from liver or germ cells, e.g. *adenocarcinomas of the stomach, pancreas, colon and lung* occasionally produce AFP but rarely in large amounts (KODAMA et al. 1981).

3.4 Function

The exact role of AFP during fetal development is still obscure. As it shows a particular affinity for oestrogen and binds to bilirubin and fatty acids, it may serve as a carrier for these substances (for review see SMITH and KELLEHER 1980). In addition, it has been considered to be involved in immune regulation mechanisms because of its ability to suppress certain immune reactions in vitro (SHEPPARD et al. 1977; GERSHWIN 1980).

4 Epithelial Membrane Antigen (EMA)

4.1 General Properties

EMA has proved to be a global marker of epithelial cells of normal and neoplastic origin. It was isolated from human milk (membranes of human fat globules; HEYDERMAN et al. 1979) and represents a high molecular weight glycoprotein (molecular weight 265 000 to 400 000 Dalton, ORMEROD et al. 1983). In analogy to CEA, EMA may belong to a family of proteins with numerous epitopes in common. EMA can be detected by the use of polyclonal antibodies (HEYDERMAN et al. 1979; SLOANE and ORMEROD 1981) and by a number of monoclonal antibodies (HMFG 2, LICR LOM/M, E29; EDWARDS and BROOKS 1984; MOLL 1986).

4.2 Immunocytochemical Spectrum

4.2.1 Normal Tissues

In normal breast tissue, EMA labels the luminal cells, while the myoepithelial cells remain negative. The staining for EMA is concentrated at the apical cell membrane. The EMA staining pattern of other exocrine glands such as pancreas, sweat glands and salivary glands (Fig. 12) is analogous to that of the mammary gland. No staining is observed in the cells of the proximal kidney tubules and in hepatocytes. In the transitional epithelium of the urogenital tract, EMA is present in the superficial layer. Squamous epithelium is often negative for

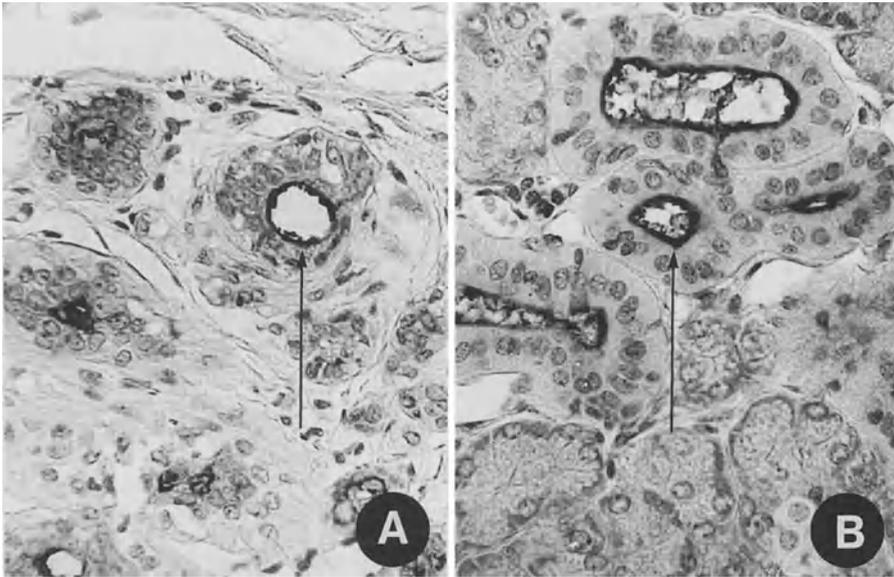


Fig. 12. Formalin-fixed, paraffin-embedded sections from mammary gland (A) and parotid gland (B), stained with polyclonal EMA antiserum. Labelling of apical border of duct cells (*arrows*). $\times 300$

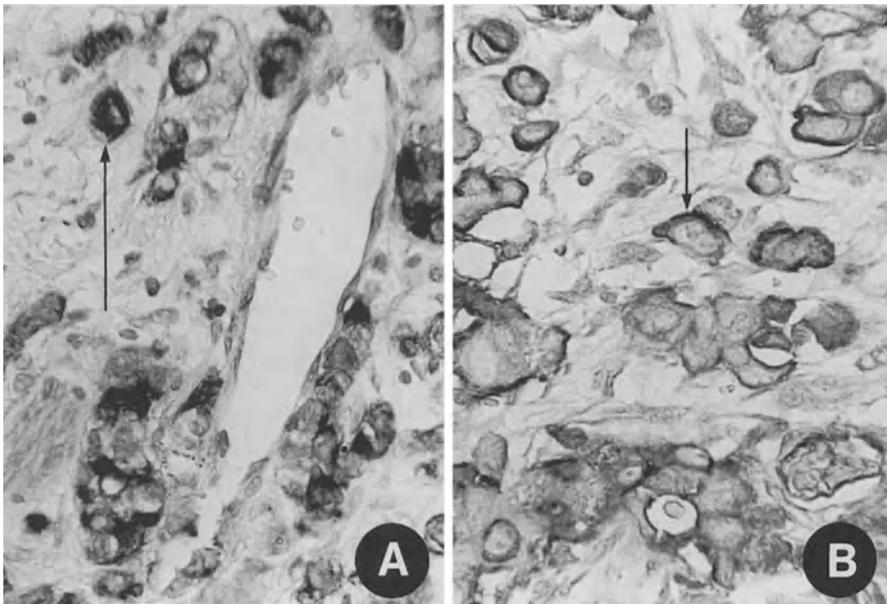


Fig. 13. Formalin-fixed, paraffin-embedded sections from signet ring cell carcinoma of stomach (A) and poorly differentiated carcinoma of ovary (B), stained with polyclonal EMA antiserum. Intracytoplasmic (*long arrow*) and apical (*short arrow*) labelling of tumor cells. $\times 300$

EMA (SLOANE and ORMEROD 1981), and mesenchymal tissues lack EMA. Non-epithelial cells containing EMA are plasma cells and transformed lymphocytes (DELSOL et al. 1984).

4.2.2 *Neoplastic Tissues*

As a rule, all tumors with glandular differentiation are EMA positive, while a few nonglandular tumors may be negative (SLOANE and ORMEROD 1981; ORMEROD et al. 1982; TO et al. 1982; EDWARDS and BROOKS 1984; PINKUS et al. 1986). The staining pattern of EMA is comparable to that of total keratin, but its variability is greater. EMA has been found in adenocarcinomas of the breast, pancreas, lung, kidney, prostate and stomach, but also in squamous cell carcinomas of the esophagus, the cervix and the skin. It is found in benign and malignant salivary gland tumors (GUSTERSON et al. 1982; CASELITZ 1987). Undifferentiated carcinomas, e.g. bronchial carcinomas of the large and small cell type, are often positive so that EMA turns out to be a valuable marker in identifying the epithelial nature of these tumors (Fig. 13). EMA is also positive in mesotheliomas. Tumors nonreactive with EMA include hepatocellular carcinomas, neuroendocrine tumors, embryonal carcinomas and sarcomas (with few exceptions). Among the malignant lymphomas, the T-cell lymphomas of the large cell variety, and plasmacytomas may stain for EMA (DELSOL et al. 1984; NORTON and ISAACSON 1986).

4.2.3 *Correlation with Keratin Markers*

EMA is most intensively expressed in those tissues and tumors where keratin 8, 18 and/or 19 are present. Since antibodies against EMA react on sections of formalin-fixed, paraffin-embedded tissue, they are a helpful adjunct in establishing the diagnosis of a carcinoma. Although the distributions of keratin and EMA are overlapping, there are still some differences. The keratins belong to the group of intermediate filaments which are located in the cytoplasm, sometimes even in a fibrillar pattern (especially in cytological specimens). Thus anti-keratin antibodies with a broad reactivity generally stain the cytoplasm of all epithelial cells. As EMA is a membrane glycoprotein, antibodies against EMA stain the cell membrane, in particular the apical border of normal glands. Only in tumors, it is occasionally detected in the cytoplasm as well (Fig. 13B).

4.2.4 *Function*

The functional role of EMA remains to be elucidated. As it seems to be a special membrane component of most glandular tissues, it might be involved in functional mechanisms like secretion or transport of secretory material (HEYDERMAN et al. 1979).

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Epithelial Tumor Markers: Special Markers of Glandular Differentiation

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1 Introduction

There are principally two types of epithelial tumor markers: (1) Normal cell products with known functional properties which may appear in increased or reduced amounts during malignant development; and (2) So-called oncodevelopmental antigens. The latter markers are mainly expressed in fetal life but may appear in increased amounts in neoplastic lesions. Examples of such antigens are alpha fetoprotein (AFP) and carcinoembryonic antigen (CEA). In the present chapter, however, only the former category of tumor markers will be

included and the discussion will be limited to salivary glands, breast and large bowel mucosa.

Immunohistochemical mapping of epithelial markers of differentiation has varied significance in different organs. Whereas contribution to increased understanding of tumor histogenesis is an important aspect in studies of salivary glands, the use of such markers in breast tumors and carcinomas or pre-cancerous lesions of the large bowel may be of diagnostic and prognostic value.

2 Normal Glandular Products and Their Epithelial Distribution

2.1 Secretory Component and Secretory Immunoglobulins

Secretory component (SC) is part of an epithelial transmembrane glycoprotein that binds J chain-containing dimeric IgA and pentameric IgM. It is thereby a key factor in the external transport of secretory IgA (SIgA) and SIgM (for review, see BRANDTZAEG 1985). Because of this receptor function of SC, its distribution in secretory epithelia will normally be mimicked by that of IgA (and to a lesser extent IgM), with the exception of the selective appearance of free SC in the Golgi zones (BRANDTZAEG 1974c). Some physicochemical properties of SC and SIgA are given in Table 1.

Recent studies have conclusively shown that SC is localized to both ductal and serous acinar elements in salivary glands (BRANDTZAEG 1977; KORSRUD and BRANDTZAEG 1982; FANTASIA and LALLY 1984). It is likewise widely distributed in mammary glands, and epithelial uptake and transport of IgA during lactation is well documented (BRANDTZAEG 1983). The columnar cells of intestinal crypts are similarly active in SC-mediated transport of IgA and to a lesser extent IgM (BRANDTZAEG 1974a; BRANDTZAEG and BAKLIEN 1977).

Table 1. Physicochemical properties of epithelial cell markers discussed in this chapter

Name	Type of protein	Molecular mass (daltons)	References
Secretory component (SC)	Glycoprotein	83000	BRANDTZAEG 1974b
Secretory IgA	Complex of dimeric IgA and SC	390000	TOMASI et al. 1965 NEWCOMB et al. 1968
Lysozyme (Muramidase)	Enzyme (EC 3.2.1.17)	14500	FLEMMING 1922 PETIT and JOLLES 1963
Amylase	Enzyme (EC 3.2.1.1)	50000	JACOBSEN et al. 1966 MUNZEL 1976
Lactoferrin	Iron-binding glycoprotein	80000	MASSON et al. 1966 BULLEN et al. 1972
MHC class I	Glycoprotein	45000	STROMINGER et al. 1980
MHC class II	Glycoprotein	33000 + 28000	STROMINGER et al. 1980

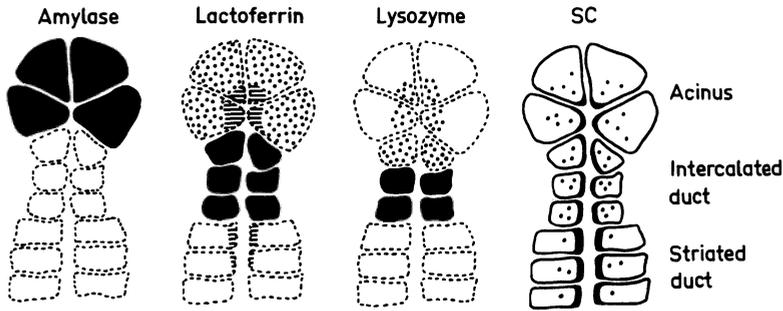


Fig. 1. Schematic distributional representation of various glandular markers in serous epithelial elements of salivary glands. The drawing is based on the average staining patterns: *dotted* (graded density), *shaded*, and *black areas* indicate, in that order, increasing occurrence and intensity. SC is particularly found apically and associated with the basolateral borders of the epithelial cells; the distribution of IgA (not shown) is similar except that SC appears selectively adjacent to the nuclei (not indicated in drawing). Adapted from KORSRUD and BRANDTZAEG (1982)

SC expression is often modulated during inflammation, and γ -interferon produced by activated T cells may be one of the stimulatory signals (SOLLID et al. 1987).

2.2 Lysozyme and Lactoferrin

Lysozyme (Ly), lactoferrin (Lf) and amylase (Am) will be discussed as tumor markers only in relation to salivary gland neoplasia. Ly is a bacteriolytic enzyme, Lf is an iron-binding glycoprotein (Table 1). Both have a broad spectrum of antibacterial properties (IACONO et al. 1980; ARNOLD et al. 1977) and are produced not only by phagocytes (MCCLELLAND and FURTH 1975) but also by certain secretory epithelia (LAI et al. 1976). In salivary glands Ly and Lf have by most authors been localized to intercalated ducts and to a much smaller extent to serous acini (REITAMO et al. 1977, 1980; KORSRUD and BRANDTZAEG 1982) (Fig. 1).

2.3 Amylase

Am is an enzyme (Table 1) produced by serous acini in salivary glands (KRAUS and MESTECKY 1971; KORSRUD and BRANDTZAEG 1982; CASELITZ et al. 1983; SEIFERT and CASELITZ 1983) (Fig. 1). Even in fetal glands it is mainly confined to primitive acini (THRANE et al. 1987a).

2.4 MHC Class I and II Molecules

Class I and II products (Table 1) of the major histocompatibility complex (MHC) play an important role in immune regulation; class I (HLA-A, -B,

-C) molecules serve as restriction elements for T cell-mediated cytotoxicity (ZINKERNAGEL and DOHERTY 1979), whereas class II (HLA-DR, -DP, -DQ) molecules are required for the presentation of foreign antigen to helper T (Th) cells (BENACERRAF 1981). Loss of class I molecules may have an important impact on the malignancy and metastatic potential of a tumor (WALLICH et al. 1985; MOMBURG et al. 1986).

Class I determinants are normally expressed in a heterogeneous manner by epithelial elements in salivary glands (THRANE and BRANDTZAEG 1987a), mammary glands (FLEMING et al. 1981) and the gut (GHOSH et al. 1986). Although class II molecules are generally confined to B lymphocytes, activated T lymphocytes, various dendritic cells and macrophages, certain epithelial and endothelial cells may likewise express such determinants. In normal salivary glands DR expression has been noted in segments of intercalated ducts (THRANE and BRANDTZAEG 1987a), and scattered duct-lining cells are likewise positive in mammary glands (BHAN and DESMARAIS 1983; WHITWELL et al. 1984). In the normal gut, epithelial DR is virtually restricted to the enterocytes of the small intestinal villi (SCOTT et al. 1980; SELBY et al. 1981; GHOSH et al. 1986). In inflammatory conditions, however, increased expression of both class I and II molecules are observed in secretory epithelial cells of all these organs (SCOTT et al. 1981; SELBY et al. 1983; GHOSH et al. 1986; POULSEN et al. 1986; THRANE and BRANDTZAEG 1987a; ROGNUM et al. 1987a).

3 Salivary Gland Neoplasia

3.1 Secretory Component and Secretory Immunoglobulins

SC-positive epithelial elements have been found in pleomorphic adenoma (KORSRUD and BRANDTZAEG 1984a; FANTASIA and LALLY 1984; SAITO et al. 1984), Warthin's tumor or adenolymphoma (KORSRUD and BRANDTZAEG 1984b), and a few adenocystic and mucoepidermoid carcinomas (SAITO et al. 1984); IgA generally appears in a similar but less extensive distribution (Table 2). Large variability in co-expression of SC and the other secretory markers (Ly, Lf and Am) occurs in pleomorphic adenomas (KORSRUD and BRANDTZAEG 1984a), probably reflecting the complexity of this tumor in terms of differentiation. The most consistent epithelial positivity in Warthin's tumor was seen for SC and IgA, whereas the three other secretory markers were either absent or faintly expressed (KORSRUD and BRANDTZAEG 1984b). The staining pattern thus resembled that of normal striated ducts and supported the notion that this neoplasia is initiated in striated duct anlagen (THOMPSON and BRYANT 1950). The number of malignant salivary gland tumors studied till now is too low to reveal any confident relation between SC expression and differentiation (Table 2).

3.2 Lysozyme and Lactoferrin

Epithelial Ly is expressed by relatively few pleomorphic adenomas (KORSRUD and BRANDTZAEG 1984a; SAITO et al. 1984; SEHESTED et al. 1985; CASELITZ et al.

1981) and Warthin's tumors (KORSRUD and BRANDTZAEG 1984b; SEHESTED et al. 1985) and it does seldom occur in malignant salivary gland tumors (CASELITZ et al. 1981; SAITO et al. 1984; SEHESTED et al. 1985) (Table 2). Positivity for Ly may thus corroborate exclusion of malignancy. Lf is more extensively distributed in both benign and malignant salivary gland tumors, although squamous cell carcinomas and anaplastic carcinomas are negative (Table 2). Since Ly and Lf may be considered as "terminal duct markers" (Fig. 1), the relatively consistent expression of Lf in pleomorphic adenomas supports a histogenetic relationship of this tumor to intercalated ducts (ERLANDSON et al. 1984) or, rather, to a duct-associated basal stem cell which may differentiate into both ductal elements and myoepithelial cells (REGEZI and BATSAKIS 1977; CASELITZ et al. 1985).

3.3 Amylase

Am is expressed almost exclusively by acinic cell carcinomas (CASELITZ et al. 1983; WARNER et al. 1985) and is present in more than half of the tumor specimens (Table 2), although in reduced amounts compared with normal parotid glands (FRANZEN et al. 1979). Its expression may depend on the degree of acinar cell differentiation (MORLEY et al. 1983). Nevertheless, Am seems to be a useful marker for acinic cell tumors.

3.4 MHC Class I and II Molecules

The neoplastic cells of pleomorphic adenoma (THRANE et al. 1987b) and Warthin's tumor (NATALI et al. 1981; THRANE and BRANDTZAEG 1987b) are virtually negative for MHC class II (HLA-DR) determinants (Table 2). These findings support the proposed histogenetic relationship between pleomorphic adenoma and myoepithelial cells (or their immediate precursors) and between Warthin's tumor and striated duct cells, respectively – i.e., cell types that under normal conditions show no DR expression (Table 2). However, pleomorphic adenomas are often rich in DR-positive dendritic histiocytic cells of apparently reactive nature (THRANE et al. 1987c).

Preliminary observations in our laboratory have indicated that epithelial elements in both pleomorphic adenoma and Warthin's tumor express MHC class I determinants. Studies of malignant salivary gland tumors are needed to see if loss of such determinants is related to malignant development.

3.5 Cell Lines and Transplantation Studies

Expression of various epithelial markers has been examined in cell lines isolated from salivary gland tumors (HAYASHI et al. 1985). Only neoplastic duct cells were found to express Lf and SC (SATO et al. 1984). Transplantation of such tumors to athymic mice gives further support for the notion that pleomorphic adenoma is derived from a multipotential duct stem cell able to differentiate

Table 2. Presence (+) or absence (-) of secretory component (SC), epithelial IgA, lysozyme, lactoferrin, amylase and MHC class I and II molecules in salivary gland epithelium and corresponding tumors

	Marker proteins							References
	SC	IgA	Lysozyme	Lactoferrin	Amylase	MHC class I	MHC class II	
Normal salivary glands								
Serous acini	+	+	-(+) ^a	-(+)	+	+	-	KORSRUD and BRANDTZAEG 1982 THRANE and BRANDTZAEG 1987a
Mucous acini	-	-	-	-	-	+/-	-	KORSRUD and BRANDTZAEG 1982 THRANE and BRANDTZAEG 1987a
Intercalated ducts	+	+	+	+	-	+	+/-	KORSRUD and BRANDTZAEG 1982 THRANE and BRANDTZAEG 1987a
Striated ducts	+	+	-	-(+)	-	+	-	KORSRUD and BRANDTZAEG 1982 THRANE and BRANDTZAEG 1987a
Salivary gland tumors								
<i>Benign tumors</i>								
Pleomorphic adenoma (59%) ^b								
	+(11/15) ^c	+(9/15)	+(2/15)	+(11/15)	-(0/5)			HODES et al. 1981
	+(16/16)				-(0/10)			MORLEY et al. 1983
	+(8/11)	+(8/11)	+(5/11)	+(6/11)	-(0/15)			KORSRUD and BRANDTZAEG 1984a FANTASIA and LALLY 1984
	+(6/7)	+(6/7)	+(5/13)	+(10/13)				SAITO et al. 1984 SEHSTEDT et al. 1985
								TOTO and Hsu 1985
Warthin's tumor (adenolymphoma) (6%)	+(27/27)	+(27/27)				+(10/10)	+(1/10)	THRANE et al. 1987c
					-(0/3)		-(0/3)	NATALI et al. 1981
	+(6/6)	+(6/6)	+(2/6)	+(6/6)	-(0/7)			HODES et al. 1981 HSU et al. 1981
	+(22/22)	+(22/22)	-(0/3)	+(2/3)	-(0/6)			MORLEY et al. 1983
						+(7/7)	-(0/7)	KORSRUD and BRANDTZAEG 1984b CASELITZ et al. 1984 SEHSTEDT et al. 1985
								THRANE and BRANDTZAEG 1987b

Malignant tumors

Malignant pleomorphic adenoma (7%)	- (0/21)	+ (7/21)		CASELITZ et al. 1981
	- (0/8)	+ (5/8)		CASELITZ et al. 1982
	+ (1/1)	+ (1/1)		SEHESTED et al. 1985
Adenocarcinoma (11%)			- (0/3)	CASELITZ et al. 1983
- Adenoid cystic carcinoma (4%)		+ (16/16)	- (0/5)	CASELITZ et al. 1981, 1982
				CASELITZ et al. 1983
	+ (7/7)			SAITO et al. 1984
	+ (24/24)	+ (24/24)	- (0/24)	CASELITZ et al. 1986
- Cystadenocarcinoma (2.5%)	- (0/7)	+ (2/4)		CASELITZ et al. 1981
	+ (24/24)	+ (3/5)		CASELITZ et al. 1982
- Salivary duct carcinoma (1%)	- (0/3)	+ (2/3)	- (0/3)	CASELITZ et al. 1981
			- (0/3)	CASELITZ et al. 1983
			+ (2/2)	HODES et al. 1981
- Acinic cell carcinoma (2.5%)			+ (0/3)	CASELITZ et al. 1983
			- (0/3)	MORLEY et al. 1983
	+ (13/15)	+ (13/15)	+ (11/15)	WARNER et al. 1985
Mucoepidermoid carcinoma (11.5%)			- (0/1)	HODES et al. 1981
		+ (3/7)	- (0/2)	CASELITZ et al. 1981, 1982
Anaplastic carcinoma (2%)	+ (3/7)	- (0/3)		CASELITZ et al. 1983
Squamous cell carcinoma (4.5%)		- (0/8)	- (0/8)	SAITO et al. 1984
				CASELITZ et al. 1981
			- (0/2)	CASELITZ et al. 1981
				CASELITZ et al. 1983

^a Infrequent finding given in parenthesis

^b Occurrence frequency (adapted from Shafer, Hine and Levy: A textbook of oral pathology, 3rd edn. Saunders, Philadelphia, USA)

^c No. of positive cases in relation to total no. tested

into both duct and myoepithelial cells (CASELITZ et al. 1985). A similar reserve duct cell has recently been proposed to be the origin of adenocystic carcinoma (CASELITZ et al. 1986).

3.6 General Remarks

Application of immunohistochemical markers has provided interesting information related to histogenesis in salivary gland neoplasia. However, further studies are needed because of the particular complexity of these tumors, especially when it comes to understanding of the nature of pleomorphic adenomas. It remains to be shown whether tumor markers will be of any value for classification and prognostic prediction in salivary gland neoplasia.

4 Breast Neoplasia

Several epithelial products have been studied in breast tumors but, with the exception of receptors for oestrogen and progesteron (MCGUIRE et al. 1975; THORESEN et al. 1982), most of these markers have been of no value for prognostic and therapeutic considerations. In this chapter the discussion will be limited to the significance of SC, SIgA and MHC class I and II molecules.

4.1 Secretory Component and Secretory Immunoglobulins

RICHMAN (1976) claimed that epithelial IgA was similarly expressed by normal and malignant glandular epithelium, but most authors have reported that normal breast tissue and benign tumors contain more SC and epithelial IgA than carcinomas (HARRIS et al. 1975; LEE and BUEHRING 1981). We found about three quarters of the carcinomas to be positive for SC with a heterogeneous expression pattern (KVALE et al. 1987a), which accorded with the report of HARRIS and SOUTH (1981). Epithelial IgA positivity correlated well with SC expression but not with the histopathological grade of the breast carcinomas (KVALE et al. 1987a). Total serum SC, present in circulating SIgA and SIgM, showed no relation to tumor SC, indicating that the spillover to blood was generally low. A major increase in circulating SC reflected, instead, the presence of liver metastasis (KVALE et al. 1987a). The same was true for colonic carcinomas (KVALE et al. 1987b).

4.2 MHC Class I and II Molecules

Several laboratories have studied the distribution of MHC class I and II determinants in breast neoplasia (FLEMING et al. 1981; BHAN and DESMARAIS 1983; ROWE and BEVERLY 1984; WHITWELL et al. 1984; LWIN et al. 1985). In contrast

to normal glandular epithelium and benign tumors, carcinomas show no consistent positivity (ROWE and BEVERLY 1984). In 28 breast carcinomas studied in our laboratory, 16 were class I-positive and 9 DR-positive (ROGNUM et al., unpublished observations). There seems to be no relation between the number and distribution of T cells and the expression of these determinants in the malignant breast epithelium (BHAN and DESMARAIS 1983; WHITWELL et al. 1984). Further studies are needed to confirm the hypothesis of SANDERSON and BEVERLY (1983), that impaired expression of class I and II molecules may be due to immunoselection of malignant cell clones.

4.3 Identification of Metastasis by Glandular Markers

Metastases from poorly differentiated or anaplastic adenocarcinomas are sometimes difficult to distinguish from malignant lymphomas by conventional histopathology. Preserved marker expression compatible with secretory epithelium may thus be of crucial value for the identification of secondary breast carcinomas in lymph nodes (BRANDTZAEG and ROGNUM 1983). It has been shown for colonic carcinomas that the original pattern of glandular markers is often preserved even after several years of tumor progression (ROGNUM et al. 1985).

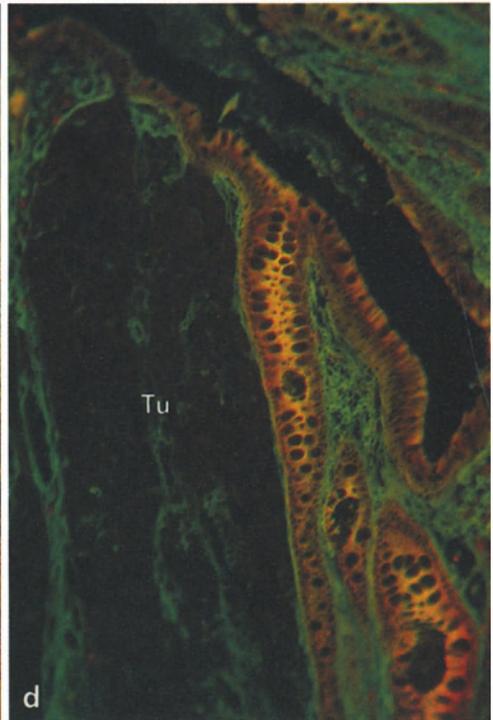
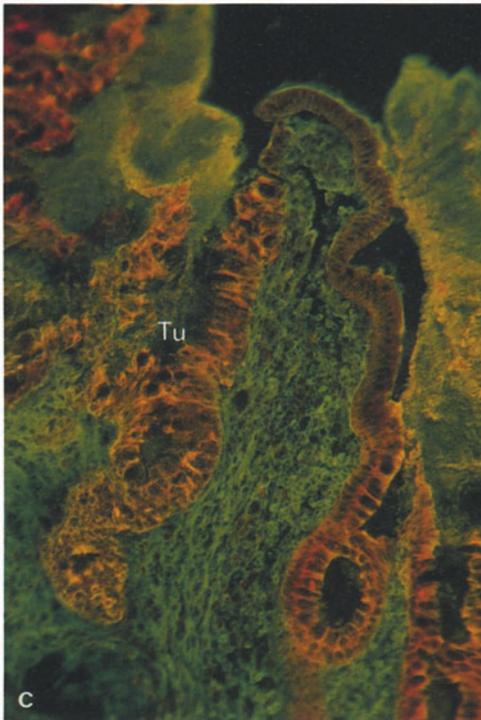
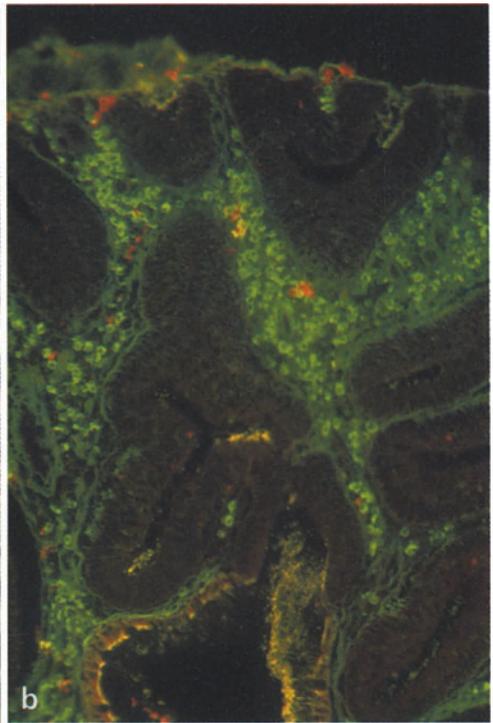
5 Large Bowel Neoplasia

5.1 Carcinoma

Several attempts have been made to define tumor characteristics of prognostic value in patients with large bowel carcinoma. There is a positive relation between histological grade and survival time (RANKIN and BRODERS 1928; REMMELE and HEINE 1981), whereas changes of tumor mucus seem to be without prognostic value (SYRJANEN and HJELT 1978). Staging is considered as the most reliable way to assess prognosis (DUKES and BUSSEY 1958), but also production of SC (ARENDS et al. 1984), DNA ploidy pattern, (WOLLEY et al. 1982; ROGNUM et al. 1982a, 1987b), and epithelial expression of class I and II determinants (DAAR et al. 1982; ROGNUM et al. 1983; CSIBA et al. 1984; MOMBURG et al. 1986) are claimed to afford prognostic information.

5.1.1 Secretory Component and Secretory Immunoglobulins

SC and epithelial IgA are well correlated in the same carcinomas (ROGNUM et al. 1980a). Concordant expression of these markers indicates preservation of a relatively high degree of functional differentiation (Figs. 2, 3); which is in harmony with histopathological observations (POGER et al. 1976; WEISZ-CARRINGTON et al. 1976; ROGNUM et al. 1980a, 1982b; ISAACSON 1982). Interestingly, ARENDS et al. (1984) reported that patients with homogeneously SC-positive carcinomas had a better prognosis than those with SC-negative ones.



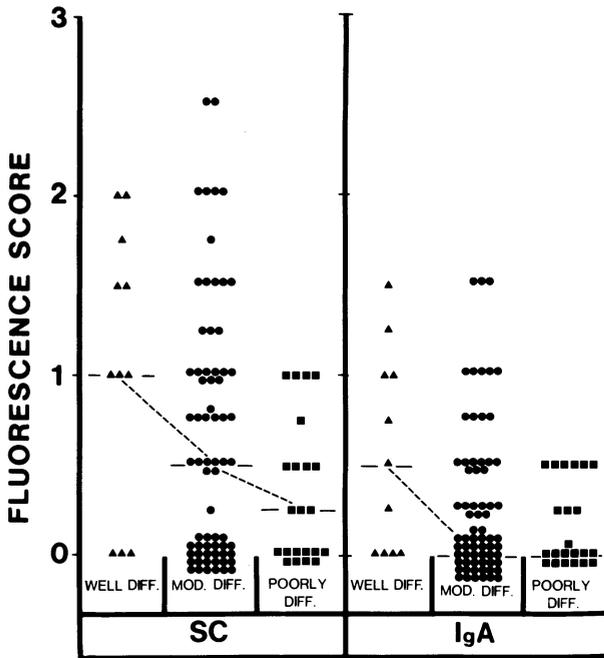


Fig. 3. Immunofluorescence scores given for epithelial staining of poorly (■), moderately (●), or well-differentiated (▲) carcinomas. Medians are connected by dashed lines. Well-differentiated carcinomas tended to express more SC and IgA than moderately differentiated ($p=0.08$ and $p=0.09$, respectively), and moderately and poorly differentiated carcinomas taken together were scored significantly lower than well-differentiated ones (SC, $p < 0.05$; IgA, $p = 0.06$). Adapted from ROGNUM et al. (1982b)

5.1.2 MHC Class I and II Molecules

Epithelial MHC class II determinants may appear in the large bowel in both inflammatory (SELBY et al. 1983) and neoplastic (ROGNUM et al. 1983; GHOSH et al. 1986) conditions. No simple relationship to tumor differentiation, clinico-pathological stage or biological behaviour has as yet emerged from studies of these markers in large bowel carcinoma (DAAR et al. 1982; DAAR and FABRE 1983; CSIBA et al. 1984; UMPLEBY et al. 1985; MOMBURG et al. 1986; GHOSH et al. 1986).

Nevertheless, for HLA-DR we found a remarkable disparity according to histological tumor grade: well-differentiated carcinomas showed great intra-

◁ Fig. 2. Immunohistochemical staining for SC (red) and IgA (green). a Normal colon mucosa; yellow indicates presence of both SC and IgA and signifies normal epithelial transport. b Large bowel adenoma with severe dysplasia; the epithelium lacks both SC and IgA although there are numerous IgA-producing immunocytes in the stroma. c Well-differentiated large bowel carcinoma; yellow in tumor epithelium (Tu) signifies preserved SC-mediated transport of IgA. d Poorly differentiated large bowel carcinoma; tumor epithelium (Tu) lacks both SC and IgA. a × 260, b, c, d × 105

tumor staining variability whereas poorly differentiated and highly aggressive carcinomas were without exception homogeneously stained – either positively or negatively (ROGNUM et al. 1983). Differences in the composition of the carcinoma material and in the recording of staining heterogeneity might explain why previous studies failed to reveal these associations with DR expression. Our observations are in agreement with the concept of clonal evolution of tumor cell populations (NOWELL 1976, 1986). According to this hypothesis tumor progression is due to an acquired genetic lability permitting stepwise selection of variant sublines of neoplastic cells. One clone may possess selective advantages and finally become predominant. Another argument for this theory is that in cases with advanced carcinoma the staining patterns for SC, epithelial IgA, and HLA-DR were found to be remarkably similar in primary and secondary tumors from the same patient (ROGNUM et al. 1985).

5.2 The “Transitional Mucosa”

The so-called “transitional mucosa” adjacent to colorectal carcinoma (FILIPE and BRANFOOT 1976) is characterized by tall and often branched crypts, dilated goblet cells and increased mucus secretion with predominance of sialomucins (DAWSON and FILIPE 1976). Moreover, the increase in sialomucins and concomitant decrease in sulfomucins is directly related to extent of invasion by the adjacent carcinomas (FILIPE and BRANFOOT 1974). These features may either reflect a pre-neoplastic phase (FILIPE and BRANFOOT 1974) or merely represent secondary changes due to toxic influences from the adjacent carcinoma. Furthermore, CEA seems to increase in the transitional mucosa with decreasing degree of tumor differentiation (ROGNUM et al. 1982b).

5.2.1 Secretory Component and Secretory Immunoglobulins

Decreased expression of SC and epithelial IgA was seen in the transitional mucosa both with decreasing degree of tumor differentiation and with increasing clinico-pathological stage (ROGNUM et al. 1982b). Expression of SC and IgA in this zone also seemed to be related to the DNA ploidy pattern of the carcinoma as less epithelial staining was observed adjacent to aneuploid than near diploid tumors (ROGNUM et al. 1982a).

5.3 Pre-cancerous Lesions in Ulcerative Colitis

Patients with long-standing total ulcerative colitis (UC) are at increased risk of developing large bowel carcinomas (LENNARD-JONES et al. 1983). This risk is related to development of epithelial dysplasia (MORSON and PANG 1967; RIDDEL et al. 1983). All patients with long-standing UC, therefore, should be subjected to regular biopsy sampling from the various segments of the large bowel to detect and select those who would benefit from prophylactic colectomy.

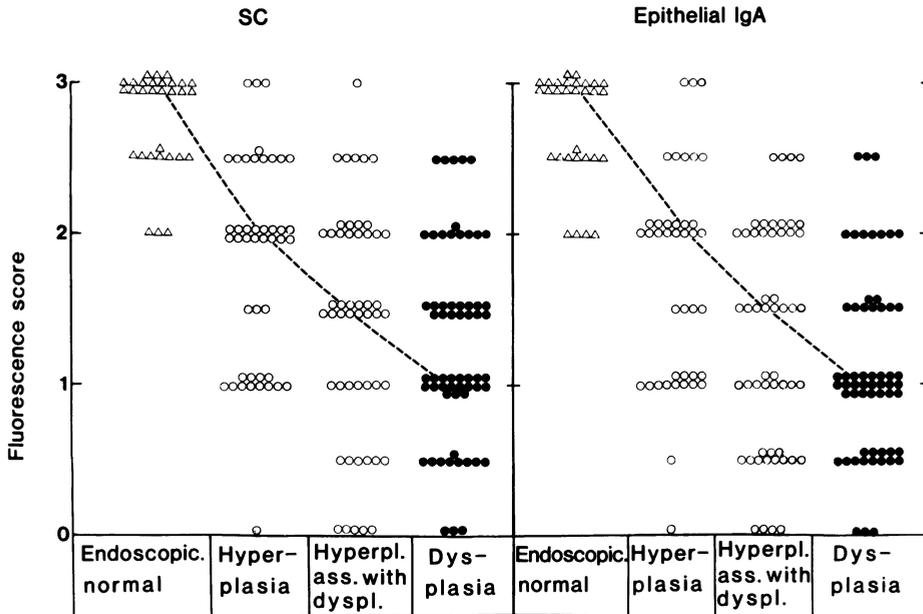


Fig. 4. Relation between immunofluorescence scores for SC and epithelial IgA in various large-bowel lesions of patients with long-standing ulcerative colitis (epithelial hyperplasia, hyperplasia associated with dysplasia in other areas, or dysplasia) compared with endoscopically normal biopsy specimens. Dashed lines connect median scores for each category. SC and IgA were scored significantly higher for normal mucosa than for the three lesions and also higher for hyperplasia than for dysplasia ($p < 0.01$). Adapted from ROGNUM et al. (1982c)

Inflammation and crypt destruction result in regenerative epithelial alterations which may imitate dysplasia (RIDDEL et al. 1983), and many attempts have been made to find markers of a truly dysplastic development. ISAACSON (1976) claimed that epithelial expression of CEA was indicative of pre-cancer but we could not confirm this observation (ROGNUM et al. 1982c). Binding of peanut lectin likewise failed to afford a precise diagnosis (JASS et al. 1986).

5.3.1 Secretory Component and Secretory Immunoglobulins

Dysplastic epithelium was reported by ISAACSON (1982) to be consistently negative for SC. Also ROGNUM et al. (1982c) found significantly reduced expression of SC and epithelial IgA compared with reactive hyperplasia, but large individual variations were observed in both types of lesions (Fig. 4) and precluded application of these markers in diagnostic work.

5.3.2 MHC Class I and II Molecules

Evaluation of heterogeneous expression of SC and class II determinants (Figs. 5 and 6) appears more promising in a diagnostic context (ROGNUM et al. 1987a).

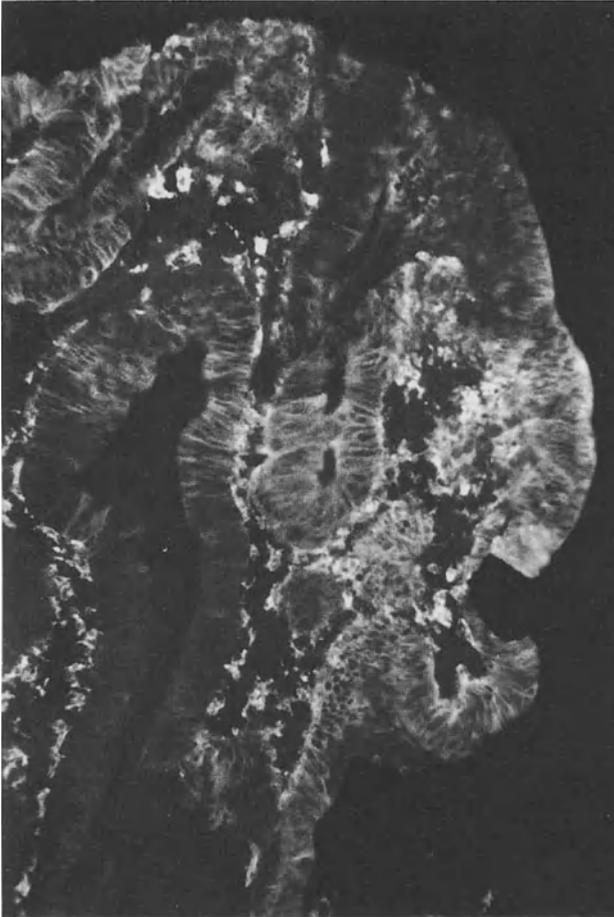


Fig. 5. Heterogeneous expression of epithelial HLA-DR determinants in ulcerative colitis with severe dysplasia. $\times 150$

In high grade dysplasia abrupt transitions between intensely positive and completely negative epithelial cells were often seen. The similarities between dysplastic lesions and early large bowel carcinomas (ROGNUM et al. 1983) indicated that such heterogeneous marker expression is characteristic of a neoplastic development in the colon epithelium. The clinical significance of this observation in distinguishing regenerative from dysplastic epithelium will have to be evaluated in a larger follow-up study.

5.4 Adenomas

The theories of an “adenoma-carcinoma sequence” (MORSON 1974) or a “dysplasia-carcinoma sequence” (MORSON and KONISHI 1980) postulate that most

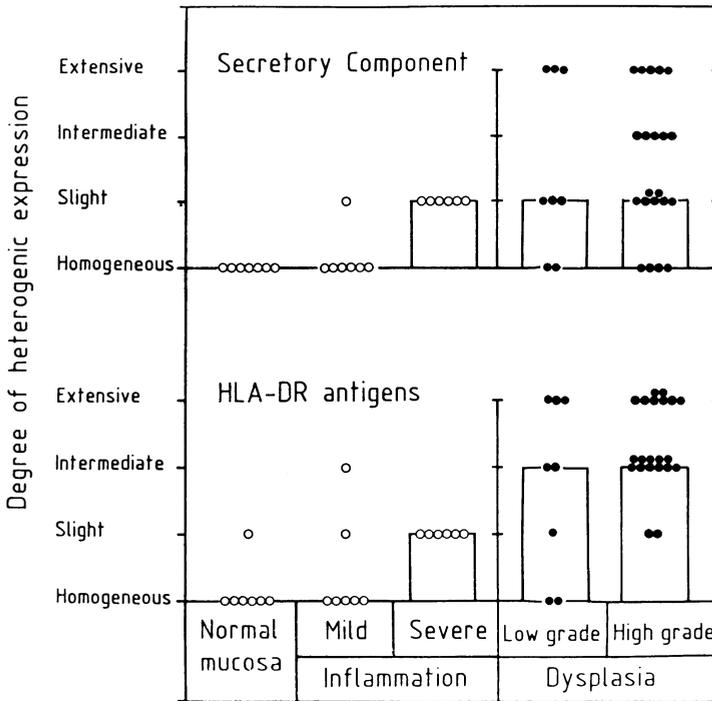


Fig. 6. Scatter diagram of degree of heterogeneous expression of HLA-DR and secretory component in normal colonic mucosa and inflammatory or dysplastic lesions of various severity. From ROGNUM et al. (1987a)

large-bowel carcinomas develop through increasing degrees of epithelial dysplasia. This notion has been supported by recent advances in the field of molecular biology. Increased expression of the *ras* oncogene in the course of neoplastic transformation is of particular interest (SPANDIDOS and KERR 1984). Studies of DNA ploidy patterns (GOH and JASS 1986), SC and epithelial IgA (ISAACSON 1982; ROGNUM et al. 1982d) may support the stepwise concept as cancer-like alterations are more pronounced in high grade dysplasia. Nevertheless, the decreased expression of SC and epithelial IgA, both with increased degree of dysplasia in adenomas (Fig. 2b) and with decreased degree of differentiation in adenocarcinomas (Figs. 2d, 3), indicates that invasiveness requires a second and as yet unknown fundamental change in the epithelial cell.

5.5 Metaplastic Polyps

The metaplastic polyp is regarded as a non-neoplastic disorder of differentiation (KAYE et al. 1973). Nevertheless, metaplastic polyps and colorectal carcinomas show several common features such as mucin alterations (BOLAND et al. 1982), increased CEA expression (ROGNUM et al. 1982d; JASS et al. 1984), and decreased SC and epithelial IgA (ROGNUM et al. 1982d; JASS and FAULDY 1985).

This does not necessarily imply that a metaplastic polyp is precancerous but rather that it develops as a result of environmental factors which may be implicated in carcinogenesis.

6 Conclusions

Markers of glandular differentiation are useful in several ways:

- they may contribute to better understanding of the histogenesis of certain tumors
- they may corroborate histopathological and clinical evaluation of tumors by enhancing the diagnostic and prognostic information
- they may be of significant value in the search for origin of anaplastic secondary tumors
- they may be an adjunct in distinguishing pre-cancerous epithelial alterations (dysplasia) from reactive hyperplasia

It is important to stress that several of these markers may likewise show either increased or decreased epithelial expression during inflammatory conditions. Such changes may imitate those found in tumors and precancerous lesions. It is crucial, therefore, to pay attention to the distribution pattern of the actual markers (e.g., homogenous vs. heterogeneous) and to the histopathological features of the lesions.

Methodological considerations have been beyond the scope of this chapter although tissue preparation and immunological staining technology are important variables in immunohistochemical work. The markers discussed above may thus be denatured or masked by routinely used formalin fixation and paraffin embedding. Some of these problems have been discussed in detail elsewhere (ROGNUM et al. 1980b; BRANDTZAEG 1981; BRANDTZAEG and ROGNUM 1984a, 1984b). With regard to the choice between immunoenzyme or immunofluorescence staining, the former method affords the possibility of evaluating morphological features in the same section. However, the observation of coexpression of two markers in a single cell is usually unreliable with paired immunoenzyme staining (VALNES et al. 1983; VALNES and BRANDTZAEG 1984). Paired immunofluorescence is superior for such studies (BRANDTZAEG 1981; BRANDTZAEG and ROGNUM 1983).

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Mesenchymal Tumor Markers: Intermediate Filaments

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1 Introduction

By far the majority of mesenchymal tumors can be classified by their typical morphological patterns in routine stains. In a minority of such tumors, and in particular those with an undifferentiated morphology, a differential diagnosis may be difficult of one relies only on the light microscopical appearance. In such situations immunohistochemical techniques using polyclonal or monoclonal antibodies can provide additional information; here we emphasize the use of antibodies specific for the different intermediate filament proteins in human tumor diagnosis and concentrate in particular on the use of antibodies to desmin and vimentin in the differential diagnosis of mesenchymal tumors.

Intermediate filaments (IF), also referred to as 10 nm filaments or 100 Å filaments, are one of the three fibrous elements of the cytoskeleton. They are present in nearly all cells of vertebrates (FRANKE et al. 1978; BENNETT et al. 1978; HOLTZER et al. 1982; OSBORN et al. 1982a; WEBER and GEISLER 1984) and seem to occur at least in some of the invertebrates. In electron micrographs they can be distinguished from the other two filament systems by their diameter (7–11 nm) which is intermediate between that of microfilaments (6 nm) and microtubules (20–22 nm). Using immunological techniques all three systems can be distinguished by the use of appropriate antisera. In the electron microscope

it is difficult to distinguish different intermediate filament types since all IFs show very similar morphologies. However immunohistological studies on normal tissues as well as biochemical and protein chemical data have demonstrated that intermediate filament proteins can be subdivided in five major categories: keratins, vimentin, desmin, GFAP and neurofilaments (see above references). The most important intermediate filaments markers for mesenchymal tumors are desmin and vimentin.

2 Definition

2.1 Desmin

Desmin (skeletin) is the intermediate filament protein typical of skeletal, visceral and some vascular smooth muscle cells. In 1977 SMALL and SOBIESZEK showed that a 55 kd band in an SDS gel from smooth muscle preparations disappeared after *in situ* proteolysis of muscle. The 55 kd protein extracted from muscle by acetic acid could also be reconstituted into filamentous arrays (for later experiments which yielded reconstituted 10 nm filaments with a morphology equivalent to those seen *in vivo*, see GEISLER and WEBER 1981b; IP et al. 1985). Since the contractility of the smooth muscle was not reduced by the removal of the 55 kd protein and in addition extraction of actin did not influence the general cell shape, this protein was assumed to have a cytoskeletal function and given the name "skeletin" (SMALL and SOBIESZEK 1977). The same protein was also shown to be present in the cytoskeleton of heart Purkinje cells and in normal myocardium (THORNELL et al. 1978, 1983). At about the same time LAZARIDES and HUBBARD (1976) demonstrated an analogous protein in chicken smooth muscle. Using antibodies specific for this protein they showed that it was present at the Z-discs of isolated skeletal myofibrils. In heart muscle cells in culture desmin was found not only in the Z-discs, but also in intercalated discs. Since this protein appeared to link myofibrils and thus resembled the desmosomes in epithelial tissues, it was named desmin (Greek: desmos = link, band). It soon became clear that desmin and skeletin were identical proteins. As the name desmin has gained more general acceptance it will be used here.

2.2 Vimentin

Vimentin filaments are the only IF type characteristically present in nonmuscular mesenchymal cells. Two groups originally described a major cellular protein different from actin and tubulin which was enriched in the detergent resistant cytoskeleton of cells grown in culture (OSBORN and WEBER 1977; BROWN et al. 1976). Rabbit autoantibodies which decorated fibres in certain permanent cell lines and which were different from microfilaments and microtubules were also described. Comparing the immunofluorescence pictures with electron microscopic data it became clear that the decorated structures were bundles of inter-

mediate filaments of the keratin type (OSBORN et al. 1977). In addition several human hepatitis sera were shown also this time to contain high levels of antibodies recognizing vimentin intermediate filaments in human fibroblasts (OSBORN, unpublished data). These studies with autoantibodies soon led to the production of antibodies raised against the different intermediate filament proteins. Thus antibodies specific for the 55 Kd band from mouse 3T3 cells clearly decorated intermediate filaments in a variety of human and animal cells (FRANKE et al. 1978). This antibody also decorated perinuclear whorls or aggregates after treatment of the cells with colcemid, a characteristic feature associated with intermediate filaments in cells, known from previous electronmicroscopic studies (GOLDMAN and KNIPE 1972; HOLTZER et al. 1975). In contrast this antibody did not react on cells of epithelial, muscle or neuronal origin. To emphasize the difference between this protein and the proteins forming other intermediate filament types it was named vimentin (latin vimentum – wickerwork), a name which also reflects the appearance of intermediate filaments in fibroblasts (FRANKE et al. 1978).

3 Biochemical and Molecular Structure of Desmin and Vimentin Intermediate Filaments

For biochemical work sources rich in only one IF protein are optimal. Desmin has been usually purified either from chicken gizzard or from pig stomach, while for vimentin porcine eye lenses are the starting material of choice. The purification schemes usually involve gel filtration and DEAE cellulose chromatography performed in the presence of 6 to 8 M urea.

The first amino acid sequence work in porcine vimentin and on desmin from chicken and pig established several points important for the later development of the IF field (GEISLER and WEBER 1981a). First vimentin and desmin were shown to be different gene products but related in sequence. Second, from the linear sequence it was clear that part of the desmin and vimentin molecules contained a seven residue repeat pattern (a–g), where the residues in a and d positions are primarily hydrophobic. The presence of these repeating heptade sequences argues for a coiled coil formation of the helical domains. Third, as already argued prior to 1981 from immunological data, tissue specificity overrode species divergence (for review see WEBER and GEISLER 1984; GEISLER and WEBER 1986).

Because the partial sequences suggested that IF molecules might contain a central α -helical rod domain conserved in sequence principle and in length, rather than in actual sequence, it was necessary to obtain a complete sequence for one IF protein. This was first done for desmin in 1982 by GEISLER and WEBER. The desmin sequence, taken together with the then available partial sequences of other IF proteins showed that IF proteins are characterized by the prototype structure shown in Fig. 1. In this structure the canonic principle is the rod domain – the central α -helical rod region some 310 residues in length. This rod domain has been documented in all of the 20–30 IF sequences currently known from protein and from DNA sequencing work (see especially HANUKOGLU and FUCHS 1982, 1983; STEINERT et al. 1983). In contrast to the rod region, the terminal domains – the head and the tail – can be hypervariable both in sequence and length. Thus those features common to all IF proteins e.g. the 21 nm repeat seen in electron microscopy for metal shadowed *in vitro* reconstituted IFs, the 7–11 nm diameter seen for filaments both *in vivo* and after *in vitro* assembly, as well as the apparently similar morphologies in the electron microscope can be attributed to the rod domain. Consequently differences in function or in stability of different IF proteins are more likely to reside in the end domains.

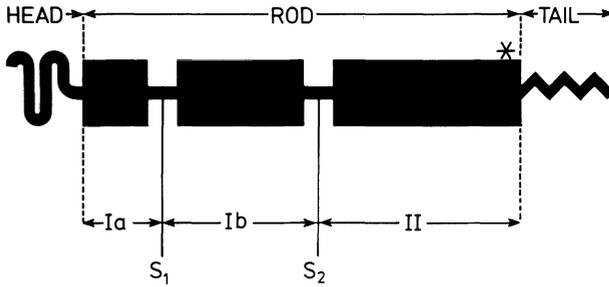


Fig. 1. A schematic representation of the domain structure of IF-proteins. The α -helical rod domain common to all IF proteins is interrupted by the nonhelical spacers S_1 and S_2 situated between helices Ia and Ib, and Ib and II respectively. The rod domain is flanked at the aminoterminal side by the nonhelical headpiece (head) and at the carboxyterminal side by the nonhelical tailpiece (tail). The asterisk marks the approximate position of the epitope of monoclonal antibody (IFA) that recognizes an epitope common to many proteins (PRUSS et al. 1981). (This figure is adapted from GEISLER und WEBER 1986)

Desmin and vimentin, together with glial fibrillary acidic protein (GFAP), form a subgroup of closely related non-epithelial, or type III, IF proteins. When sequence identities of the rod region of these IF polypeptides are compared identity within this group is around 63% whereas when the same sequences are compared either to the keratin type I or to the keratin type II sequences, the identity drops to around 30% (GEISLER and WEBER 1982; WEBER and GEISLER 1984; HANUKOGLU and FUCHS 1982, 1983). Although all three neurofilament proteins also share the same structural motif the identity between the rod regions of NF-L and vimentin is only 53%. A comparison of the DNA gene sequences shows that while the introns are very strongly conserved in number and position between keratin types I and II and the non-epithelial desmin, vimentin and GFAP group, NF-L (LEWIS and COWAN 1986) and NF-M show a totally different intron arrangement. Thus currently desmin, vimentin and GFAP are assigned to a IF group III separate from neurofilament proteins which are placed in group IV. Complete DNA sequences have been provided for hamster desmin and for hamster vimentin by QUAX et al. 1983, 1984, 1985. Hybridization studies suggest that vertebrates have only one gene for desmin and one for vimentin. (for review see BLOEMENDAL et al. 1985; for chicken m-RNA see NGAI et al. 1985; ZEHNER and PATERSON 1985).

The considerations important in arriving at a model of the 10 nm filament have been detailed elsewhere. Cross linking experiments, performed on a desmin derivative containing the whole rod region, showed that this was a tetramer and not a trimer (GEISLER and WEBER 1982). Thus the triple stranded model (STEINERT 1978) that had dominated IF structure for almost a decade could be laid to rest. The inference that the tetramer arose from double stranded coiled coils was soon confirmed by other methods. In the electron microscope the tetramers are indeed rod-shaped and have a length of around 50 nm (GEISLER et al. 1982). Decoration of these structures with Fab fragments from a desmin monoclonal antibody suggested that the two coiled coils in the tetramer are

antiparallel since antibody molecules could be seen on both ends (GEISLER et al. 1985). Although details of the exact arrangements of the tetramer units, and of the importance of the tetramer arrangement for filament assembly are still under discussion, it is generally agreed that each 10 nm filament contains on average around 8 tetramers per diameter or 32 polypeptide chains/diameter (GEISLER et al. 1985; for review see IP et al. 1985; GEISLER and WEBER 1986).

The principles important in deciding the filament structure are assumed to be the same for all IF proteins with one important exception. Whereas the non-epithelial proteins in groups III and IV (desmin, vimentin, GFAP and the NF-L polypeptide) form homopolymers i.e. each protein can form a 10 nm filament by itself, the keratins are obligatory heteropolymers requiring one type I and one type II polypeptide for filament formation (for keratin nomenclature see HANUKOGLU and FUCHS 1982, 1983).

4 IF Arrangement in Vitro

Certain cell types can contain more than one IF polypeptides (for review see OSBORN et al. 1982a). In such cells it is interesting to know whether the different IF polypeptides are present in the same or in different 10 nm filaments. Several approaches have been used to try to answer this question. In some cell types it is already clear at the light microscopical level (OSBORN et al. 1980) that keratin and vimentin are present in different IFs a fact that has been confirmed by immunoelectron microscopy. However when the non-epithelial proteins occur together in the same cell e.g. desmin and vimentin in the rhabdomyosarcoma cell line RD (Fig. 2), or GFAP and vimentin in a glioma cell line, double labelling at the light microscopic level usually shows very similar profiles for the two IF proteins. Using the electron microscope it could be shown that individual 10 nm filaments in the glioma line contained both GFAP and vimentin (SHARP et al. 1982) and that individual 10 nm filaments in the RD cells contained both desmin and vimentin (TÖLLE et al. 1986). These experiments, suggesting that type III IFs can copolymerize into the same 10 nm filament are supported by chemical cross linking experiments (QUINLAN and FRANKE 1982, 1983) and also by experiments in which antibodies specific for a single intermediate filament type III protein have been microinjected. Thus for example if antibodies specific for either vimentin or desmin are microinjected into a cell line containing both IF proteins, perinuclear caps are formed in which the two different IF proteins colocalize, again suggesting that the two intermediate filament proteins were present in the same 10 nm filament (TÖLLE et al. 1986).

When cell types which have only vimentin filaments are injected with antibodies that recognize vimentin, the intermediate filaments are cross linked and within 2–4 hours retract into perinuclear caps. Cells with such caps seem perfectly capable of carrying out basic cellular functions such as cell division and movement over a substratum (GAWLITTA et al. 1981; KLYMKOWSKY 1981; LIN and FERAMISCO 1981). These experiments as well as the discovery of cell lines which lack IFs and yet divide without problem, argue strongly that IF function is more subtle than that of the other two filament systems present in the cell, i.e. microfilaments and microtubules. Perhaps, therefore, it may be more appropriate to search for a putative function of IFs in tissues rather than cells. Undoubtedly in certain situations IFs, through their links to the plasma membrane and the nucleus, contribute to tissue integrity, and the combined IF-membrane linked system may then serve to restrict the mobility of individual cells. Monoclonal antibodies specific for vimentin (OSBORN et al. 1984a; GOWN and VOGEL

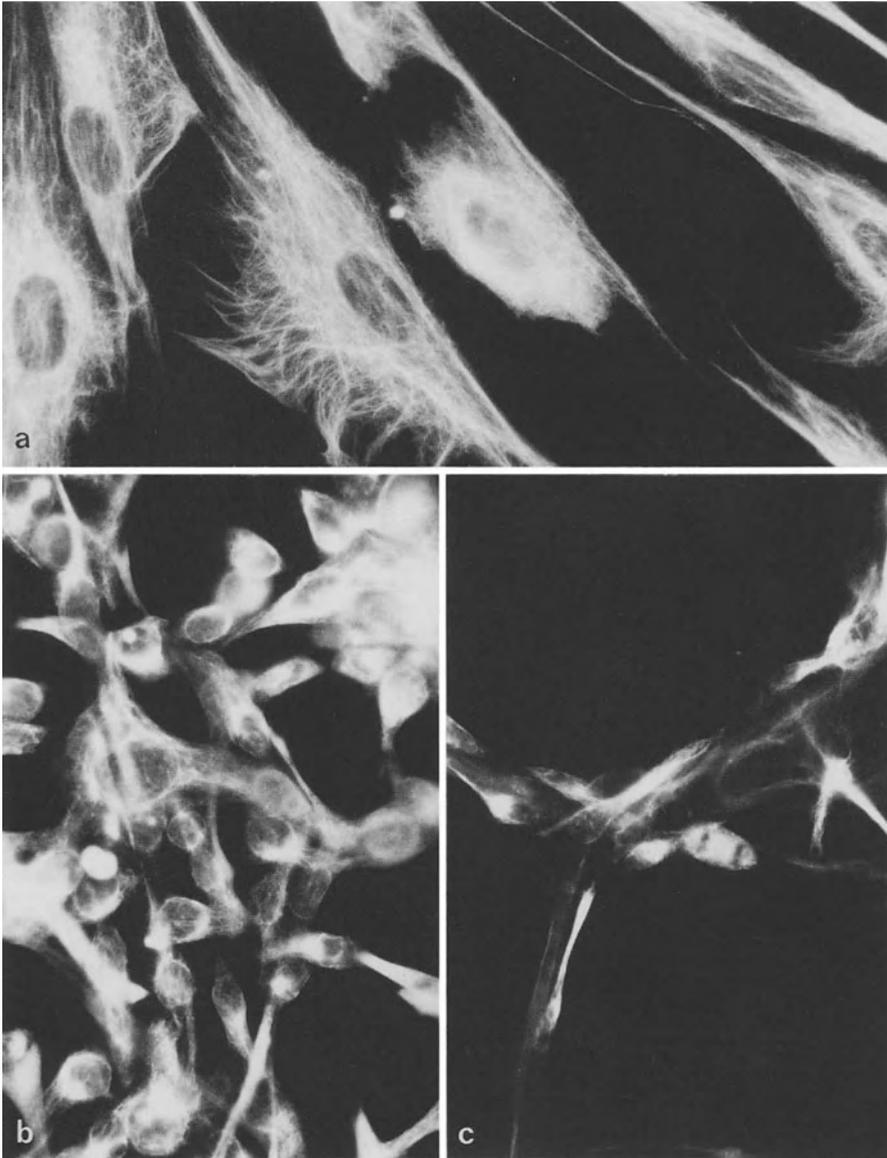


Fig. 2. Human cell lines labelled by antibodies to intermediate filaments. Fibroblast cell line (HS 27) stained by vimentin antibodies (a) and human rhabdomyosarcoma (RD) cell line incubated with antibodies against vimentin (b) and desmin (c). The fibroblast cell line expresses vimentin. In the RD cell line all cells are stained by the vimentin antibody (b), while the majority of cells additionally coexpress desmin (c) (a-c, FITC) (polyclonal antibodies against vimentin and desmin)

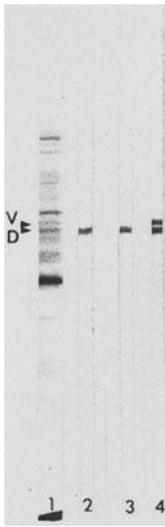


Fig. 3. Lanes 1–4 show an extract of the human rhabdomyosarcoma line RD stained with Coomassie Blue (lane 1) and immunoblots of the same RD extract after transfer to nitrocellulose and reaction with the desmin monoclonal antibodies DE-A-7 (lane 2), DE-C-3 (lane 3) and a mixture of the desmin monoclonal antibody DE-B-5 together with a vimentin monoclonal antibody (lane 4). V,D. indicate the positions of vimentin and desmin respectively (for further details see DEBUS et al. 1983a). Note that the RD cell line contains both vimentin and desmin as shown by immunoblotting

1984; VIRTANEN et al. 1985) or specific for desmin have been described (DEBUS et al. 1983) (Fig. 3). Whether all monoclonal vimentin antibodies display the same specificity when tested on a large number of normal cells and tumors has not been definitely determined (see below).

5 Distribution of Desmin and Vimentin IFs in Normal Tissue

5.1 Vimentin

In normal tissue almost all mesenchymal cells express either vimentin or desmin or simultaneously both intermediate filament proteins. The majority of nonmuscular cell types are characterized by a positive reaction with antibodies against vimentin. This is true for the “cytic” as well as the “blastic” cell forms i.e. vimentin positive cell types include: fibrocytes, fibroblasts (Fig. 4), osteocytes and osteoblasts, chondrocytes and chondroblasts (FRANKE et al. 1978; OSBORN et al. 1984b). Expression of vimentin filaments seems to be independent of germ layer derivation and even cells coming from the neuroectoderm express only vimentin, e.g. Schwann cells (Fig. 5c) (OSBORN et al. 1982b, 1986a). The situation appears more complex in bone marrow. Using polyclonal vimentin antibodies DELLAGI et al. (1983) have shown that mature cells of erythropoiesis and thrombopoiesis express neither vimentin nor other intermediate filaments types. In addition in primary cultures of plasma cells 30% of the cells could not be labeled with a vimentin antibody.

Currently the question of whether all lymphatic cells express vimentin cannot be answered with certainty. Because of the close spatial relationship of hetero-

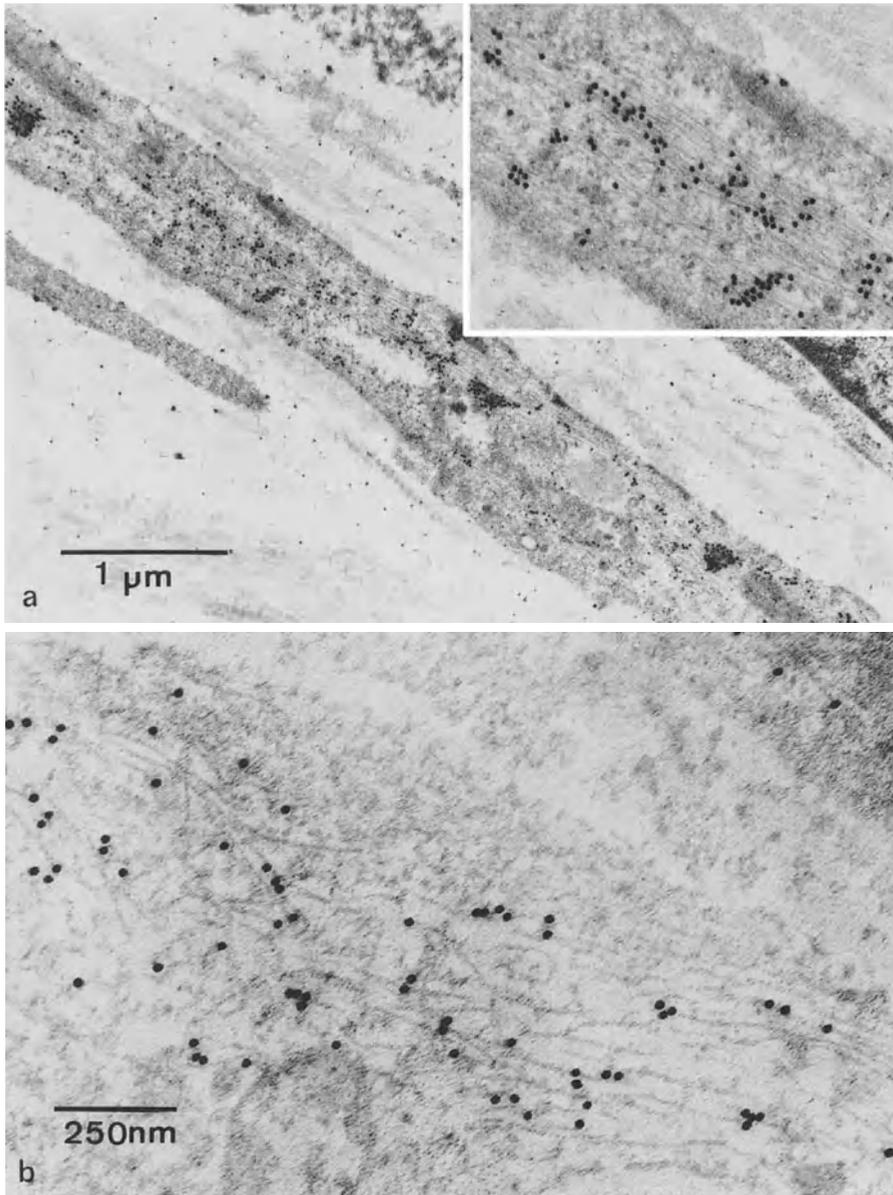


Fig. 4. Ultrastructural immunogold staining of vimentin. Tissue from an infantile fibromatosis was fixed in phosphate buffered saline containing 1% glutaraldehyde and 0.2% picric acid. It was then embedded at low temperature in Lowicryl K4M by standard procedures. Thin sections were incubated with the vimentin monoclonal antibody V 9 (diluted 1:100 in 1% ovalbumin in PBS), with rabbit antiserum to mouse IgG_s [Dako, diluted 1:100 (a) and 1:1000 (b) in 1% ovalbumin in PBS, and subsequently with protein-A-colloidal gold complex, particle size 15 nm (Janssen Life Science Products) diluted 1:25]. The sections were counterstained with uranyl acetate and lead citrate. **a** Fibroblast, $\times 21\,600$, *inset* $\times 48\,900$, **b** fibroblast, $\times 66\,600$. (Courtesy R. ROMANOWSKI, Department of Pathology, University of Giessen)

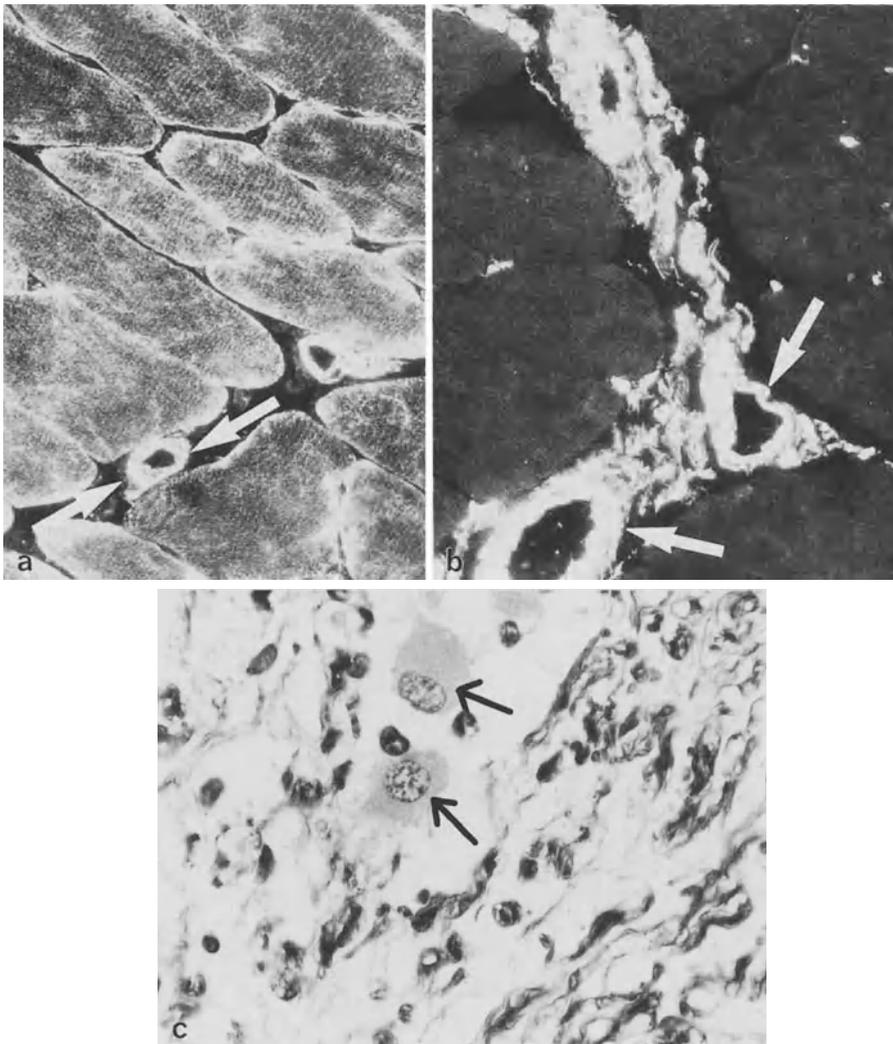


Fig. 5. Distribution of desmin and vimentin in skeletal muscle. Muscle fibres are desmin positive (a) and vimentin negative (b). In contrast fibrous tissues are not stained by the desmin antibody but are clearly labelled by the vimentin antibody (b). Note the coexpression of desmin and vimentin in vascular smooth muscle (a, b, arrow). c Schwann cells in a case of ganglioneuroblastoma also express vimentin; however the ganglion cells (arrow) are unstained (a, b FITC; c peroxidase) (polyclonal antibodies against desmin and vimentin)

genous cell populations in lymph nodes biochemical investigation using microdissection is not possible. Thus all studies are at the moment based on immunohistological findings using polyclonal or monoclonal antibodies against vimentin and it remains to be demonstrated whether the vimentin antibodies used in such studies have equivalent specificities. On the one hand, GIORNO (1985) has stated that nearly all lymphatic cells in the lymph node showed no staining

with the vimentin antibody he used; on the other hand our preliminary results on lymph nodes using several different vimentin antibodies, show that the majority of lymphatic cells do have vimentin filaments although there is a subset of lymphocytes which are not stained by such antibodies. This problem can only be fully solved by double labelling of the cells with monoclonal antibodies against vimentin and with antibodies directed against appropriate surface markers to define the lymphatic cells. Vimentin is also the only intermediate filament type present in granulosa cells of the ovary (CZERNOBILSKY et al. 1985; MOLL et al. 1986), Sertoli cells of the testis, as well as in Langerhans cells (LÖNING et al. 1982) and in melanocytes of skin (CASELITZ et al. 1983).

5.2 Desmin

Desmin is the intermediate filament protein characteristic of skeletal, cardiac and visceral smooth muscle where it is the only IF-type usually present in mature muscle cells (for review see THORNELL et al. 1983) (Fig. 5a, b). The situation is different in vascular smooth muscle. Many vascular smooth muscle cells are vimentin-positive and desmin-negative; others coexpress desmin and vimentin while a very small fraction show only desmin and no vimentin (FRANK and WARREN 1981; GABBIANI et al. 1981a; SCHMID et al. 1982). The relative number of each cell type present varies with the particular blood vessel examined. Thus for example in the aortic arch of the rat the vast majority of vascular smooth muscle cells are vimentin positive and desmin negative, while in the femoral and the external iliac arteries many cells coexpress desmin and vimentin (OSBORN et al. 1981). Antibodies to desmin clearly stain the Z-line of skeletal muscle, and in heart stain additionally the intercalated discs (LAZARIDES and HUBBARD 1976; HOLTZER et al. 1982; THORNELL et al. 1978; ALTMANNBERGER et al. 1982, 1985). Desmin filaments seems to be anchored at the intercalated discs of cardiac muscle as well as at the cell boundary between cells of the Purkinje fibre where they abut on the desmosome-like structures (KARTENBECK et al. 1983). Currently it is not yet clear whether desmin is restricted in skeletal muscle only to the Z-discs or whether it is mainly localized in the interfibrillary space (see TOKUYASU et al. 1983 for further discussion).

6 Intermediate Filament Typing in Mesenchymal Tumors

6.1 Desmin-Positive Tumors (Tables 1, 2; Fig. 6)

A positive reaction with desmin antibodies is characteristic for rhabdomyosarcoma, rhabdomyoma, leiomyosarcoma and leiomyoma (for review see ALTMANNBERGER et al. 1986b, c). We have investigated 31 cases of embryonal and alveolar rhabdomyosarcomas including the sarcoma botryoides subtype, and have shown that polyclonal or monoclonal antibodies against desmin positively identify tumor cells irrespective of their state of differentiation (ALT-

Table 1. Desmin-positive tumors

1. Rhabdomyosarcoma	(ALTMANNBERGER et al. 1982, 1985; MIETTINEN et al. 1982c)
2. Leiomyosarcoma, leiomyoma	(EVANS et al. 1983; MIETTINEN et al. 1982b, c)

Table 2. Desmin positive tumors (own results, cryostat sections or ethanol-fixed and paraffin-embedded material)

Diagnosis	No. of cases	Keratin ^b KL1/ polycl.	Vimentin V 9/ polycl.	Desmin DE-B-5/ polycl.	Neuro- filaments ^b NR 4/ polycl.
Leiomyoma (stomach/small intestine)	7	—	(+) ^a	+	—
Leiomyosarcoma	6	—	(+) ^a	+	—
Embryonal rhabdomyosarcoma	26	—	(+) ^a	+	—
Alveolar rhabdomyosarcoma	6	—	(+) ^a	+	—
Sarcoma botryoides	2	—	(+) ^a	+	—

^a In some tumor cells a coexpression of desmin and vimentin is seen. The percentage of cells in which such coexpression is observed varies with the tumor (see ALTMANNBERGER et al. 1985)

^b Not all tumors have been examined with all IF antibodies. However when they have been tested they proved negative

Polyclonal antibodies see OSBORN et al. (1982a)

Monoclonal antibodies V 9 OSBORN et al. (1984a); DE-B-5 DEBUS et al. (1983a); NR 4/G-A-5 DEBUS et al. (1983b); KL 1 VIAC et al. (1983)

MANNBERGER et al. 1985). In alveolar rhabdomyosarcomas a coexpression of desmin and vimentin is consistently found in nearly all tumor cells (ALTMANNBERGER et al. 1982, 1985; MIETTINEN et al. 1982c). Pleomorphic rhabdomyosarcomas are extremely rare and thus far no studies on intermediate filaments in this entity have been published. MOLENAAR et al. (1985b) have reinvestigated tumors in which the first diagnosis done by routine light microscopic stains was pleomorphic rhabdomyosarcoma. All the tumors that were reinvestigated could not be labelled by the desmin antibody but clearly express vimentin. Since these tumors expressed 1-antitrypsin and 1-antichymotrypsin the authors reclassified these tumors as malignant fibrous histiocytomas, although it has to be emphasized that both markers can also be found in other mesenchymal tumors and even in carcinomas (ROHOLL et al. 1985a-c). The results on human rhabdomyosarcomas can be further supported by results in an animal model system. Rhabdomyosarcomas can be specifically induced by implantation of different heavy metals, including nickel or nickel-sulfide. When the heavy metal is injected into skeletal muscle rhabdomyosarcomas develop, but when it is implanted in bone or subcutaneous tissue fibrosarcomas are observed (see ALTMANNBERGER et al. 1985). The histologic features of the tumors induced by injection of nickelsulfide were of three different types. In the rhabdomyoblastic

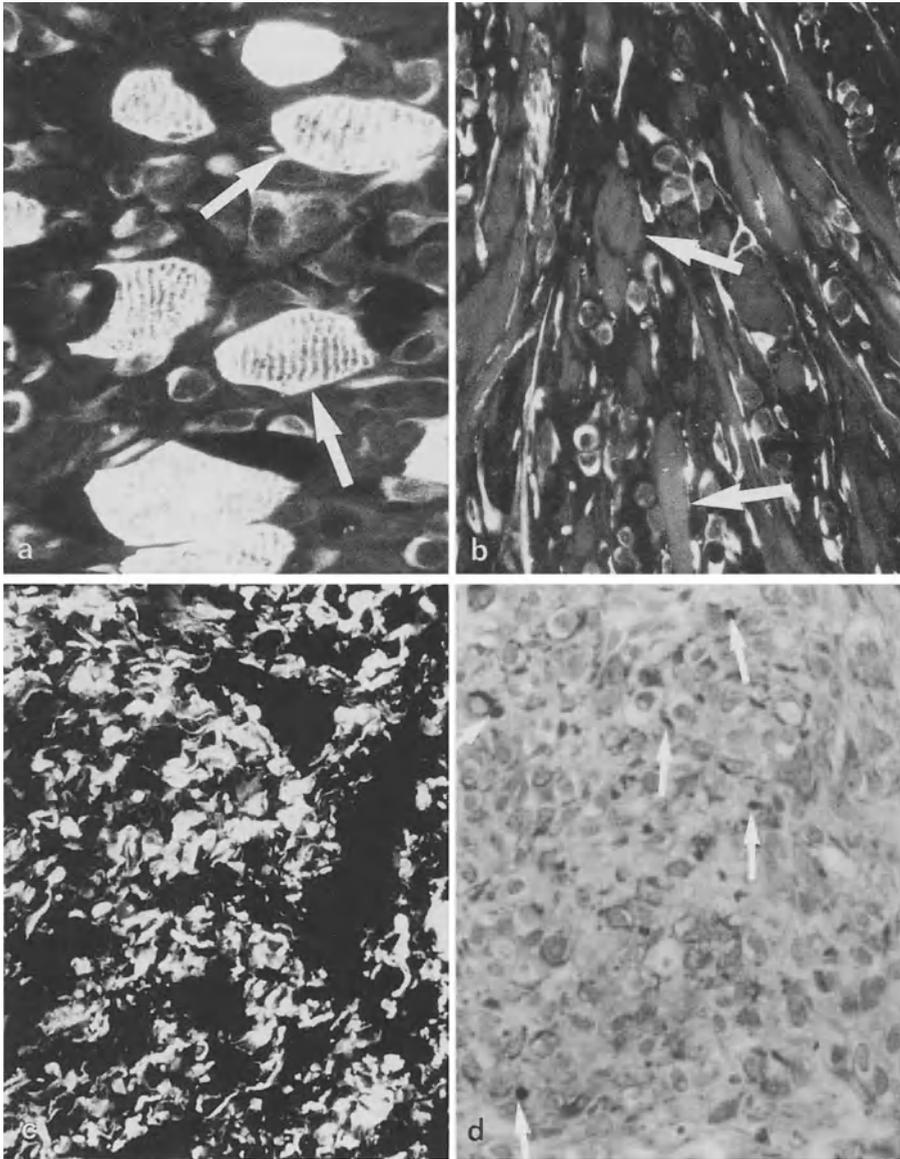


Fig. 6. Human (a, b) and rat rhabdomyosarcoma (c) as well as a case of epithelioid leiomyosarcoma stained by antibodies to desmin (a, c, d) and vimentin (b). The human embryonal rhabdomyosarcoma (a, b) infiltrates preexisting skeletal muscle (a, b, arrow), while the tumor cells coexpress desmin and vimentin. The preexisting muscle is desmin positive and vimentin negative. Rat rhabdomyosarcomas (spindle cell types) are clearly labeled by the desmin antibody. In a case of epithelioid leiomyosarcoma desmin antibody stains intracytoplasmic globules (d, arrow) (a-c, FITC; d, peroxidase labeled second, antibodies; polyclonal desmin antibody)

type tumor cells were highly differentiated mimicking rhabdomyoblasts, and in a few scattered tumor cells cross striations could even be detected. The spindle cell type was characterized by elongated nuclei and a small rim of eosinophilic cytoplasm, whereas the round cell type, showed oval cells with eccentrically located nuclei. Both the spindle as well as the round cell type correspond to a lower degree of differentiation. A small percentage of tumors was built up of only a single histological type. In the majority of tumors the histologic type changed from area to area when these tumors were studied by immunohistology using antibodies against desmin and vimentin. We found using polyclonal desmin antibodies that all tumor cells were desmin-positive. Even in the less differentiated round and spindle cell type desmin expression could be documented. In contrast vimentin staining depended on the histologic type. It was consistently seen in the spindle and round cell type but was restricted to a few small cells in the rhabdomyoblastic type. Thus also in nickelsulfide-induced rat rhabdomyosarcomas undifferentiated tumors coexpressed desmin and vimentin, while in the more differentiated tumors only desmin was detected. These findings should be considered in relation to a similar pattern of IF expression in skeletal muscle embryogenesis where in early stages of development desmin and vimentin are coexpressed, but in mature muscle only desmin is observed.

Our results with human and rat rhabdomyosarcomas do not support the idea of a class of primitive rhabdomyosarcomas, which are vimentin positive and desmin negative. In all 31 cases of rhabdomyosarcomas which we have examined, a positive desmin staining was observed, regardless whether frozen sections, or ethanol fixed and paraffin embedded material (ALTMANNBERGER et al. 1981, 1985) or smears were investigated. The advantages of desmin as a marker for rhabdomyosarcomas has been confirmed in studies from other laboratories (MIETTINEN et al. 1982c; MOLENAAR et al. 1985a) although some authors have not always seen desmin in undifferentiated tumor cells of rhabdomyosarcomas (GABBIANI et al. 1981b; DENK et al. 1983; KAHN et al. 1983). These negative reactions may be attributable to the fact that different desmin antibodies were used and, more importantly perhaps, to the different fixation methods used in the studies cited above. Thus MOLENAAR et al. (1985a) detected desmin in primitive undifferentiated tumor cells of one rhabdomyosarcoma when frozen sections were used, but another portion of the same tumor fixed in formaldehyde and embedded in paraffin was desmin-negative. Since in the same study in well differentiated rhabdomyosarcomas desmin could be detected also after formalin fixation, the different reactivities may reflect quantitative differences in the amount of desmin present.

When desmin is compared with other markers used in the differential diagnosis of muscle sarcomas, it is important to note that desmin expression is an early event in muscle differentiation, as shown by its presence in replicating cell lines of muscle origin, such as the hamster cell line BHK and the human RD rhabdomyosarcoma line (FRANK et al. 1981; OSBORN et al. 1982a). From studies of muscle myogenesis in culture it seems clear that desmin is expressed in the first generation of myoblasts and its synthesis probably precedes that of sarcomeric specific proteins such as the sarcomeric specific isoforms of actin

and myosin (HOLTZER et al. 1982) as well as of titin, a high molecular weight constituent protein of sarcomeric muscle (WANG et al. 1979; HILL and WEBER 1986). Myoglobin expression seems to occur at an even later time point in sarcomeric muscle development. We suggest that the protein complement seen in different rhabdomyosarcomas can be used to assay how differentiated the tumor is. Undifferentiated tumors express desmin as well as in some cases muscle specific forms of actin, myosin and also the high molecular weight protein titin (OSBORN et al. 1986b). Moderately and well differentiated rhabdomyosarcomas are characterized by increasing levels of the muscle specific forms of actin and myosin and by myoglobin expression. In the last few years there are several reports in which antibodies against actin and myosin have been used in the diagnosis of rhabdomyosarcoma (BUSSOLATTI et al. 1980; DONNER et al. 1983; TSOKOS et al. 1983). Unfortunately by far the majority of these antibodies react not only with the sarcomeric isoforms of actin or myosin respectively, but also crossreact with other actin and myosin isoforms, which form a part of the microfilament system in all cells of the organism. This crossreactivity is not surprising, since for example the isoforms of actin in vertebrates have nearly identical sequences (VANDEKERCKHOVE and WEBER 1979). Using such antibodies only a small percentage of rhabdomyosarcomas can be labeled but other mesenchymal tumors and even some epithelial neoplasms also show a strong positive reaction (BUSSOLATTI et al. 1980; DONNER et al. 1983). Polyclonal antibodies to sarcomeric myosin and actin cross absorbed on non-sarcomeric actin or myosin are probably skeletal muscle specific (DE JONG et al. 1984, 1985), but again these antibodies only label mature forms of rhabdomyosarcoma, because the tumor cells in undifferentiated cases have not started to produce the sarcomeric specific proteins. However a recent report by TSUKADA et al. (1987) encourages further trials with actin specific antibodies. Although both myoglobin and titin are specific for sarcomeric muscle they share the disadvantage that they are expressed relatively late in muscle development and consequently are only detected in mature forms of rhabdomyosarcoma (KINDBLOM et al. 1982; MUKAI et al. 1979; OSBORN et al. 1986b).

Thus desmin currently appears to be the best available marker for diagnosis of rhabdomyosarcomas especially for undifferentiated neoplasms.

Desmin antibodies do not allow the separation of rhabdomyosarcomas from smooth muscle tumors, since leiomyomas and leiomyosarcomas in different locations also express desmin (MIETTINEN et al. 1982b; BONAZZI DEL POGETTO et al. 1983; EVANS et al. 1983). The distinction of rhabdomyosarcoma from leiomyosarcoma generally can be done on the grounds of morphology and from the clinical history. Preliminary data suggest that antibodies to titin may also allow a distinction, since expression appears characteristic of skeletal muscle and its tumors, but not of smooth muscle and its tumors. Spindle cell tumors in the gastric wall which are classified as smooth muscle tumors when routine stains are used are of particular interest. Surprisingly these tumors are desmin-negative and vimentin-positive and in addition show positive staining with the S-100 protein. Thus they should better be classified as Schwann cell tumors (MAZUR and CLARK 1983).

Table 3. Vimentin-positive tumors

1. All nonmuscular soft tissue tumors (ALTMANNBERGER et al. 1986b, c) including granular cell tumor and glomus tumor
But: synovial sarcoma and epithelioid sarcoma coexpress K and V
2. Bone tumors (LÖNING et al. 1985)
But: adamantinoma and chordoma K⁺
3. Lymphomas and leukemias in some cases heterogenous staining (GABBIANI et al. 1981b)
4. Malignant melanoma (CASELITZ et al. 1983)

K = Keratin; V = Vimentin

Table 4. Vimentin Positive Tumors (own results, cryostat sections or ethanol-fixed and paraffin-embedded material)

Diagnosis	No. of cases	Vimentin V 9/ polycl.	Desmin DE-B-5/ polycl.	Neuro-filaments ^b NR 4/ polycl.	Keratin ^b KL 1/ polycl.
Non-Hodgkin lymphoma (LB, CB, IB, CC-CB, LP-immunocytoma)	19	(+) ^a	—	—	—
Hodgkin lymphoma	6	+	—	—	—
Malignant histiocytosis	2	+	—	—	—
Ewing's sarcoma	10	+	—	—	—
Osteosarcoma	2	+	—	—	—
Chondrosarcoma	2	+	—	—	—
Soft tissue sarcoma (without muscle sarcomas, Synovial sarcomas, Epithelioid sarcoma)	25	+	—	—	—

^a In some cases not all lymphatic cells are stained

^b Not all tumors have been examined with all IF-antibodies. However when they have been tested they proved negative

^c For polyclonal antibodies see OSBORN et al. (1982a)

Monoclonal antibodies V9 OSBORN et al. (1984a); DE-B-5 DEBUS et al. (1983a); NR 4/G-A-5 DEBUS et al. (1983b); KL 1 VIAC et al. (1983)

6.2 Vimentin-Positive Mesenchymal Tumors (Tables 3, 4; Fig. 7)

These tumors are listed in Tables 3 and 4.

The overwhelming majority of soft tissue tumors and of bone tumors (LÖNING et al. 1985; ALTMANNBERGER et al. 1986b) are vimentin-positive corresponding to their proposed origin from mesenchymal cells. Additionally in tumors such as glomus tumor and granular cell tumor (MIETTINEN et al. 1983c, 1984; SLOOTWEG et al. 1983), in which for the former a derivation from muscle and for the latter a neural origin was assumed by ultrastructural studies, vimentin is the only intermediate filament protein present. Exceptions expressing addi-

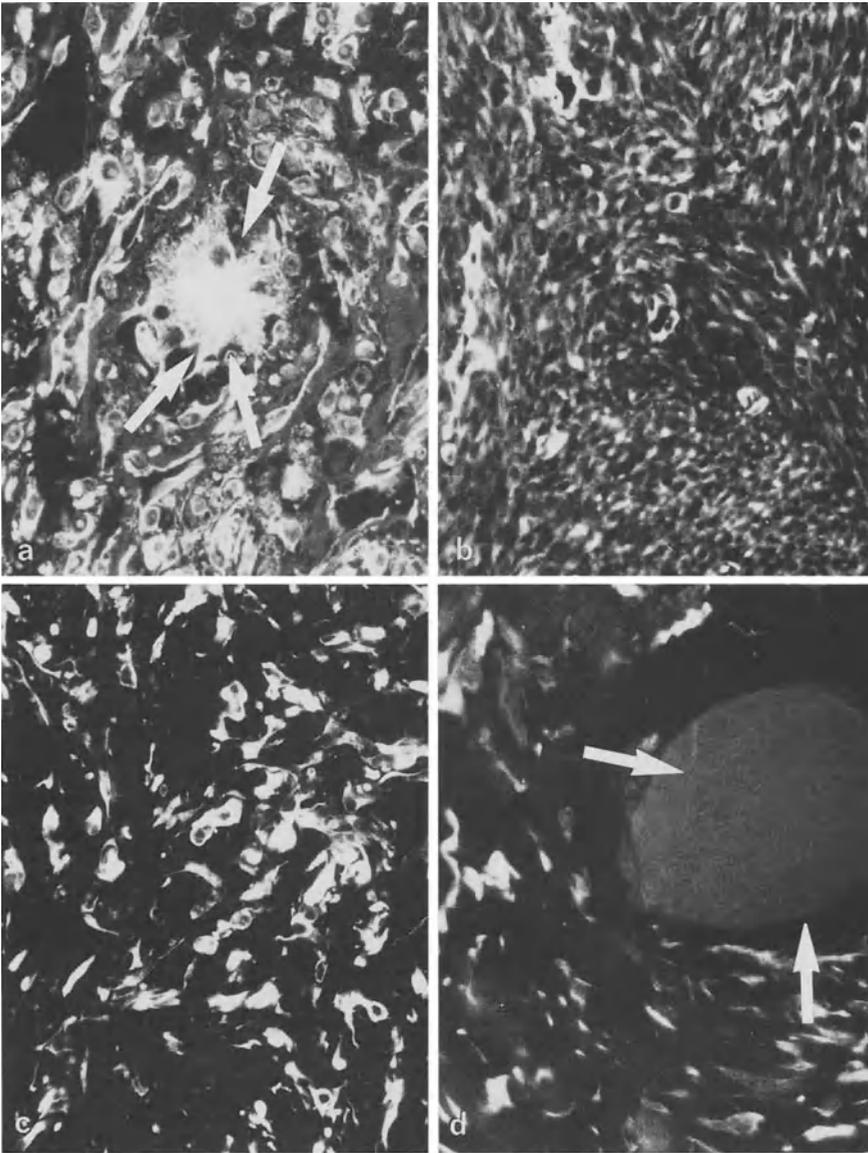


Fig. 7. Liposarcoma (a), fibrosarcoma (b), malignant fibrous histiocytoma (c) and fibromatosis (d) stained by a polyclonal vimentin antibody. Note the positive staining of lipoblasts in liposarcoma (a, arrow). Muscle fibres in fibromatosis (d, arrow) are negative. (Polyclonal vimentin antibody: a-d FITC)

tionally keratins include synovial sarcomas (MIETTINEN et al. 1982a; MIETTINEN and VIRTANEN 1984) and epitheloid sarcomas (CHASE et al. 1984; CHASE and ENZINGER 1985) in the group of soft tissue tumors, and chordomas (MIETTINEN et al. 1983b) as well as adamantinomas (ROSAI and PINKUS 1982) in the group of bone tumors.

Most Hodgkin and Non-Hodgkin lymphomas are vimentin-positive, but in some cases the staining is heterogenous and not all tumor cells are stained. It cannot be excluded that there is a group of Non-Hodgkin lymphomas lacking intermediate filaments, because in normal lymph nodes there are lymphocyte subsets which appear unstained by one or the other monoclonal vimentin antibodies. Our own data thus far do not support the findings of GIORNO (1985), and GIORNO and SCIOTTO (1985), who state that the majority of lymphomas express neither vimentin nor other intermediate filament polypeptides. It seems more likely that the negative reaction on these tumors was caused by the limited reactivity pattern of the vimentin antibody used by these authors, since this antibody did not react with any lymphatic cell in normal lymph nodes.

Further differential diagnosis of the entities in the group of vimentin-positive tumors requires the use of additional markers. Suitable markers which can yield further diagnostic information include endothelial markers (ALLES and BOSSLET 1986) S-100 (e.g. WEISS et al. 1983) histiocytic markers (ROHOLL et al. 1985a-c) or leucocyte common antigen (for review see ALTMANNBERGER et al. 1986b, c).

6.3 Keratin-Positive Mesenchymal Tumors (Tables 5, 6; Fig. 8)

The third main group is the keratin-positive mesenchymal tumors. The existence of this group was a surprising finding, because originally keratin-positivity ap-

Table 5. Keratin-positive mesenchymal tumors

1. Synovial sarcoma K + V + (MIETTINEN and VIRTANEN 1984)
Monophasic synovial sarcoma most cells V + K -, some cells coexpress V and K (MIETTINEN et al. 1983a)
2. Epithelioid sarcoma K + V + (CHASE et al. 1984)
3. Chordoma K + V + (MIETTINEN et al. 1983b)

K = Keratin; V = Vimentin

Table 6. Keratin-positive mesenchymal tumors (own results, cryostat sections or ethanol-fixed and paraffin-embedded material)

Diagnosis	No. of cases	Keratin KL 1/ polycl.	Vimentin V 9/ polycl.	Desmin DE-B-5/ polycl.	Neuro-filaments NR5/NF-MIX
Biphasic synovial sarcoma	2	+ ^a	+ ^b	-	-
Monophasic synovial sarcoma	5	(+)3/5	+5/5	-	-
Epithelioid sarcoma	1	+	+	-	-

^a Epithelial part + ^b Mesenchymal part +
+ 95% of tumor cells positive; (+) 5% tumor cells positive
Polyclonal antibodies see OSBORN et al. (1982a)

Monoclonal antibodies V9 OSBORN et al. (1984a); DE-B-5 DEBUS et al. (1983a); NR4/G-A-5 DEBUS et al. (1983b); KL 1 VIAC et al. (1983)

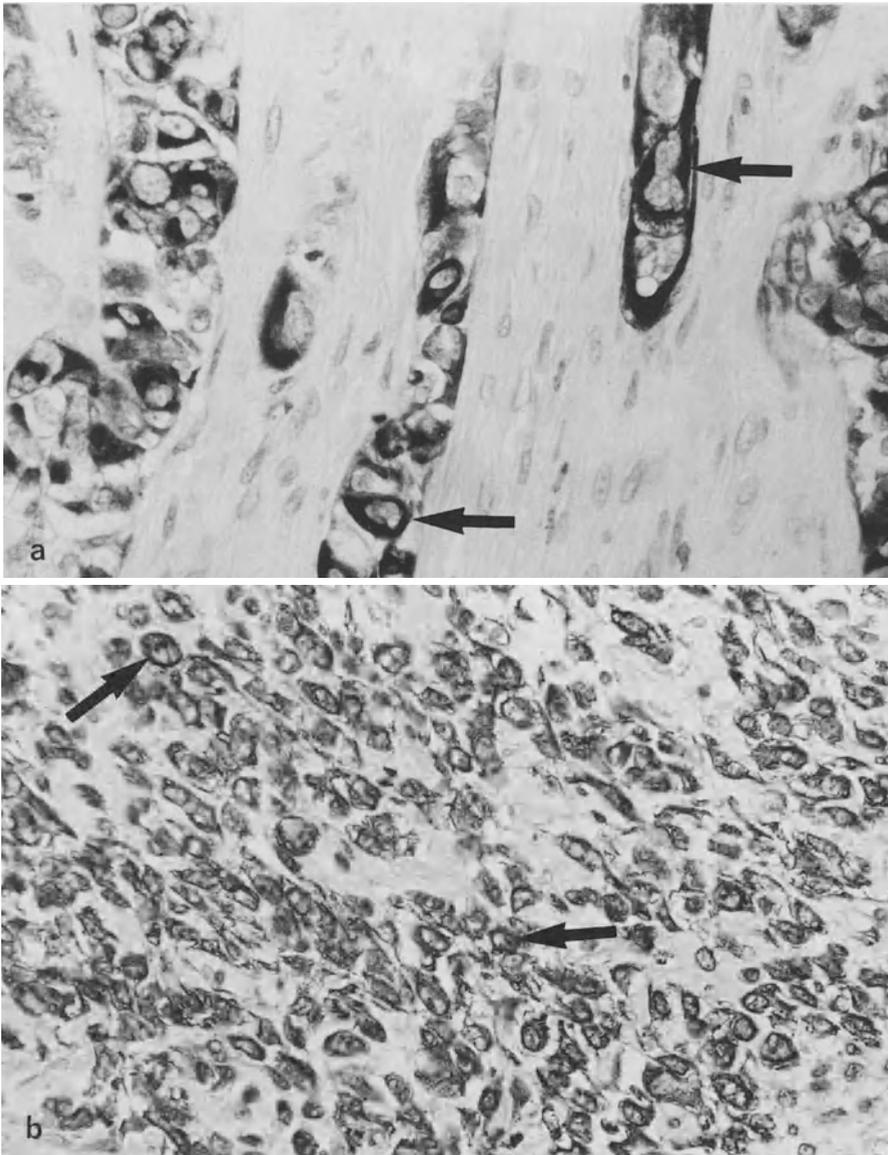


Fig. 8. Epithelioid sarcoma (a) and biphasic synovial sarcoma (b) stained by the monoclonal keratin antibody *KLI*. Tumor cells are positive (arrow), mesenchymal cells are negative (a, b peroxidase labeled second antibodies)

peared restricted to carcinomas (ALTMANNBERGER et al. 1982, 1986a, 1987; OSBORN and WEBER 1983; MOLL et al. 1982, 1986). However keratin was soon detected in synovial sarcomas (MIETTINEN et al. 1982a; MIETTINEN and VIRTANEN 1984; SALISBURY and ISAACSON 1985) in epithelioid sarcomas (CHASE et al. 1984; CHASE and ENZINGER 1985), in adamantinoma of tibia (ROSAI and PINKUS

1982) and in chordomas (MIETTINEN et al. 1983b). Most of these tumors co-express keratin and vimentin. In monophasic synovial sarcomas the majority of tumor cells were vimentin-positive with a few scattered tumor cells expressing keratin and vimentin (MIETTINEN et al. 1983a).

The transformation of mesenchymal cells to epithelial cells is perhaps best studied in nephroblastomas. In a first step cells of the undifferentiated blastema only synthesize vimentin. In a second step keratin and vimentin are coexpressed and finally when tubules are formed vimentin expression is lost and keratin is the only intermediate filament system found (ALTMANNBERGER et al. 1984).

6.4 Alveolar Soft Part Sarcoma

Alveolar soft part sarcoma is a tumor of unknown histogenesis. The hope that intermediate filament typing would have solved the problem, has not yet been fulfilled. Controversial results have been published by two different groups. We have investigated two cases, one of which was negative when tested with different intermediate filament antibodies (OSBORN et al. 1982b) whereas the second case showed a focally positive reaction with the vimentin antibody (JUNDT et al. 1984). This is in contrast to two other reports, which demonstrated coexpression of desmin and vimentin and suggested an origin from muscle cells for alveolar soft part sarcoma (DENK et al. 1983; MÜLLER and STUTTE 1984).

7 Conclusions

There is abundant evidence that intermediate filaments can be used as cell type specific markers both for normal tissue and for tumors. The results of intermediate filament typing in mesenchymal tumors and its diagnostic relevance is shown above. This system can also be used to solve other problems in surgical pathology (for review see OSBORN and WEBER 1983). One of the most useful applications of intermediate filament typing is to differentiate the round cell tumors of children, thus rhabdomyosarcomas are desmin positive, the majority of malignant lymphomas as well as Ewing sarcomas contain only vimentin and neuroblastomas show positivity for neurofilaments (OSBORN et al. 1986a).

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Mesenchymal Tumor Markers: Special Proteins and Enzymes*

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1 Introduction

The differential diagnosis of sarcomas is difficult. Different sarcomas can exhibit almost identical histological features when examined with light microscopic techniques. The visualization of structural and functional proteins in the tumor cells, using immunohistochemical methods, is an important factor in establishing a precise diagnosis. However, it is imperative to be familiar with the reaction range of the antibodies of the different tumors. The literature does not provide uniform findings with regard to the reaction range of "histiocytic" markers and thus far, there are no systematic investigations for ferritin and transferrin when applied to soft tissue tumors. This is why, in addition to the literature, we rely on our investigations carried out on 73 soft tissue sarcomas and 16 benign soft tissue lesions. Antisera have been used against the following proteins: desmin (Euro Diagnostics), myoglobin, lysozyme, α_1 -antitrypsin, α_1 -antichymotrypsin, ferritin, transferrin, factor VIII-related antigen, and S-100 protein (all from Dako). Myosin was identified on frozen sections of selected tumors with monoclonal antibodies.

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2 Myosin

Myosin is a globular enzyme acting both as ATPase and a fibrous structural protein simultaneously. Myosin from smooth muscle and myosin from striated muscle have slightly different molecular properties which account for their different contractile regulatory mechanisms. A single monomeric myosin molecule always contains two identical heavy chains (MW 200 kD) and two pairs of light chains of two different types (MW \approx 22 kD). Each heavy chain consists of a globular headpiece and a long rodlike α -helical part. Bound to each myosin head are the two molecules of light chains, one of each type. Movements of the head are important in generating the force of contraction in muscle. Myosin reversibly binds to actin. The regular array of actin and myosin filaments form the element of a myofibril (DARNELL et al. 1986).

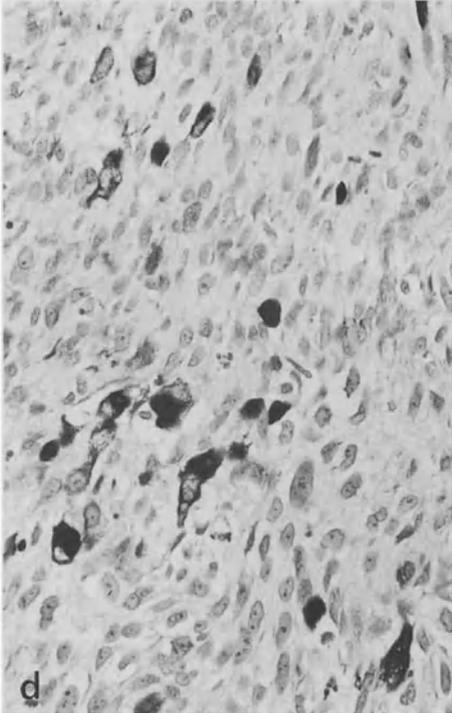
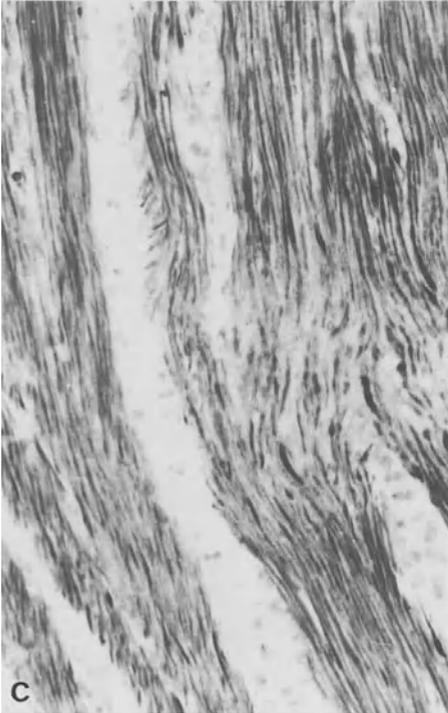
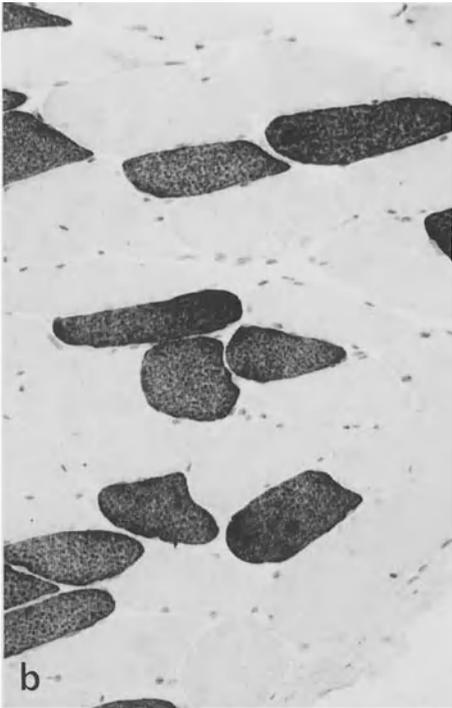
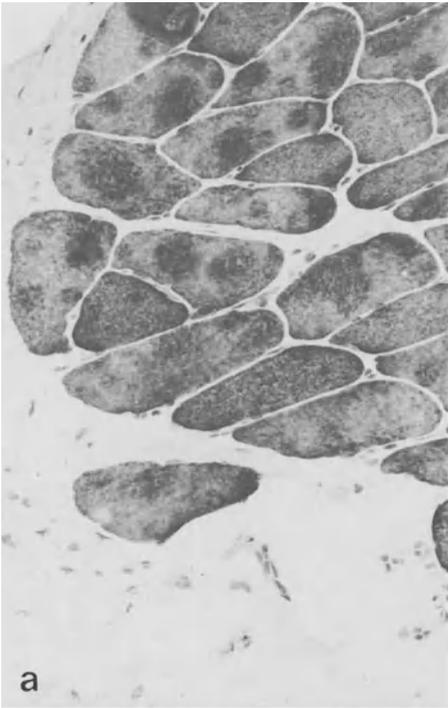
Myosin has been isolated from smooth muscle (PINKUS et al. 1986) and non-muscle cells (WEBER and GROESCHEL-STEWART 1974), although myosin filaments are not visible in the latter cells by using standard electron microscopic techniques (LONGTINE et al. 1985). Since myosins have a high variability in primary structure and enzymatic activity in different cell types (SYVORY 1979), antibodies have been raised. They discriminate among myosins (LONGTINE et al. 1985; SAKU et al. 1985). Anti-myosin antibodies have then been used in diagnostic pathology (HIRAMOTO et al. 1961; UNSICKER et al. 1978; MACARTNEY et al. 1979; DRENCKHAHN et al. 1980; DONNER et al. 1983; LEMANSKI and TU 1983; DE JONG et al. 1984; HAYASHI et al. 1984; SAKU et al. 1985; SCHIAFFINO et al. 1986; SCUPHAM et al. 1986).

The heterogeneity of myosins and antibodies hampers the analysis of the data in literature concerning the diagnostic value of myosin in mesenchymal tumors. In the diagnosis of rhabdomyosarcoma, anti-myosins have been found superior to anti-myoglobins (see below); (DE JONG et al. 1984) since they were found in both the well-differentiated and poorly-differentiated variants; the latter failed to show staining for myoglobin. Pleomorphic rhabdomyosarcomas were also positive for myosin, while malignant fibrous histiocytomas, liposarcomas, schwannomas, and leiomyosarcomas were negative. Others, however, found myoglobin in leiomyomatous tumors (BURES et al. 1981). Even epithelia reacted with some of the antisera (DRENCKHAHN et al. 1980; TSOKOS et al. 1983). In recent studies (SCUPHAM et al. 1986; TSOKOS and TRICHE 1986) the superiority of myosin to myoglobin was questioned since myosin failed to be detectable in poorly differentiated rhabdomyosarcomas.

Apart from the nosological or diagnostic question of whether and to what extent myoglobin occurs in myogenic tumors, there is now a problem in specifying the antibodies described and used. Those monoclonal antibodies that are sufficiently characterized (LONGTINE et al. 1985; PINKUS et al. 1986) are not yet commercially available. Therefore, the diagnostic and differential diagnostic value of myosin is still unclear.

Our own experience with monoclonal antibodies to myosin is very preliminary. Four monoclonal antibodies, kindly provided by H.A. Katus and his co-workers (Dept. of Internal Medicine III (Cardiology) at Heidelberg Universi-

Fig. 1. **a** Myosin immunoreactivity in normal skeletal muscle fibers by the monoclonal antibody \triangleright 1B2/1H4; \times 160. **b** Myosin immunoreactivity in normal skeletal muscle is restricted to some fibers; anti-myosin 4B8/1A7; \times 160. **c** Demonstration of myosin microfilaments in the muscle layer of small intestine; monoclonal anti-myosin antibody 5C9/3E2; \times 160. **d** Rhabdomyosarcoma: desmin immunoreactivity is seen in some cells; \times 250. **a-d** PAP method; AEC; haematoxylin counterstain



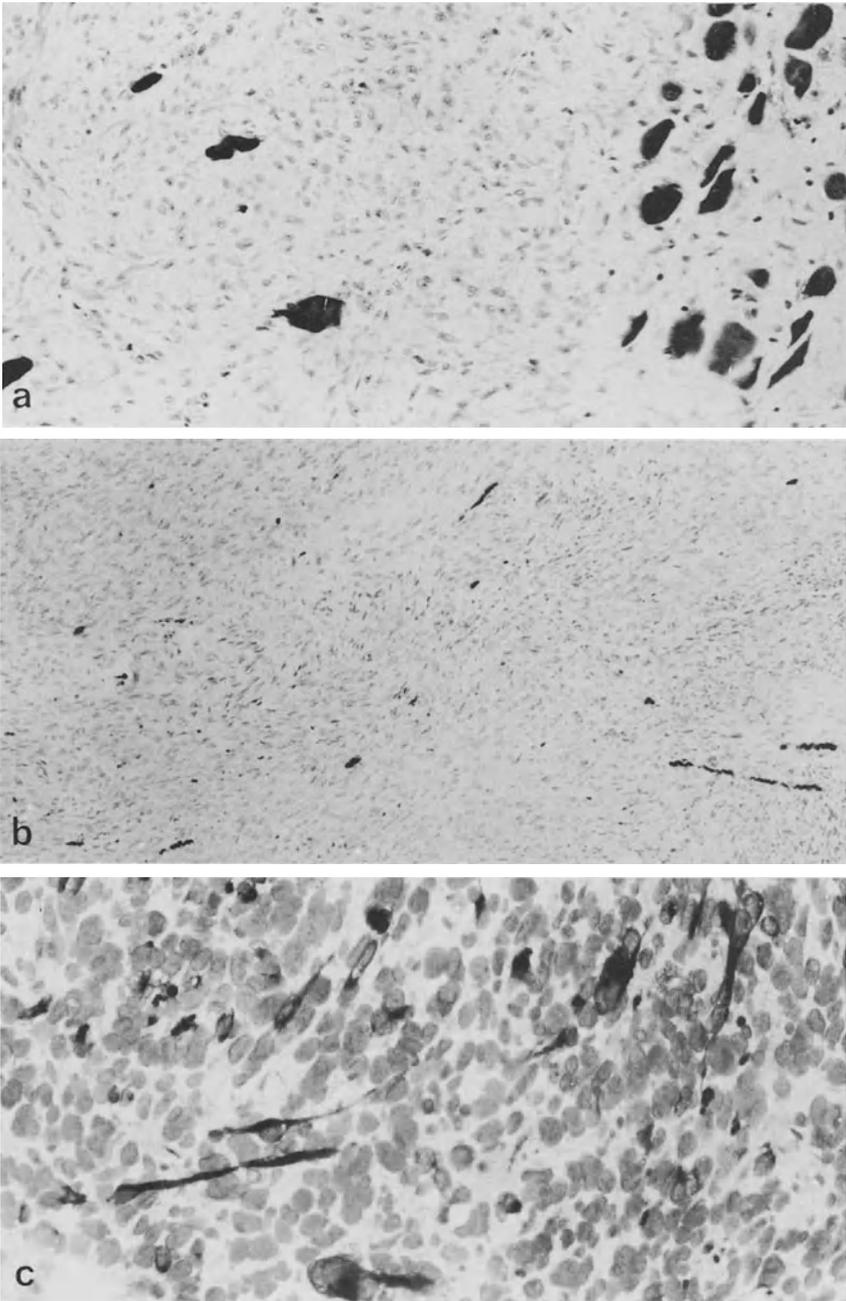


Fig. 2. **a** *Aggressive fibromatosis*: myoglobin reaction in the underlying muscle fibers; tumor cells negative; $\times 250$. **b** Same tumor as **a**: note the myoglobin immunoreaction in normal skeletal muscle fibers; $\times 160$. **c** *Rhabdomyosarcoma*: myosin immunoreaction in some spindle-shaped or strap-like tumor cells; $\times 250$. **a-c** PAP method; AEC; haematoxylin counterstain

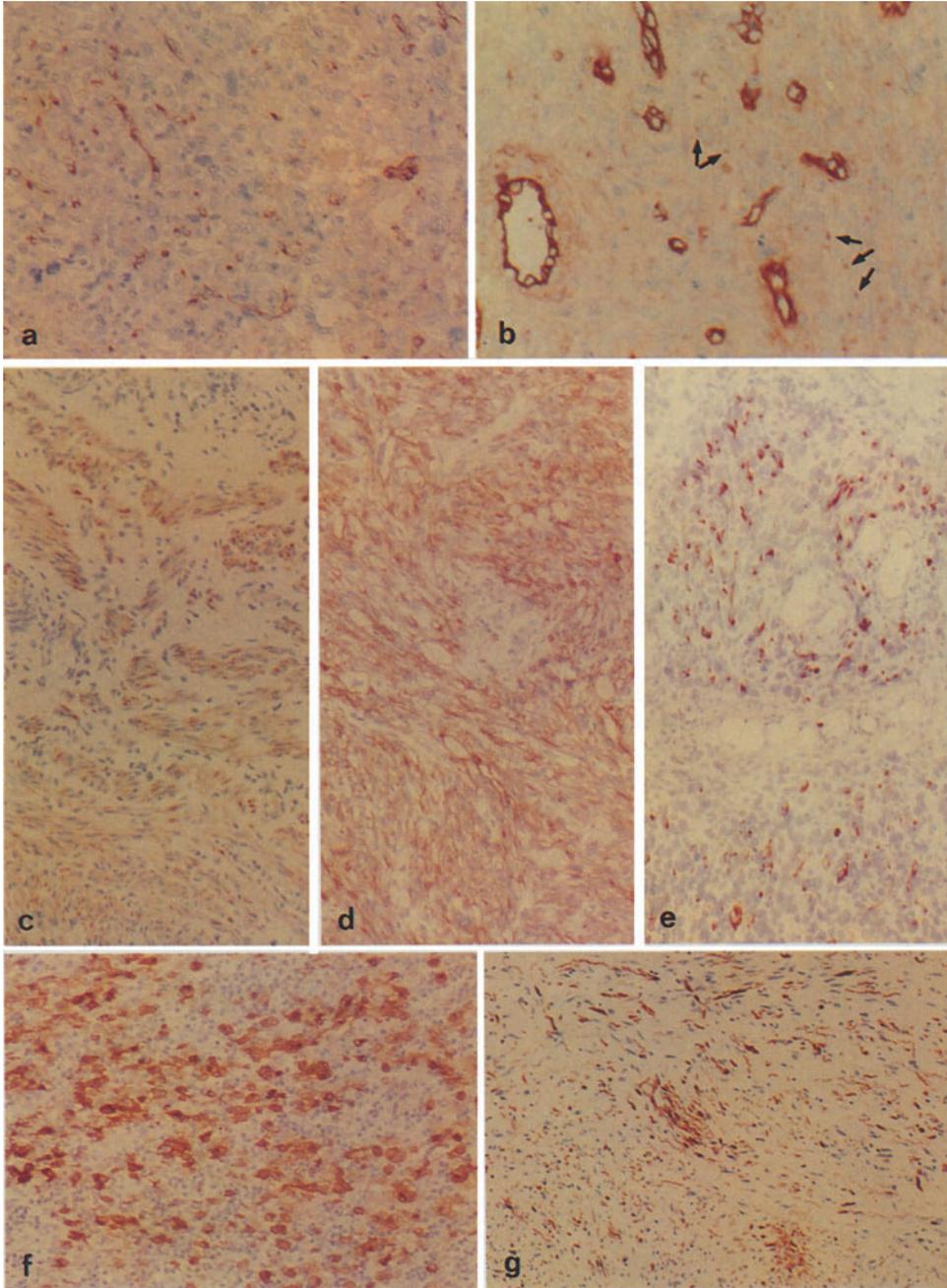


Fig. 3. **a** Immunoreaction of factor VIII-associated antigen in a malignant haemangioendothelioma; $\times 140$. **b** Kaposi sarcoma (note the discontinuity of swollen endothelial cells): FVIIIaAg in the endothelium as well as in the cytoplasm of some tumor cells (arrows); $\times 140$. **c, d** Desmin immunoreaction in leiomyosarcomas; $\times 140$. **e** Myosin immunoreaction in embryonal rhabdomyosarcoma is restricted to some cells; $\times 140$. **f** Protein S-100 immunoreactivity in interdigitating reticulum cells of a dermatopathic lymphadenitis; $\times 140$. **g** Neurofibroma with numerous protein S-100-positive Schwann cells; $\times 90$. **a-g** PAP method; AEC; haematoxylin counterstain

Table 1. Myogenic marker proteins

	Desmin	Myoglobin	Myosin
Rhabdomyoma			
Rhabdomyosarcoma	+	(+)	+/- ^a
Leiomyoma			
Leiomyosarcoma	+	-	+/- ^a

+ positive in most cases; (+) positive in the minority of cases

^a depends on myosin subtype and specificity of antibody

ty) were initially raised against human cardiac myosin (KATUS et al. 1982, 1984; KHAW et al. 1982, 1983). The tissue specificity was determined by using immunohistological methods on frozen sections. The antibody 5C9/3E2 is restricted in binding to smooth muscle of the gastrointestinal, bronchial and urogenital tract, excluding vascular smooth muscle cells (Fig. 1c). It does not react with myocardium. The antibody 1B2/1H4 always recognizes striated muscle cells and binds to atrial myocardium and to the myocardium of the right ventricle, whereas it does not recognize the muscle fibres of the left ventricle. The monoclonal antibodies 4B8/1A7 and 2D7/1C2 have a very similar binding pattern in normal organs: smooth muscle cells are not stained, while myocardium is completely stained, but only 50% of the skeletal muscle fibres show a specific binding (Fig. 1b). No cross-reactivity with non-myogenic cells was noted. With these four reagents we tested three embryonic rhabdomyosarcomas and one proliferating leiomyoma. The leiomyoma was stained by neither antibody. The rhabdomyosarcomas contained a varying number of positive tumor cells that were, however, scarce or even nearly absent (Figs. 2c, 3e). Best results for the positive staining were obtained by 1B2/1H4, the antibody that binds to 100% of the skeletal muscle fibres (Fig. 1a). A control series of liposarcomas, fibrosarcomas and malignant fibrous histiocytomas was completely unreactive, indicating that myosin, if ever expressed, seems to be specific for myogenic tumors. However, more data will have to be collected to support this view. For this reason, statements concerning the diagnostic value in comparison to other myocytic markers should be postponed (Table 1).

3 Myoglobin

Myoglobin as a monomeric oxygen-binding protein is evolutionarily related to haemoglobins (BLANCHETOT et al. 1983). It has a molecular weight of 17.8 kD and is a cytoplasmic constituent of normal skeletal and cardiac muscle fibres. Although all skeletal muscle fibres contain myoglobin, its concentration varies. The type I (red) fibres are rich in myoglobin and show a slow-sustained contraction, whereas type II (white) fibres exhibit a rapid short contraction and are poor in myoglobin (KAGEN 1973). Since most muscles contain a mixture of fibre types, immunohistology gives rise to a checkerboard pattern for myoglobin.

Since the first observation of myoglobin in a myogenic tumor (MUKAI et al. 1979) it has since been proclaimed as a marker for tumors of skeletal muscle differentiation (CORSON and PINKUS 1981; BROOKS 1982), although the sera applied showed, at least in some hands, cross-reactivity with nonmuscular cells (EUSEBI et al. 1984). Using antisera to myosin, MUKAI et al. (1980a) succeeded in demonstrating rhabdomyoblastic cells in 40% of 25 uterine and ovarian müllerian tumors. Thus, the immunohistochemical method was superior to the conventional proof of cross-striations which are indicative of this special differentiation. Likewise, GIANGASPERO et al. (1981) found myoglobin in rhabdomyoblastic cells of Wilms' tumors. DICKINSON et al. (1983) successfully applied anti-myoglobin to demonstrate rhabdomyoblastic differentiation in a medulloblastoma. DAIMARU et al. (1984) used myoglobin antisera to stain the rhabdomyoblastically differentiated parts of "Triton" tumors.

The data on frequency of myoglobin containing rhabdomyosarcomas vary considerable in literature: CORSON and PINKUS (1981): 13/17; BROOKS (1982): 25/28; KAHN et al. (1983b): 16/53 of the embryonal vs. 8/12 of the alveolar type; KAGAWA et al. (1983): 13/26; JONG et al. (1984): 11/23; ROYDS et al. (1985) 18/26; EUSEBI et al. (1986): 10/15.

Our data, based on 73 soft tissue sarcomas and 16 benign lesions with a polyclonal antiserum (Dakopatts, Denmark), illustrate the high specificity but low sensitivity of myoglobin as a marker for malignant skeletal tumors. Only 2/8 rhabdomyosarcomas were specifically stained in a considerable but minor part of the neoplastic cell population, while normal skeletal muscle regularly served as intrinsic or extrinsic positive control. It is important not to regard stained cells as positive when they are apparently necrotic or are adjacent to areas of necrosis, since this can be observed with a multitude of antisera. On the other hand, specifically stained fibers or cellular buds must not be regarded as intrinsic parts of the tumor while they are actually degenerating or regenerating non-neoplastic muscle fibers (Fig. 2a). This is not always easy to assess. In order to exclude errors, the direction of stained fibers proved to be helpful (Fig. 2b). In case of unidirectional arrangement, specifically stained fibers or single cells ought to be regarded as remnants of normal muscle. Keeping these pitfalls in mind, we had no false positive results in our series.

It is still controversial whether myoglobin is a more sensitive marker for rhabdomyosarcoma than myosin or vice versa. Some authors (JONG et al. 1984; EUSEBI et al. 1986) who have anti-fast myosin antibodies at their disposal, claim that this special type of myosin is superior to myoglobin in detecting rhabdomyoblasts. ROYDS et al. (1985) favour β -enolase and KAHN et al. (1983b) prefer creatine kinase to myoglobin. There is, nevertheless, agreement that desmin is the most reliable marker for myogenic tumors (see also chapter 7). In our series, 10/11 tumors of this category stained for desmin, and only 1 rhabdomyosarcoma was unreactive. However, our series contained 2 leiomyosarcomas and one myogenic sarcoma that could not further specified (Figs. 1d, 3c, d). This indicates that the desmin reaction cannot distinguish between leiomyogenic and rhabdomyogenic tumors (GABBIANI et al. 1981; ALTMANNBERGER et al. 1982; MIETTINEN et al. 1982; DENK et al. 1983).

4 Lysozyme

Lysozyme (muramidase), first described by FLEMING (1922) as a bacteriolytic substance, is a carbohydrase acting on cell wall of susceptible bacteria through cleavage of $\beta(1-4)$ linkages between N-acetyl muramic acid and N-acetyl glucosamine (STROMINGER and TIPPER 1974). Among mesenchymal cells, lysozyme is found in monocytes (FLANAGAN and LIONETTI 1955), mature myeloid cells (BRIGGS et al. 1966) and histiocytes (OSSERMAN 1975). Since the classical immunohistochemical studies of KLOCKARS and REITAMO (1974) and MASON and TAYLOR (1975) on the tissue distribution of lysozyme, it is regarded as a reliable marker indicating the myelomonocytic origin of mononuclear cells in the lymphoid system and in inflammatory tissues.

There are reports of marked positivity for lysozyme in neoplastic histiocytes of malignant histiocytosis (MEISTER et al. 1980; MENDELSON et al. 1980; VILPO et al. 1980; BURGDORF et al. 1981a; ROHOLL et al. 1985). Juvenile xanthofibroma was found to consist predominantly of lysozyme-positive histiocytic cells (SONODA et al. 1985; SEO et al. 1986). While BROOKS et al. (1984) detected lysozyme in the tumor cells of a malignant giant cell tumor of the bone, LING et al. (1986) found lysozyme only in the giant cell tumors of the tendon sheath among the 24 giant cell tumors they examined. The tumor cells of histiocytosis X, however, do not contain lysozyme (MOTOI et al. 1980; NAKANISHI et al. 1982). In proliferative and neoplastic fibrohistiocytic lesions, lysozyme expression turned out to be a rare phenomenon. NAKANISHI et al. (1982) detected lysozyme-positive cells in 13/100 dermatofibromas, 1/4 xanthogranulomas and 8/33 malignant fibrous histiocytomas; 13 cases of dermatofibrosarcoma protuberans were unreactive. FLETCHER et al. (1985) confirmed the lack of lysozyme in dermatofibrosarcoma protuberans. In the series of ROHOLL et al. (1985), again, only 2/77 malignant fibrous histiocytomas were classified as lysozyme-positive, in the series of KINDBLUM et al. (1982) only 1/31 cases. Lysozyme could thus be characterized as a marker reliable only for monocytes/macrophages. When raising the question of whether tissue histiocytes are of myeloid origin or not, WOOD et al. (1985) emphasized that the histiocytic cells of malignant fibrous histiocytoma do not express the antigenic or enzyme-histochemical features of the myelogenic macrophages and are thus derived from primitive mesenchymal cells as suggested by electron microscopy (FU et al. 1975; TAXY and BATTIFORA 1977; ALGUACIL-GARCIA et al. 1978). This handsome concept, however, is challenged by recent data on the expression of differentiation antigens detected by monoclonal antibodies (STRAUCHEN and DIMITRIU-BONA 1986).

Using a well-established antiserum (Dakopatts, Copenhagen, Denmark), we observed in our series (Table 2) scattered lysozyme containing tumor cells in 2/5 malignant fibrous histiocytomas, and in considerable proportions of atypical cells in 2/5 sarcomas not further specified, 1 sarcoma of spindle-cell type, and 1 sarcoma of pleomorphic type (Fig. 4d). In this context, it is noteworthy that 1/3 cases of dermatofibrosarcoma protuberans were clearly lysozyme-positive. Nevertheless, all our myogenic, lipogenic and fibrogenic tumors were unreactive. It is important not to misinterpret intermingled phagocytic histiocytes, mastocytes and granulocytes as part of the neoplastic population. Furthermore, areas of necrosis of necrotic cells may be artifactually stained.

Table 2. Pattern of reactivity of 73 soft tissue sarcomas and 16 benign lesions with antisera to lysozyme, AAT, ACT, ferritin, and transferrin

Diagnose ^a	Lysozyme		AAT		ACT		Ferritin		Transferrin	
	n	n:pos. ^b	n:pos.	n:pos.	n:pos.	n:pos.	n:pos.	n:pos.	n:pos.	
Lipoma	1	0	0	0	0	0	0	0	0	
Leiomyoma	1	0	0	0	0	0	0	0	0	
Fibroma	1	0	0	0	0	0	0	0	0	
Dermatofibroma/cut. histiocytoma	3	0	2	2	2	1	1	1	1	
Fasciitis	2	0	0	0	0	1	1	0	0	
Neurofibroma	1	0	0	1	1	1	1	0	0	
Schwannoma	1	0	0	1	1	0	0	1	1	
Liposarcoma	23	0	6	22	22	12	9	9	9	
Leiomyosarcoma	2	0	0	1	1	0	0	0	0	
Rhabdomyosarcoma	8	0	1	5	5	3	4	4	4	
Myogenic sarcoma, n.f.sp. ^b	1	0	0	1	1	0	1	1	1	
Fibrosarcoma	2	0	1	1	1	1	1	1	1	
Dermatofibrosarcoma protuberans	3	1	0	2	2	1	1	1	1	
Aggressive fibromatosis	3	0	0	2	2	2	3	3	3	
Malignant fibrous histiocytoma	5	2*	4	5	5	4	4	4	4	
Infantile hemangiopericytoma	1	0	0	1*	1*	0	0	0	0	
Hemangiopericytoma	2	0	1	2	2	1	0	0	0	
Malignant hemangioendothelioma	1	0	1	1	1	1	1	1	1	
Malignant synovialoma	2	0	0	2	2	0	1	1	1	
Neurogenic sarcoma	2	1	0	1	1	0	0	0	0	
Neuroblastoma	1	0	0	0	0	0	0	0	0	
Epithelioid sarcoma	2	0	0	0	0	2	0	0	0	
Spindle cell sarcoma, n.f.sp.	11	1	4	6	6	4	6	6	6	
Pleomorphic sarcoma, n.f.sp.	4	1	2	3	3	1	2	2	2	
Sarcoma, n.f.sp.	6	0	0	3	3	3	3	3	3	
Total	89									

^a Diagnosis made on conventional histological grounds

^b n.f.sp.: not further specified; n: pos.: number of tumors characterized as "positive"; *: weak reactivity

To conclude, we regard lysozyme as a marker which, among mesenchymal cells, is generally restricted to the monocyte/macrophage/epithelioid cell system (MASON and TAYLOR 1975). It is very useful in detecting myeloid metaplasia of lymph nodes in the course of myelomonocytic leukaemia and chloromas in other sites (Fig. 5d). Lysozyme may become the most reliable conventional marker for "true" histiocytic lymphomas, since α_1 -antitrypsin has recently lost its alledged cell type specificity among the cellular constituents of the immune system (STEIN et al. 1984). In initial immunodiagnosis of soft tissue tumors, lysozyme is a marker of second choice, because of its low sensitivity (Figs. 4d, 5a, b). In case of positive immunoreactivity, however, it may be an aid in discriminating between malignant fibrous histiocytoma and other tumors of comparable morphology (Table 2).

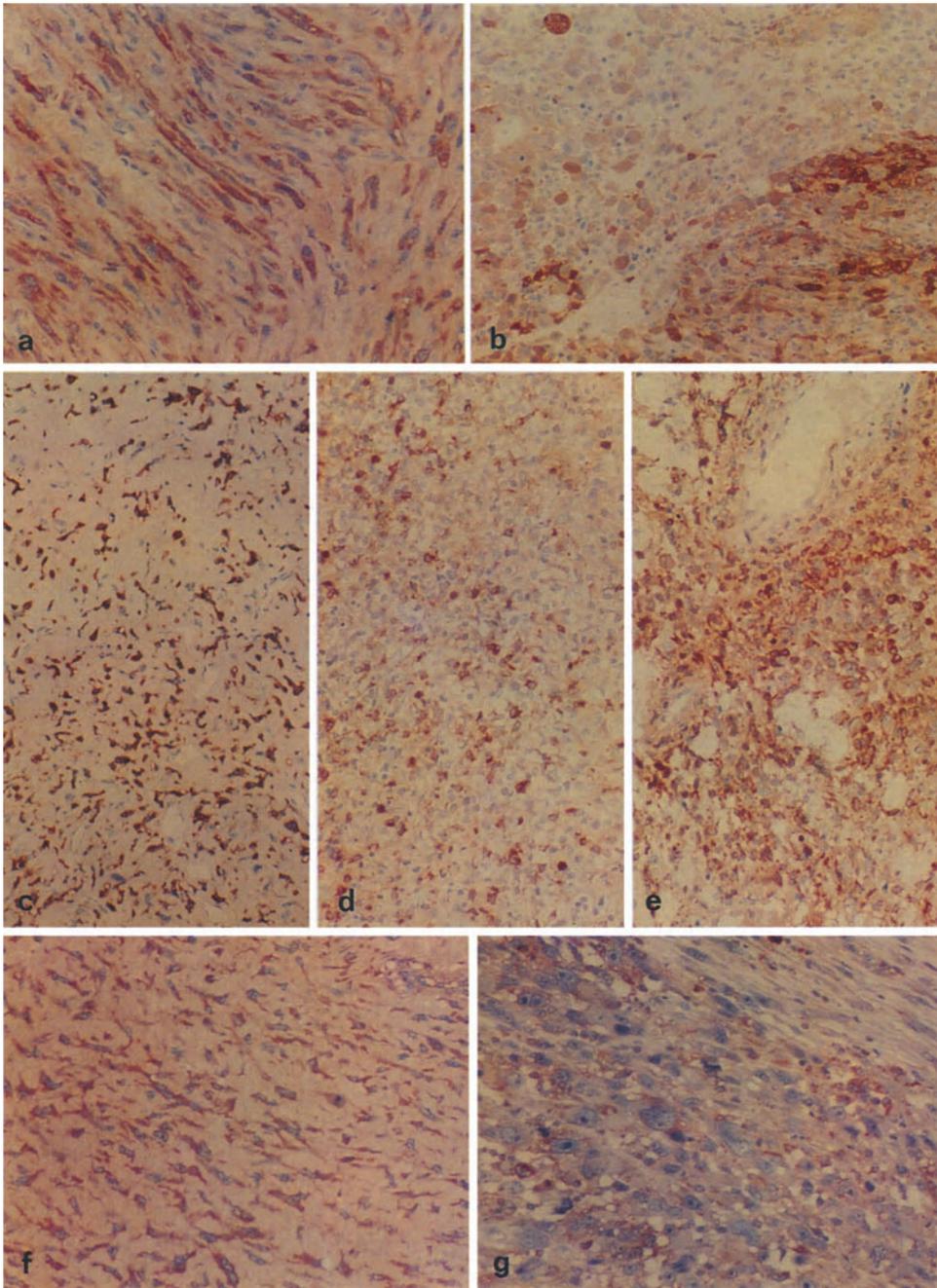


Fig. 4. **a** *Transferrin* reaction in a *rhabdomyosarcoma*; $\times 140$. **b** *Ferritin* immunoreactivity in individual cells of a *pleomorphic liposarcoma*; $\times 140$. **c** *Ferritin* immunoreaction in the majority of tumor cells of a *malignant fibrous histiocytoma*; $\times 90$. **d** *Lysozyme* reaction in randomly distributed cells of a *malignant fibrous histiocytoma*; $\times 160$. **e** *ACT* reaction in most cells of a *round cell liposarcoma*; $\times 90$. **f** *AAT* immunoreaction in slender processes of *fibrosarcoma* cells; $\times 140$. **g** *AAT* in a *pleomorphic liposarcoma* is restricted to some cells; $\times 140$. **a-g** PAP method; AEC; haematoxylin counterstain

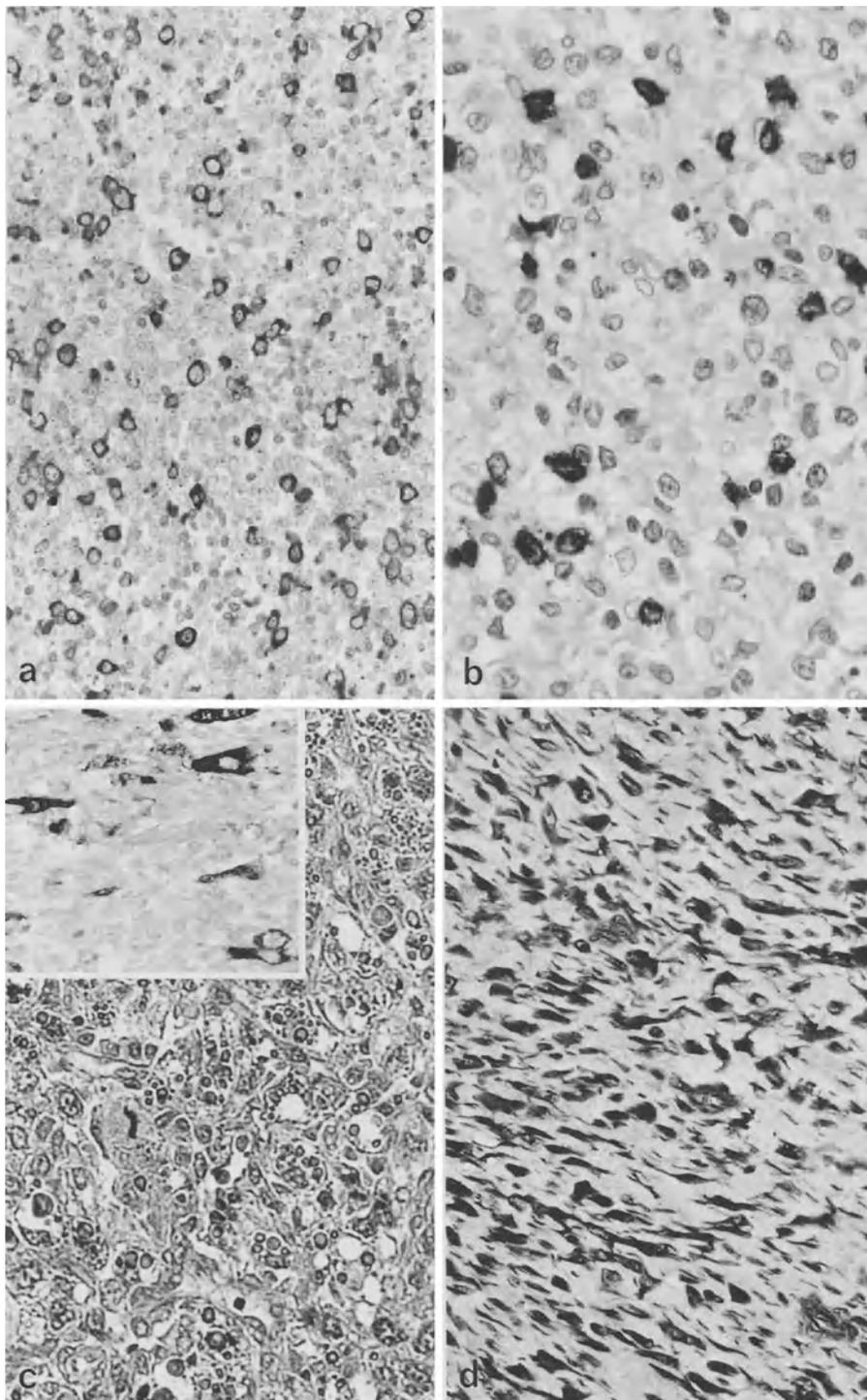


Fig. 5. **a** Tumor-forming subtype of *myelo-monocytic leukaemia*: *lysozyme* in scattered leukaemia cells; $\times 250$. **b** *Malignant histiocytosis* of the intestine: *lysozyme* immunoreactivity in some tumor cells; $\times 400$. **c** *Angiogenic sarcoma*, H & E stain, $\times 250$; *inset*: Demonstration of *AAT* in individual tumor cells; $\times 250$. **d** *Fibrosarcoma*: *ACT* immunoreaction in a large number of spindle-shaped tumor cells; $\times 250$. **a-d** PAP method; AEC; haematoxylin counterstain

5 Alpha-1-Antitrypsin

α_1 -antitrypsin (AAT), a glycoprotein of 51 kD (CARRELL et al. 1982), is the major component of the α_1 -electrophoretic band of the human plasma proteins and is capable of inhibiting a variety of proteolytic enzymes, such as trypsin, chymotrypsin, kallikrein and the neutral proteases of polymorphonuclear leukocytes (MORSE 1978). By this effect, AAT inhibits the inflammatory process, in the course of which these enzymes are released by various participating cell types (BREIT et al. 1982). Furthermore, AAT inhibits chemokinesis and chemotaxis of neutrophils and monocytes (BREIT et al. 1983). Reduced levels of AAT as a consequence of a genetic deficiency are associated with pulmonary emphysema and juvenile cirrhosis of the liver (for review see CARRELL et al. 1982). This protein is synthesized by liver cells (ASOFSKY and THORBECKE 1961; ALPER et al. 1980), monocytes (WILSON et al. 1980; VAN FURTH et al. 1983) and macrophages (GUPTA et al. 1979; ISAACSON et al. 1979, 1981), but not in normal peripheral lymphocytes (VAN FURTH et al. 1983). This is why AAT was regarded for several years as a marker – among mesenchymal cells – to be specific for monocytes and histiocytes.

Since the tumor cells contained AAT, a highly malignant lymphoma of the intestine was called “malignant histiocytosis” (ISAACSON and WRIGHT 1978; ISAACSON et al. 1981, 1982). AAT was then detected in Hodgkin and Sternberg-Reed cells (POPPEMA et al. 1978; PAYNE et al. 1982), a finding interpreted as a consequence of their histiocytic origin. At present, both concepts are challenged since STEIN et al. (1984) claimed to have detected AAT in malignant T cells.

Like α_1 -antichymotrypsin, AAT turned out to be of minor value in the differential diagnosis of soft tissue tumors. ROHOLL et al. (1985) found both markers in the majority of cases of “true” malignant histiocytosis, whereas they were detectable only in the minority of cases of malignant fibrous histiocytoma. Within the latter group, cases of the storiform subtype were less frequently stained than the pleomorphic or giant cell subtypes. These data are in sharp contrast to the results of KINDBLOM et al. (1982) who found AAT in 18/22 pleomorphic fibrous histiocytomas, but in 0/5 spindle-cell types of this tumor. NATHRATH and REMBERGER (1986) discussed whether some rare cases of granular cell tumor were of histiocytic origin since they found AAT in 1/5 such cases. In our series (Table 2) we observed AAT in soft tissue tumors of different types including rhabdomyosarcoma, liposarcoma, hemangiopericytoma, endothelioma and even Schwannoma (Figs. 4f, g, 5c). At present we doubt whether this marker is likely to be a helpful tool for a specific differential diagnostic problem.

6 Alpha-1-Antichymotrypsin

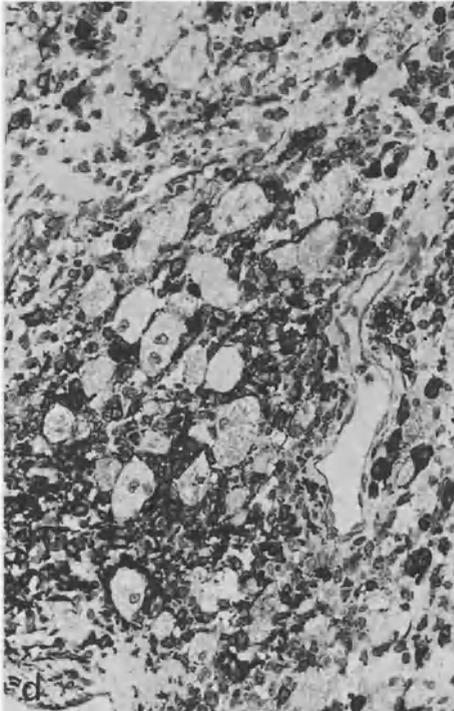
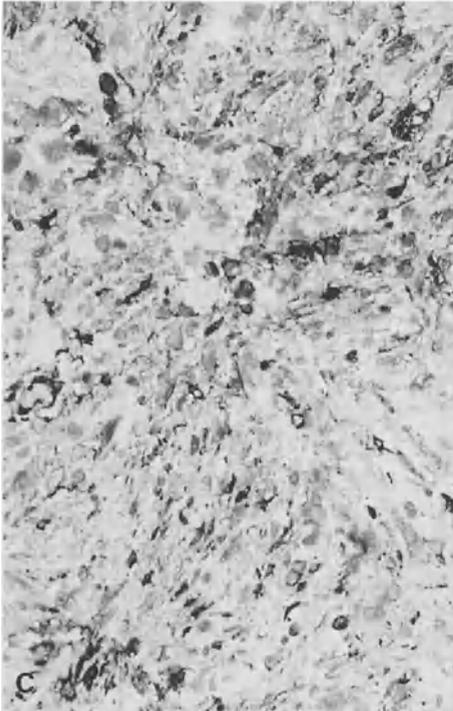
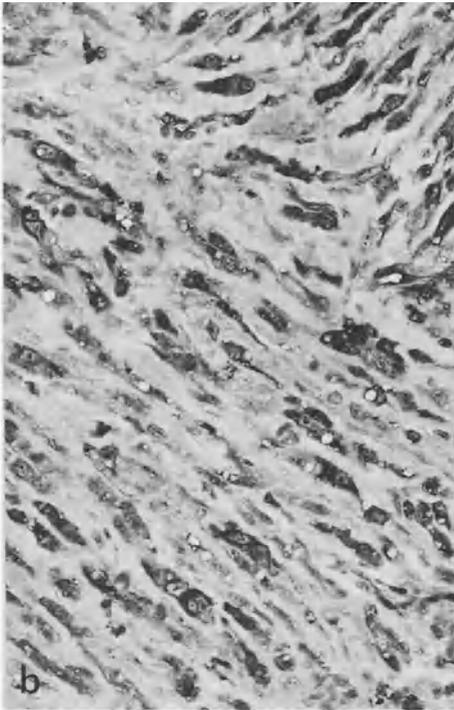
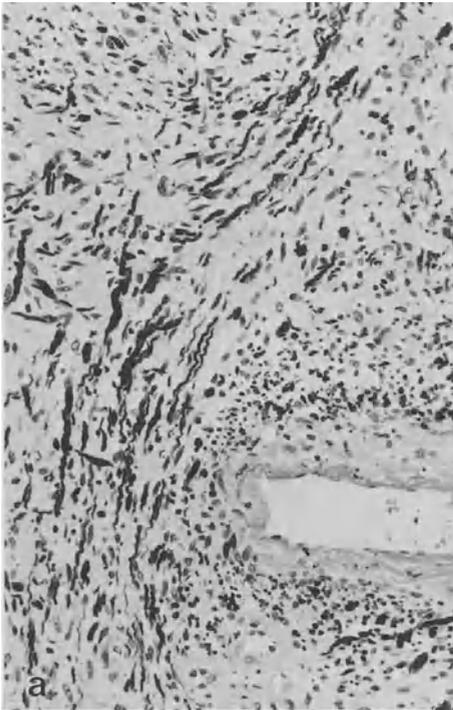
α_1 -antichymotrypsin (ACT) like AAT acts as a protease inhibitor on chymotrypsin, cathepsin D, elastase, collagenase, urokinase, renin, kallikrein, Hageman-factor, thrombin and plasmin (TRAVIS et al. 1978; BEATTY et al. 1980). Among non-epithelial cells, ACT is presently regarded as a monocyte/macrophage marker (PAPADIMITRIOU et al. 1978). However, there are very few reports on this issue in the literature (BRAUNHUT et al. 1984; MEISTER et al. 1980). Cytoplasmic presence of ACT was used as an additional argument to support theories

on the monocyte origin of Hofbauer cells in the placenta (BRAUNHUT et al. 1984) and of Hodgkin cells (PAPADIMITRIOU et al. 1978). ROHOLL et al. (1985) found co-expression of ACT and AAT in "true" malignant histiocytosis, and ISAACSON et al. (1982) in malignant histiocytosis of the intestine. Only a minority of the malignant fibrous histiocytomas examined by ROHOLL et al. (1985) contained ACT-positive tumor cells. In our series of soft tissue tumors ACT, unlike AAT, proved to be synthesized by 5/5 malignant fibrous histiocytomas, 2/3 dermatofibromas, in 2/3 cases of dermatofibrosarcoma protuberans, 5/8 rhabdomyosarcomas, 22/23 liposarcomas, in 2/2 haemangiopericytomas and others (Table 2; Figs. 4e, 5d). Therefore, ACT was of no help in discriminating among these. Our numbers were too small to allow statements concerning the negative tumors (epithelioid sarcomas, neuroblastoma, myoma, fibroma, lipoma). If these tumor types were consistently ACT-negative, ACT could claim some differential diagnostic relevance. This point needs further study.

7 Protein S-100

In 1965, MOORE was able to isolate a protein from rat brain which has been called "S-100" due to its solubility in 100% ammonium sulfate at neutral pH. The heterogeneous protein fraction consists of two subunits (α and β) which form heterodimers in solutions (ISOBE et al. 1978, 1981, 1983). From ox brain, three S-100 isoforms can be purified, i.e. S-100 a₀, S-100a and S-100b that are characterized by the same molecular weight (21 kD) and similar pH (about 4.3). They differ, however, in their subunit composition ($\alpha\alpha$, $\alpha\beta$ and $\beta\beta$, respectively; ISOBE et al. 1978, 1981, 1983). The physiological role of this dimerization is not clear. Amino acid sequence analysis of the α - and β -subunits (ISOBE et al. 1978, 1981) demonstrated that both are characterized by a sequence of 24 amino acids highly homologous to the calcium-binding domain (TUFTY and KRETSINGER 1975), suggesting that the S-100 protein is evolutionarily related to the family of calcium-binding proteins such as calmodulin. A crude S-100 fraction containing S-100a and S-100b has been shown to be involved in the Ca^{2+} -mediated control of the assembly and disassembly of brain microtubule proteins in vitro (BAUDIER et al. 1982; DONATO 1983; ENDO and HIDAOKA 1983). In addition, S-100 (a+b) interferes with the nucleation and the elongation of microtubule proteins by interacting with tubulin (DONATO 1984a, b). Furthermore, S-100 (a+b) is a Zn^{2+} -binding protein. Recent reports by DONATO and co-workers (1985) demonstrate that single S-100 isoforms have similar, if not identical, effects in the presence of Ca^{2+} , i.e. the inhibition of assembly and promotion of disassembly of microtubule proteins. In the presence of Zn^{2+} , rat S-100 and ox S-100a and S-100b inhibit assembly, while S-100a₀ yields no effect.

There have been many efforts to demonstrate the cellular and tissue distribution of S-100 by immunocytochemical methods. Originally regarded as a brain-specific protein (BOCK 1978; ZOMZELY-NEURATH and WALKER 1980) and demonstrated in astrocytes, oligodendrocytes and ependymal cells (MATUS and MUGHAL 1975; YAMAGUCHI 1980; LUDWIN et al. 1981), protein S-100 was then observed in Schwann cells and supportive cells of the peripheral (NAKAJIMA et al. 1982; STEFANSSON et al. 1982b) as well as in the autonomic nervous system (COCCHIA and MICHETTI 1981; FERRI et al. 1982; KONDO et al. 1982). Since that time, an increasing number of mesenchymal and epithelial cell types are reported to show S-100 immunoreactivity such as interstitial cells of the pineal gland (MOLLER et al. 1978), stellate cells of the adenohypophysis (NAKAJIMA et al. 1980), melanocytes (NAKAJIMA et al. 1982), satellite cells of the adrenal medulla (COCCHIA and MICHETTI 1981), chondrocytes (STEFANSSON et al. 1982c), fat cells (MICHETTI et al. 1983), interdigitating reticulum cell in T-dependent areas of peripheral lymphoid tissue (Tabl. IIf) and in human thymic medulla (TAKAHASHI et al. 1981; NAKAJIMA et al. 1982; MECHTERSHEIMER et al. 1986), Langerhans cells of the epidermis (NAKAJIMA et al. 1982) and tubular epithelium of rat kidney (MOLIN et al. 1985) as well as salivary gland epithelium (MOLIN et al. 1985; NAKAZATO et al. 1985).



In correlation with its distribution in normal cells, protein S-100 immunoreactivity has been found in neuroectodermal and Schwann cell tumors, malignant melanomas, lipo- and chondrosarcomas, chordomas, histiocytosis X as well as salivary gland tumors (for reviews see: HAGLID et al. 1973; NAKAJIMA et al. 1982; STEFANSSON et al. 1982a; KAHN et al. 1983a; NAKAMURA et al. 1983; WEISS et al. 1983; TERENGI et al. 1984; NAKAZATO et al. 1985; SCHMIDT et al. 1985; SHIMADA et al. 1985; KIMURA et al. 1986).

Within the spectrum of soft tissue tumors, protein S-100 immunoreactivity has been detected in benign tumors of the nerve sheath including neurinoma, schwannoma, neurofibroma (Figs. 3g, 6a), nerve sheath myxoma and traumatic neuroma (NAKAJIMA et al. 1982; STEFANSSON et al. 1982a; WEISS et al. 1983; KAHN et al. 1983a; ANGERVALL et al. 1984). This finding is highly important concerning the differential diagnosis of protein S-100 negative meningiomas and leiomyomas which can exhibit similar or nearly identical histological features and a very similar topographical distribution (YAMAGUCHI 1980; NAKAJIMA et al. 1982; STEFANSSON et al. 1982a; KAHN et al. 1983a).

In malignant schwannomas and neurogenic sarcomas, however, protein S-100 shows a wide range of expression including neoplasms completely lacking this protein. In neurogenic sarcomas associated with von Recklinghausen's disease and malignant nerve sheath tumors originating from a nerve trunk, the great majority of tumors expresses protein S-100 when investigated with immunocytochemical methods (WEISS et al. 1983; HERRERA and PINTO DE MORAES 1984; NAKAJIMA et al. 1984; DAIMARU et al. 1985; MATSUNOU et al. 1985). In neurogenic sarcomas which were exclusively diagnosed on histological grounds the number of tumor cells containing S-100 protein was markedly reduced (DAIMARU et al. 1985; MATSUNOU et al. 1985). In such cases, the majority of S-100-positive cells corresponded to the wavy spindle cells in the loosely arranged areas. These cells were frequently interspersed among cells forming characteristic palisading, plexiform, storiform, or nodular patterns and tactile differentiation. Protein S-100 can be completely negative in the fibrosarcoma-like areas of neurogenic sarcomas. Since, on the other hand, non-neurogenic spindle cell soft tissue tumors such as fibrosarcomas, leiomyosarcomas, synovial sarcomas and dermatofibroma protuberans completely lack protein S-100, the demonstration of a small number of S-100-positive cells can be regarded in accordance with the clinical data and their morphology as indicative of a neurogenic sarcoma (NAKAJIMA et al. 1982; STEFANSSON et al. 1982a; WEISS et al. 1983). Although granular cell tumors show a characteristic morphology, the consistent immunoreactivity of protein S-100 can be a valuable contribution for the diagnosis. This finding is used to support the presumptive Schwann cell derivation of these tumors (NAKAJIMA et al. 1982; NAKAZATO et al. 1982; STEFANSSON et al. 1982a; ARMIN et al. 1983; MUKAI 1983).

◁ **Fig. 6. a** *Neurofibroma*: protein S-100 reaction in the majority of spindle-shaped wavy Schwann cells; $\times 160$. **b** *Rhabdomyosarcoma*: transferrin reaction in most, possibly all tumor cells; $\times 250$. **c** *Unclassified sarcoma*: ferritin in some scattered tumor cells; $\times 160$. **d** *Pleomorphic liposarcoma*: ferritin in most tumor cells; $\times 250$. **a-d** PaP method; AEC; haematoxylin counterstain

Table 3. Pattern of reactivity of 73 soft tissue sarcomas and 16 benign lesions with antiserum to S-100 protein

Diagnosis	positive/ cases	Diagnosis	positive/ cases
Fasciitis	0/2	Leiomyoma	0/1
Aggressive fibromatosis	0/3	Leiomyosarcoma	0/2
Fibroma	0/1	Rhabdomyosarcoma	0/8
Dermatofibrosarcoma protub.	0/3	Myogenic sarcoma, n.f.sp.	0/1 ^a
Fibrosarcoma	0/2	Malignant synovialoma	0/2
Dermatofibroma/cut. histiocyct.	0/3	Epithelioid sarcoma	0/2
Malignant fibrous histiocyctoma	2/5 ^a	Spindle cell sarcoma, n.f.sp.	4/11 ^a
Neurofibroma	1/1	Pleomorphic sarcoma, n.f.sp.	0/4
Cellular schwannoma	0/1	Unclassified sarcoma	2/6 ^a
Neurogenic sarcoma	0/2	Hemangiopericytoma	0/3
Neuroblastoma	0/1	Malignant hemangioendothelioma	0/1
Lipoma	1/1		
Liposarcoma	8/23 ^a	Total	88

^a few scattered cells

n.f.sp.: not further specified

In the literature, the expression of S-100 protein in lipomas and liposarcomas is controversial (NAKAJIMA et al. 1982; COCCHIA et al. 1983; KAHN et al. 1983a; WEISS et al. 1983; HASHIMOTO et al. 1984). In lipomas of various differentiation, a rather consistent S-100 immunoreactivity can be demonstrated. In liposarcomas, protein S-100 is found in mature adipocytes, vacuolated cells and lipoblasts, predominantly. Our series, which includes 23 liposarcomas among 73 soft tissue sarcomas, showed scattered S-100 positive tumor cells in 8 sarcomas (Table 3). In accordance with the literature, myxoid and spindle-cell areas were negative, and, 2 of 5 malignant fibrous histiocyctomas exhibited rare positive cells. These data suggest that protein S-100 is not a very reliable marker to identify less differentiated liposarcomas. In spite of the exceptions mentioned above, S-100 protein may be of some value in the differential diagnosis between the myxoid variant of liposarcoma and the myxoid type of malignant fibrous histiocyctoma (HASHIMOTO et al. 1984).

Since its first detection in tissue cultures of malignant melanomas (GAYNOR et al. 1980), protein S-100 has been regarded as a marker for malignant melanomas (GAYNOR et al. 1981; STEFANSSON et al. 1982a; SPRINGALL et al. 1983; WEISS et al. 1983; KINDBLOM et al. 1984; RODE and DHILLON 1984). According to these reports, protein S-100 is a relatively consistent marker of malignant melanomas including both the epithelioid and spindle cell type. Protein S-100 can therefore be used as a reliable marker in differential diagnosis between malignant melanoma and carcinoma. In this context it is interesting that also the clear-cell sarcoma of aponeuroses and tendon sheaths, which is sometimes interpreted as a variant of malignant melanoma, expresses protein S-100 (KINDBLOM et al. 1983; WEISS et al. 1983).

For principal and diagnostic reasons it is important that the histiocytosis X cells are S-100-positive (WATANABE et al. 1981, 1983; NAKAJIMA et al. 1982).

This finding allows the correlation of histiocytosis X cell to the T-zone histiocyte and the separation from lysozyme- and non-specific cross-reacting antigen-positive histiocytes of the monocyte-macrophage system (WATANABE et al. 1981). S-100 positive variants of histiocytosis X can be distinguished from juvenile xanthogranuloma.

Despite an increasing number of protein S-100 positive cell types and tumors, the immunocytochemical demonstration may be helpful in selected problems of tumor differential diagnosis, e.g. (1) the distinction between benign nerve sheath tumors and leiomyomas or meningiomas, (2) cellular schwannomas and neurogenic sarcomas, (3) neurogenic sarcomas and other spindle-cell sarcomas, (4) histiocytosis X and juvenile xanthogranuloma. Scattered S-100 positive cells in spindle cell sarcomas can be used to support the diagnosis of neurogenic sarcoma; negative immunoreaction, however, does not exclude this diagnosis. Some S-100-positive cells in liposarcomas are of limited value for the differential diagnosis, since in malignant fibrous histiocytomas rare S-100-positive cells can also be identified by immunocytochemical methods.

8 Ferritin

Ferritin, a protein containing more than 20% iron, was first isolated by LAUFBERGER (1937) from horse spleen. It is widely distributed in mammalian tissues. The ferritin molecule's inner core is about 55 Å in diameter and consists of ferric hydroxid micelles surrounded by an almost spherical shell of protein with a molecular weight of 462 kD. The overall diameter of the molecule is approximately 110 Å. Because of its electron dense iron core, ferritin has been used as a tracer in electron microscopy since the early sixties. Since abnormally high levels of ferritin are associated with early stages of many malignant neoplasias (for review see DRYSDALE 1979) and, since tissue specific iso-ferritins have been isolated (BULLOCK et al. 1980), the question has been raised whether ferritins might be exploited as tumor markers.

So far, there have been few attempts to use ferritin as tumor marker in immunohistochemistry. Ferritin was demonstrated in hepatocellular carcinoma (COHEN et al. 1984a; IMOTO et al. 1985) and in testicular seminoma (JACOBSEN and JACOBSEN 1983; COHEN et al. 1984b). SEHESTED et al. (1985) detected ferritin in both epithelial and mesenchymal components of pleomorphic adenomas, but not in normal parotid tissue. The only report on in situ demonstration of ferritin in soft tissue tumors we could find was that published by KINDBLOM et al. (1982). The authors investigated malignant fibrous histiocytomas and detected ferritin in 12/22 of the pleomorphic and 3/5 tumors of the spindle cell type.

In our series of soft tissue tumors, ferritin could be detected in a considerable number of cases of nearly every type of benign and malignant lesions (Table 2; Figs. 4b, c, 6c, d). Accordingly there seems to be no possibility of using ferritin as a diagnostic aid. This phenomenon, however, does not exclude the possibility of existing differences in tumor biology between the tumors that contain high amounts of ferritin and those that do not. This issue as well as the practical relevance of tissue specific iso-ferritins, against which at present there are no specific antibodies available, remain to be elucidated.

9 Transferrin

Transferrin is one of the β_1 serum globulins. It has a molecular weight of 88 kD and is synthesized in liver cells (VASSY et al. 1984). Each molecule carries two binding sites with probably different functions. Its main function is iron transfer. The iron-free, apoferritin, binds to Fe^{3+} ions that form ferrotferrin. All growing cells express surface transferrin receptors that bind ferrotferrin which is then subjected to receptor-mediated endocytosis (IACOPEITA et al. 1983). The iron atoms are afterwards transferred to cytoplasmic ferritin and within a few minutes, apoferritin is secreted from the cell (WILLINGHAM and PASTAN 1985). The transferrin receptor is a homo-dimeric transmembrane glycoprotein (MW 180 kD) (SCHNEIDER et al. 1984). One antibody with a binding affinity for it is OKT9. These phenomena make it evident that a cytoplasmic localization of transferrin does not reflect a synthesis of this protein but the degree of transmembranous iron transfer (PARMLEY et al. 1979). In neoplastic cells, an additional effect might account for a high cytoplasmic transferrin content, i.e. its accumulation as a consequence of a defective or functionally impaired transferrin receptor. An immunohistochemical proof of increased cytoplasmic transferrin content in a tumor cell is thus a finding that cannot be further interpreted and that might explain the absence of data on tissue distribution of transferrin in tumors.

As can be seen in Table 2, we observed transferrin in many different malignant soft tissue tumors (Figs. 4a, 6b) whereas the benign tumors did not contain detectable intracytoplasmic transferrin. Our data, however, do not suffice to determine whether cytoplasmic transferrin might be an indicator of malignancy, but it is quite obvious that transferrin is of little diagnostic value because it does not discriminate between diverse types of sarcomas.

10 Factor VIII Associated Antigen

Together with the clot-promoting factor and the von Willebrand-factor, the factor VIII associated antigen (FVIIIaAg) which is synthesized by endothelial cells (JAFFE 1977; GIDDINGS 1982), forms the antihemophilic agent factor VIII. The antigen, a glycoprotein with a molecular weight of approximately 1100 kD (McCOMB et al. 1982), has been detected within these cells (HOYER et al. 1973; MUKAI et al. 1980b), and also in platelets, megakaryocytes and mast cells (PIOVELLA et al. 1978; KINDBLUM 1982). FVIIIaAg has been used as a marker for tumors of vascular origin (FEIGL et al. 1976; BURGENDORF et al. 1981b; SEHESTED and HOU-JENSEN 1981), of fibrous hematopoietic tumors (MEISTER and GOESSNER 1983) and Kaposi's sarcoma (NADJI et al. 1980; GUARDA et al. 1981; MODLIN et al. 1983; FLOTTE et al. 1984; MILLARD and HERJET 1985; BECKSTEAD et al. 1985).

In the literature, FVIIIaAg is regarded as a reliable marker for highly differentiated angiogenic tumors, excluding lymphangioma (LITTLE et al. 1986; BURGENDORF et al. 1981b). However, staining was most marked in endothelial cells of normal vessels and was much weaker in neoplastic endothelia of poorly differentiated angiosarcoma and in the proliferating spindle cells of Kaposi's sarcoma which irregularly exhibit a dot-like or granular cytoplasmic staining for FVIIIaAg (BECKSTEAD et al. 1985; MILLARD and HERJET 1985; LITTLE et al. 1986). Some authors (HOSAKA et al. 1985; LITTLE et al. 1986) prefer *Ulex europaeus* lectin I (UEA I) as a more sensitive marker.

In our series of 89 mesenchymal tumors, we could readily demonstrate the vasculature of each tumor, but found FVIIIaAg-containing tumor cells only three times: in a poorly differentiated angiosarcoma and in two additional cases of Kaposi's sarcoma (Fig. 3a, b). The latter contained spindle cells with a granu-

lar positivity or a dot-like staining pattern for this marker. However, in the cases of haemangiopericytoma, the tumor cells were FVIIIaAg-negative. We suggest that in a case of poorly differentiated sarcoma, the detection of clearly FVIIIaAg-positive tumor cells might suffice for a diagnosis of malignant endothelioma. It is, however, important not to regard collapsed atypical capillaries in the "stromal" compartment of the tumor as part of the neoplastic cell population. Since FVIIIaAg stains platelets as well, areas of intratumoral haemorrhage should be avoided in evaluation. Apart from the fact that abnormal cells often produce smaller amounts of a cytotypical product than does the normal counterpart, a weak or negative staining result may be caused not only by a decrease in antigenic density alone but also by a low affinity of the antiserum. We agree with LITTLE (1986) that UEA I is a far more sensitive marker but that it has the great disadvantage of reacting with diverse epithelial cell types (MÖLLER et al. 1984; WIRBEL et al. 1984). Additionally, UEAI recognizes lymphatic endothelium at least within areas of inflammation and sclerosis (MÖLLER and LENNERT 1984), whereas FVIIIaAg does not. Therefore, the combination of these two markers might facilitate the differential diagnosis between lymphangioma and haemangioma.

11 Conclusions

Routine light microscopy, together with special stains, is a prerequisite for the classification of soft tissue tumors. Additional immunohistological investigations can either substantiate the diagnosis or lead to a decision between two alternatives if the antigenic pattern of respective tumors differs. The negative result of a reaction does not necessarily exclude a diagnosis and a histogenetic diagnosis is confirmed by a positive result only if the antibody is specific. Desmin, myosin and myoglobin are antibodies with a good specificity for myogenic tumors. Desmin, the most sensitive antibody, can recognize tumors with smooth muscle as well as skeletal muscle differentiation whereas myoglobin only recognizes tumors with skeletal muscle differentiation and only highly differentiated tumor cells. Myosin is also a reliable myogenic marker, but it is not yet commercially available. Protein S-100 is a valuable adjunct in the diagnosis of benign and malignant nerve sheath tumors, histiocytosis X-related lesions, and malignant melanomas. The factor VIII-associated antigen is highly specific for tumors originating from blood vessel endothelium, while it cannot always be detected in poorly differentiated tumors. Lysozyme, AAT, ACT, ferritin and transferrin, because of their low specificity, are only of limited value for differential diagnosis of soft tissue tumors.

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Proteoglycans and the Intercellular Tumor Matrix

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1 Introduction

The intercellular matrix is a highly organized, dynamic system that dictates the overall shape and structure of organs. The spatial and chemical signals which are present in the extracellular matrix modulate the phenotypic expression of epithelial and mesenchymal cells. In turn, these cells regulate the chemistry and structural organization of the extracellular matrix. This active interplay between cells and their products leads to the formation of a highly complex environment in which normal and neoplastic cells can thrive and proliferate. Proteoglycans are suitable candidates for mediating this constant exchange of information. For instance, they influence the diffusion of molecules across tissues, the migration of cells along defined pathways, the surface properties of cells, and the structure of biological filters. These effects are primarily mediated by the polyanionic nature of the proteoglycan, by their expanded configuration in tissues and body fluids, and by their ability to interact with a variety of important matrix macromolecules. They are indeed constituents of both the stromal matrix and the cell surface (Fig. 1), and their biosynthesis, secretion and metabolism are highly regulated by intrinsic and extrinsic signals. These molecules are composed of a protein backbone to which a number of glycosaminoglycan chains and oligosaccharides are covalently attached, much like the branches to a tree. The proteoglycan-rich intercellular matrix can thus be conceived as a microcosmic forest, the properties of which depend primarily on the number, size and structure of the various glycosaminoglycan chains and their complex intermolecular affiliations. This "forest" is in a state of continuous remodeling particularly during embryogenesis and cancer development, and is finely regulated by a combination of biosynthetic and catabolic events. Thus, it would be easier to imagine the extracellular matrix as exerting

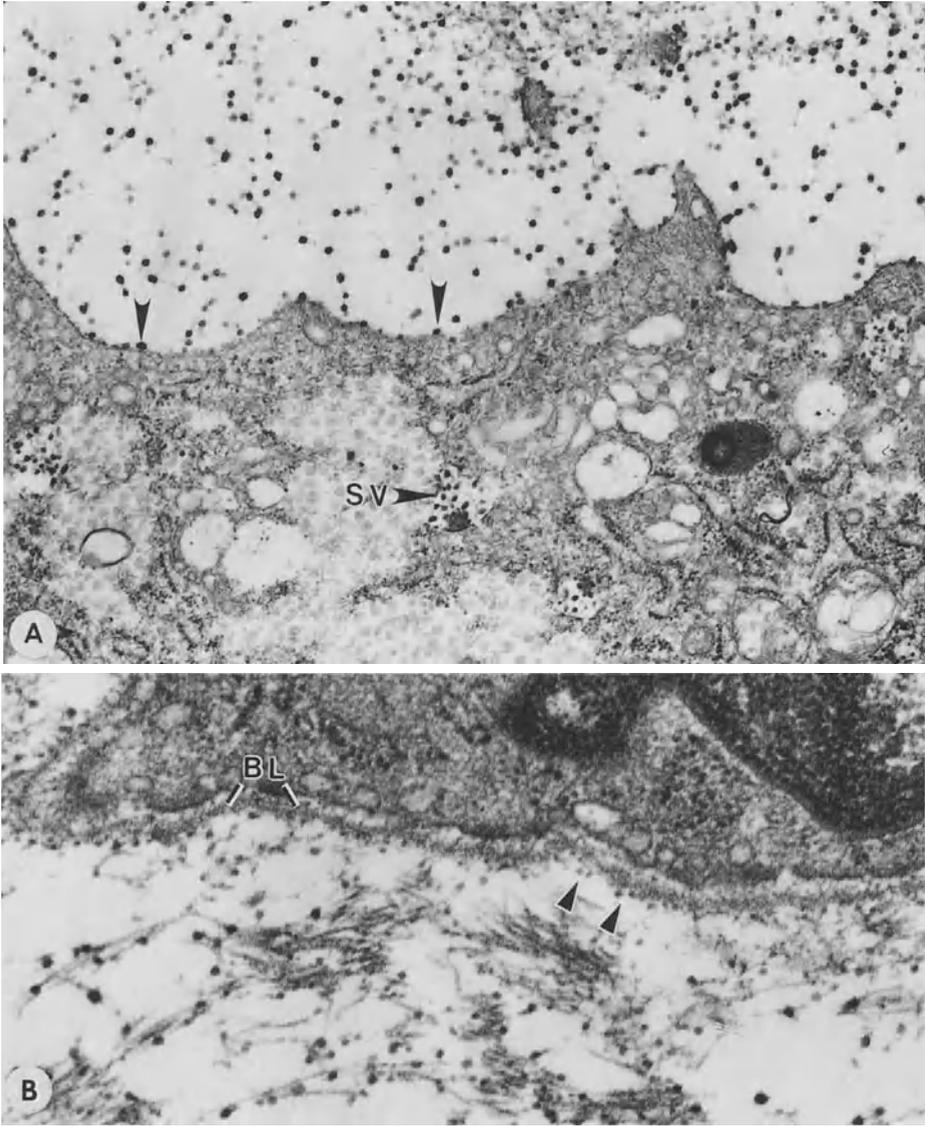


Fig. 1A, B. Electron micrographs of human chondrosarcoma fixed and processed in the presence of ruthenium red, a cationic dye that retains proteoglycans. **A** shows portion of a chondrosarcoma cell containing numerous secretory vesicles (*SV*) filled with proteoglycans which appear as round, electron dense particles. Note the association of the proteoglycans with the cells surface (*arrowheads*) and the presence of the same particles dispersed throughout the extracellular matrix. **B** shows the association of proteoglycans (*arrowheads*) with the basal lamina (*BL*) of an endothelial cell and with fibrillar matrix constituents. **A** $\times 23000$; **B** $\times 34000$

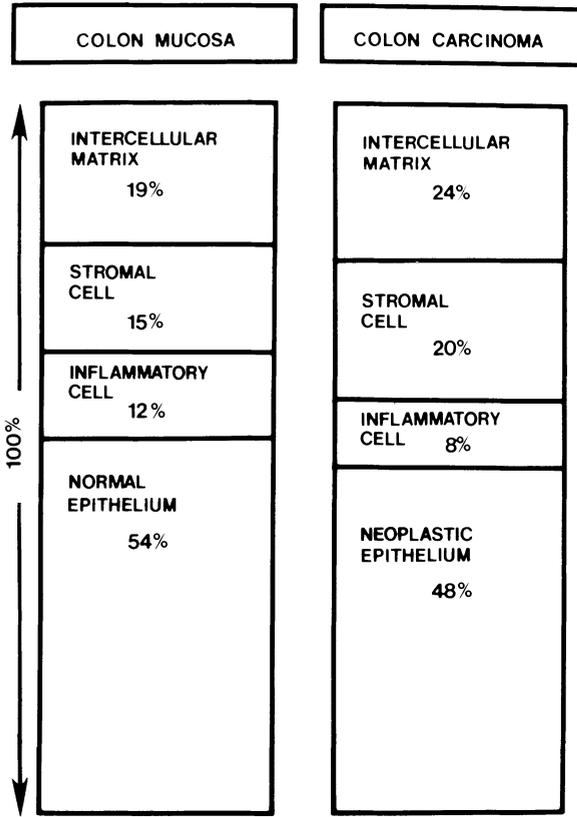


Fig. 2. Stereologic analysis of human colon mucosa and colon carcinoma. The data represent the summary of seven different cases (Iozzo et al. 1982) and show that about half of the volume in both the normal and neoplastic tissue is composed of stromal tissue and intercellular matrix rich in proteoglycans and collagen. This figure stresses the concept that in certain human malignancies, particularly those of epithelial origin, the associated connective tissue stroma occupies a significant proportion of the “tumor”

an active role, permissive or restrictive, on neoplastic growth and invasion, a far cry from the traditional notion of its being an amorphous ground substance.

This chapter will briefly review the alterations of proteoglycans and glycosaminoglycans in cancer, and it will describe some of the mechanisms through which neoplastic cells modulate their extracellular environment. For extended reviews on proteoglycan structure and function please see previous published work (HASCALL and HASCALL 1981; HEINEGÅRD and PAULSSON 1984; IOZZO 1985a).

2 Proteoglycans and Neoplasia

If one analyzes quantitatively the various compartments of human colon carcinoma, one of the most common malignant neoplasms in the Western world, one finds that surprisingly only about half of the tissue is composed of neoplastic cells (Fig. 2). The other half is composed of an acellular matrix, rich in proteoglycans, and a cellular compartment including fibroblasts, endothelial and

smooth muscle cells and a variety of immunocompetent cells. It follows that each component must contribute, in either a negative or positive way, to the growth and progression of the neoplastic cells. Two fundamental questions arise from the above considerations: a) is the tumor connective tissue a specific response of the host, and b) how is this response regulated? In answer to the first question, indirect evidence suggests that this response is specific, since it can be produced *in vitro* by factors released by the tumor cells (IOZZO 1985b) and it is found only in certain tumors, particularly those of epithelial origin. In response to the second question, current research (LIOTTA et al. 1983; IOZZO 1984a, 1985a; WEWER et al. 1986) indicates that neoplastic cells modulate their extracellular milieu by the following three, often interrelated, mechanisms: a) abnormal production of extracellular matrix by tumor cells, b) enhanced destruction of surrounding matrix, and c) neoplastic induction of matrix biosynthesis. These three mechanisms can be present in the same tumor, or in different locations of the various neoplasms where one could be predominant over the others. The significance of these basic processes and the implications in cancer growth and metastasis is the focus of the next sections.

3 Altered Proteoglycan and Glycosaminoglycan Levels in Tumors

It has been frequently observed that tumor cells grow and move within a hydrated interstitial space, analogous to the migrating cells during embryogenesis (TOOLE 1981). The ultrastructural appearance of the proteoglycans in these matrix spaces varies widely in size, arrangement and degree of packing (Fig. 3). Furthermore, these spaces are often different from normal tissues in that they contain abnormal amounts of specific proteoglycan or glycosaminoglycan (Table 1). For example, elevated amounts of hyaluronic acid in pleural effusion of mesotheliomas have been known for about fifty years (MEYER and CHAFFEE 1939), and detection of this glycosaminoglycan has been a useful marker in establishing the diagnosis of mesothelioma (THOMPSON et al. 1969). Furthermore, proteolytic extracts of human mesothelioma have been shown to contain elevated concentrations of hyaluronic acid (WAXLER et al. 1979). However, the

Table 1. Reported proteoglycan changes in human tumors^a

Mesothelioma	1. elevated hyaluronic acid in tissue and effusions 2. elevated chondroitin sulfate in tissue
Wilms' tumor	elevated hyaluronic acid in tissue, blood and urine
Chondrosarcoma	decrease in keratan sulfate content and chain length
Colon carcinoma	elevated chondroitin sulfate proteoglycan, with smaller chains
Hepatoma	abnormal levels of heparan sulfate with lower degree of sulfation
Plasmocytoma	abnormal circulating levels of heparan sulfate with anticoagulant activity

^a Additional information about each individual abnormality and references are provided in the text

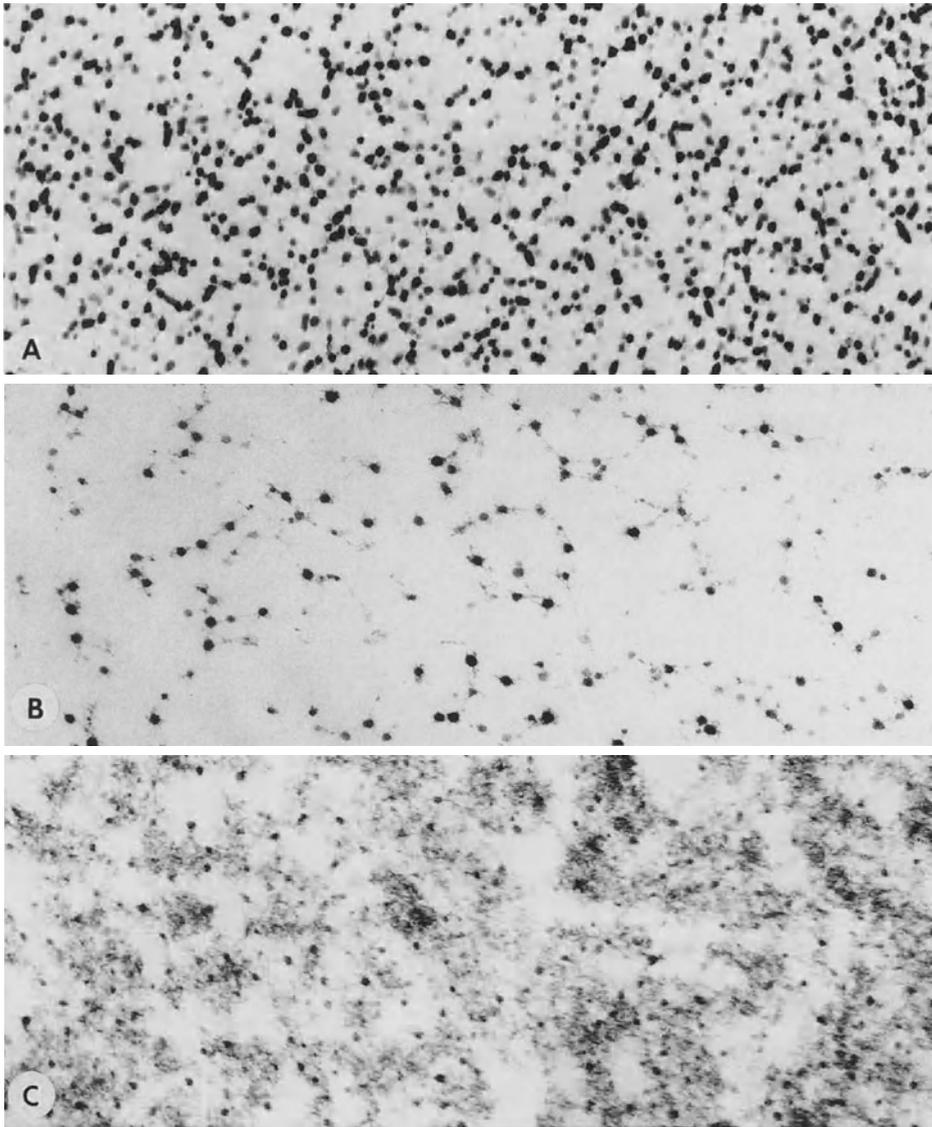


Fig. 3. Electron micrographs of ruthenium red-stained extracellular matrix from human chondrosarcoma (A), normal human colon (B) and human colon carcinoma (C). Notice the marked difference in the density and size of the proteoglycan granules among these three conditions. The three micrographs were taken at the same magnification of $\times 36000$

specificity of hyaluronic acid as a marker for mesothelioma is questioned by the following contrasting reports: a) there are cases of mesothelioma that apparently lack the high levels of hyaluronic acid (THOMPSON et al. 1969); b) neoplastic tissues other than mesothelioma may express high levels of this glycosaminoglycan (CHIU et al. 1984); c) pleural effusions unrelated to mesothelioma can con-

tain high levels of hyaluronic acid (CASTOR and NAYLOR 1977). We have reported (IOZZO et al. 1981) that tissue extracts of pleural mesothelioma contain large amounts of chondroitin sulfate and we have proposed the existence of a biochemical variant for this neoplasm. Indeed, recent studies (KAWAI et al. 1985; NAKANO et al. 1986) support the latter view since it has been found that in a group of twelve cases there was an elevation in either hyaluronic acid or chondroitin sulfate, or both. Therefore, characterization of the glycosaminoglycan composition in this neoplasm may represent a useful tool in establishing the diagnosis of mesothelioma.

Wilms' tumor (nephroblastoma), a renal neoplasm affecting primarily children, has been shown to be associated with high levels of hyaluronic acid in the blood and urine, in contrast to the trace-amount levels of this substance in normal hosts (MORSE and NUSSBAUM 1967). The concentration of circulating hyaluronic acid can reach levels of 2 mg/dl, but it falls dramatically to normal levels after surgical removal of the affected kidney (POWARS et al. 1972). This fact together with the demonstration that tissue fragments, cultured cells or cell-free preparations of Wilms' tumor synthesize hyaluronic acid at high rates (HOPWOOD and DORFMAN 1978) support the view that the abnormal blood and urinary levels of this glycosaminoglycan derive from the tumors cells.

Another example of altered proteoglycan structure is provided by human chondrosarcoma, a neoplasm that varies widely in the degree of differentiation and metastatic behavior. Specifically, the amount and chain size of keratan sulfate decrease significantly in poorly differentiated chondrosarcomas (PAL et al. 1978; THONAR et al. 1979), whereas well-differentiated neoplasms have a proteoglycan profile similar to normal cartilage. On the basis of these results, it has been proposed that there might be an inverse relationship between the length of the keratan sulfate chains and the biological behavior of the chondrosarcoma (PAL et al. 1978; THONAR et al. 1979). Inasmuch as the assessment of the degree of malignancy in human chondrosarcoma is based purely on morphological grounds, the use of alternative prognostic indicators, i.e. biochemical assessment of keratan sulfate, could be of great biological importance (ROSENBERG et al. 1979). In addition, a variety of epithelial and mesenchymal neoplasms express abnormal levels of chondroitin sulfate, and correlations between tumor cell properties and this glycosaminoglycan have been proposed (IOZZO 1984, 1985a).

Two general conclusions emerge from the above studies. First, proteoglycan changes do indeed occur in human malignancy and may be closely related to some biological properties of the tumor-matrix complex. Second, it appears that a common alteration in proteoglycan structure is not universally shared by all the different neoplasms; rather, the changes summarized above appear to be tissue specific.

4 Abnormal Synthesis of Proteoglycans by Neoplastic Cells

A vast body of literature on the abnormal biosynthesis of proteoglycans and glycosaminoglycans has been accumulating over the past years. Indeed, *in vitro*

studies of either virally or spontaneously transformed cells have elucidated several aspects of the biosynthetic pathways of proteoglycans as well as provided important clues as to what might occur in an *in vivo* situation. Since a comprehensive review of the literature falls outside the scope of this chapter, only a few examples will be described below.

One of the most consistent qualitative abnormalities in proteoglycan structure which is directly related to the transformed phenotype is a reduced degree of sulfation in the heparan sulfate molecule. Over a decade ago, UNDERHILL and KELLER (1975) reported this change in 3T3 cells transformed with simian virus 40. Since then, a number of investigators (WINTERBOURNE and MORA 1981; DAVID and VAN DEN BERGHE 1983; ROBINSON et al. 1984) have reported similar findings in different cell systems. Specifically, the degree of sulfation of the heparan sulfate chains is markedly reduced upon transformation, and the reduced sulfation occurs in the 0-sulfate groups (KELLER et al. 1980). The link between the neoplastic transformation and these structural abnormalities is supported by the findings that cells selected for high tumorigenicity also produce heparan sulfate with lower degree of sulfation (WINTERBOURNE and MORA 1981). Interestingly, *in vivo* studies of human hepatoma (NAKAMURA and KOJIMA 1981) have shown the presence of undersulfated heparan sulfate, a finding also reported in ascites hepatoma (NAKAMURA et al. 1978; HURST et al. 1981) or hepatoma cells *in vitro* (ROBINSON et al. 1984). Finally, spontaneously transformed mouse mammary cells synthesize undersulfated basement membrane heparan sulfate (DAVID and VAN DEN BERGHE 1983). Two major questions arise from the above reports: a) what is the specific cause of this alteration, and (b) what are the possible functional implications of it? Although the specific cause of this molecular alteration is not clear, it is likely that transformation may generate some inhibitor or reduce the activity of sulfotransferases involved in the transfer of sulfate to the growing polymer or in the level of 3'-phosphoadenosine-5'-phosphosulfate (PAPS), the major sulfate donor in most of the cells (WINTERBOURNE and MORA 1981). Indeed, it has been demonstrated that the availability of PAPS plays a fundamental role in the sulfation of glycosaminoglycans (SUGAHARA et al. 1980). In regard to the functional consequences, suffice it to say that qualitative changes in the cell-associated heparan sulfate may have profound effects on the cellular ability to interact correctly with other cells or matrix constituents. In fact, this molecule is a highly charged polymer strategically located at the cell surface where most of the cellular interactions take place. Furthermore, the structural changes may have an effect on the molecular interactions normally occurring during the assembly of the basal lamina and may be directly implicated in the abnormal basal lamina produced by neoplastic cells (DAVID and VAN DEN BERGHE 1983). It is possible, therefore, that the lack of an ordered microenvironment around tumor cells could be essential for the full expression of the neoplastic phenotype (GALLAGER and HAMPSON 1984). If these reported alterations do indeed reflect the *in vivo* situation, these qualitative changes in molecular structure may then contribute to the abnormal growth behavior of the tumor cells.

Another example of tumor-associated abnormality in heparan sulfate proteoglycan has been reported occurring in patients with plasma cell disorders,

i.e. multiple myeloma (KHOORY et al. 1980; PALMER et al. 1984). In this case the abnormality appears to be a quantitative rather than a qualitative one; that is an increased level of circulating heparan sulfate with anticoagulant activity. In fact, functional coagulation studies showed that the circulating heparan sulfate had antithrombin III cofactor activity that could be neutralized by protamine or platelet factor 4 (PALMER et al. 1984). It is well established that the anticoagulant activity in heparin, a closely related molecule, depends on the negative charge density of sulfate and carboxyl groups, and on the amount of L-iduronic acid which are all present in heparan sulfate, though to a lesser extent than in heparin (LINDAHL et al. 1986). Therefore, it is not surprising that abnormally high levels of circulating heparan sulfate may inhibit the coagulation cascade and induce fatal bleeding. Interestingly, a similar process has been recently described in an infant with acute monoblastic leukemia (RUSSEL et al. 1984). Although the specific cellular source of this heparan sulfate has not been definitively established, it is likely that neoplastic cells do contribute in a significant way to its biosynthesis and release. Alternatively, it has been proposed (RUSSEL et al. 1984) that the circulating heparan sulfate proteoglycan derives from the endothelial cells damaged by the chemotherapy. In any event, it is quite clear that overproduction and release of an otherwise normal heparan sulfate proteoglycan can have catastrophic consequences because of its potential anticoagulant activity.

It is apparent from the examples described above that the process of transformation markedly affects the mechanisms involved in the control of proteoglycan biosynthesis and secretion. Both qualitative and quantitative abnormalities may thus significantly affect the life of the host, and may contribute to the abnormal growth behavior or other oncogenic properties of the transformed cells.

5 Matrix Degradation by Neoplastic Cells

Malignant cells are confronted with several natural barriers during the invasion of adjacent structures and distal colonization of tissues (WOOLLEY 1984). The extracellular matrix and the basement membranes represent the most important biological barrier and their active degradation is thought to facilitate invasive growth and metastatic potential (LIOTTA et al. 1983). These features are two of the most important prognostic indicators in cancer. There is evidence to indicate that malignant cells can digest matrix constituents either directly or indirectly by inducing the production and release of degradative enzymes in host mesenchymal cells (LIOTTA et al. 1982, 1983). The collagenases are probably the most important extracellular enzymes since they are capable of degrading cross-linked collagen fibers at neutral pH (WOOLLEY 1984). The end result is either complete or partial collagenolysis with generation of small fragments which are eventually phagocytosed and degraded completely within lysosomes by the cathepsins, usually operating at a more acidic pH (WOOLLEY 1984). LIOTTA et al. (1979) have identified a metalloproteinase from a metastatic tumor that preferentially digests type IV collagen, the major constituent of basement membranes (KEFALIDES et al. 1979). Subsequently, a correlation between the

metastatic potential of malignant cells and the secretion of various collagenases has been demonstrated (LIOTTA et al. 1980; TARIN et al. 1982; TURPEENNIEMI-HUJANEN et al. 1985), thus supporting the concept that collagenolysis facilitates tumor cell invasion. It is notable that in the transformation of a benign tumor into a frankly invasive carcinoma of the breast, the immunoreactivity of the basement membrane progressively declines to eventually become undetectable at the invasive front and in the metastatic foci (BARKSKY et al. 1983a). Concurrently, type IV collagenase immunoreactivity has been detected in invasive breast carcinoma (BARKSKY et al. 1983b). Furthermore, immunohistochemical studies have shown the presence of collagenases in a variety of neoplasms, including metastatic melanoma and cancers of the skin, stomach and breast (for a review see WOOLLEY 1984). Another well studied enzyme that is associated with the malignant phenotype is cathepsin B, which is capable of digesting both collagen and proteoglycans. This enzyme has been localized at the invasive front of rabbit V2 carcinoma (GRAF et al. 1981), and increases several fold in breast carcinoma as compared to fibroadenoma (POOLE et al. 1978) and its activity has been correlated with enhanced metastatic potential (SLOANE et al. 1981).

Another important enzymatic step that may be operational in invasion is offered by endoglycosidase synthesized by melanoma (KRAMER et al. 1982) or lymphoma (VLODAVSKY et al. 1983) cells. This enzyme is capable of cleaving the heparan sulfate present in the extracellular matrix and basement membrane of endothelial cells (KRAMER et al. 1982; KRAMER and VOGEL 1984; NAKAJIMA et al. 1983, 1984). Specifically, B16 melanoma cells degrade the heparan sulfate chains into fragments approximately one-third of their original size. This enzymatic cleavage is observed only when the prelabeled matrix is incubated with the tumor cells, indicating that the tumor may express this trait *in vivo*. Additionally, melanoma cells with high propensity for lung metastasis degrade purified heparan sulfate from the mammalian lung at significantly higher rates than cells with lower metastatic potential (NAKAJIMA et al. 1983, 1984). Therefore, it is possible that the ability to extravasate and colonize a distant organ such as the lung may reside, at least in part, in the expression of this endoglucuronidase.

One of the most important concepts in modern cancer biology is the recognition that primary neoplasms are composed of heterogeneous cell populations (POSTE and FIDLER 1980). The expression of degradative enzymes is likely to be important for tumor progression, a process that is due to the sequential appearance of genetically altered subpopulations of cells with new characteristics within the neoplasms (NOWELL 1986). The expression of degradative enzymes would thus confer a selective advantage to a specific subpopulation. An open question, however, is whether these enzymatic activities reflect a continuous phenotypic trait or just a transient phase of enzyme production which depends on local tissue factors (WOOLLEY 1984) and cell interactions.

6 Neoplastic Induction of Matrix Biosynthesis

Desmoplasia, the excessive production of connective tissue around invasive neoplasms, has long been recognized as a phenomenon associated with certain

types of human tumors. However, little has been done to answer some of the important questions about this response, such as: a) is there any specific qualitative or quantitative change in matrix constituents, b) what is the cellular source of matrix production, c) what is the role played by the cancer cells and d) is desmoplasia a specific response? In the past few years, we and others have attempted to answer some of these questions. For instance, BARSKY et al. (1982) have demonstrated that human desmoplastic breast carcinomas exhibit a ten-fold increase in the concentration of type V collagen, a product present only in minute amounts in normal breast or fibrocystic disease. Interestingly, an elevated number of host myofibroblasts or activated smooth muscle cells has been recognized in desmoplastic breast carcinomas (SEEMAYER et al. 1979) suggesting that these cells may be responsible for the excessive production of matrix (SCHÜRCH et al. 1981). Furthermore, we have shown (IOZZO et al. 1982) that human colon carcinoma tissue contains markedly elevated concentrations of chondroitin sulfate when compared with the normal counterpart, an observation also reported in a transplantable colon carcinoma of the rat (ISEMURA et al. 1982). In subsequent studies, (IOZZO and WIGHT 1982) we have demonstrated that human colon carcinoma in organ culture synthesizes primarily a small chondroitin sulfate proteoglycan, whereas normal colon synthesizes primarily heparan sulfate and only trace amounts of chondroitin sulfate proteoglycan. Several lines of evidence indicate that the stromal elements around the tumor represent the main cellular source for desmoplasia. First, the matrix around colon carcinoma incorporates the majority of the radiolabeled sulfate and retains affinity for alcian blue even at high magnesium chloride concentrations (Fig. 4), indicating that the stromal cells actively synthesize and accumulate proteoglycans. This phenotypic trait can also be retained in metastatic foci (Fig. 4e), suggesting that it may be a specific process closely related to the type of neoplastic cells. Second, human colon carcinoma cells in vitro synthesize a large heparan sulfate proteoglycan but no detectable amounts of chondroitin sulfate (IOZZO 1984b; IOZZO et al. 1986). Third, human colon fibroblasts are stimulated to synthesize chondroitin sulfate proteoglycan when cultured in the presence of medium conditioned by colon carcinoma cells. Partial characterization of the conditioned medium (IOZZO 1985b) showed that the stimulatory activity comprises a family of polypeptides that act on proteoglycan metabolism without significantly stimulating cell replication. The work of KNUDSON et al. (1984) and MERRILEES and FINLAY (1985), supports these observations. They have demonstrated that the co-culture of carcinoma and mesenchymal cells or media conditioned by tumor cells can stimulate the production of both hyaluronic acid and sulfated glycosaminoglycans in normal fibroblasts. In agreement with our studies, MERRILEES and FINLAY (1985) have found that human colon carcinoma cells, when cultured alone, synthesize primarily heparan sulfate, but are capable of stimulating the synthesis of both hyaluronic acid and chondroitin sulfate in human fibroblasts. Further support for the neoplastic modulation of extracellular matrix is provided by the discovery that mammary carcinoma contains collagen-synthesis stimulating polypeptides (BANO et al. 1983). It has been proposed that these stimulatory activities may favor tumor growth, since proline analogues, which selectively block collagen deposition, can also block

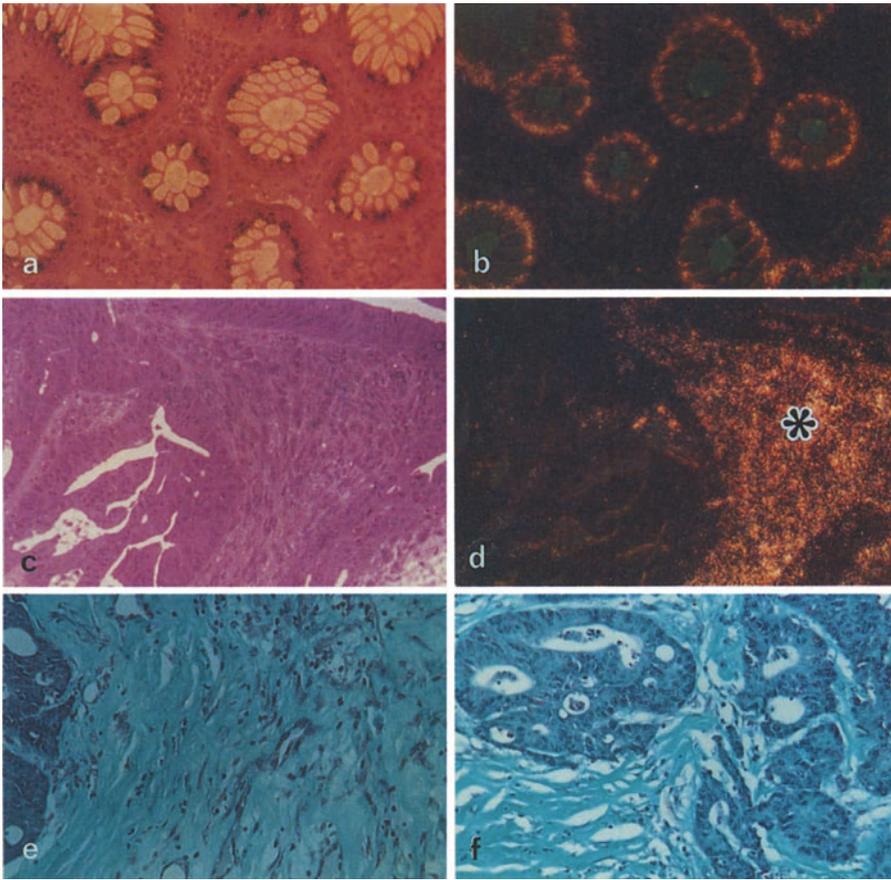


Fig. 4. Gallery of light and dark field micrographs of normal colon (**a, b**) and colon carcinoma (**c-f**). The top four panels represent autoradiographs of tissue incubated in an organ culture system with radiosulfate. Notice that in control (**a, b**) the radiosulfate is incorporated nearly exclusively by the epithelium, whereas in the tumor (**c, d**) the radiosulfate is incorporated primarily by the connective tissue stroma (*asterisk*). The accumulation of sulfated material in the peritumoral extracellular matrix is further shown by staining of the primary (**e**) or metastatic (**f**) colon carcinoma matrix with alcian blue at high (0.3 M) magnesium chloride concentrations. Methacrylate sections stained with H&E were subjected to autoradiography (Iozzo et al. 1982) and studied by light (**a, c**) and dark field (**b, d**) microscopy. Panels **e** and **f** represent paraffin sections of alcian blue-stained colon carcinoma at the primary site and at a metastatic pulmonary focus, respectively. **a-f** $\times 140$

the growth of mammary carcinoma (LEWKO et al. 1981). In a recent study, we (IOZZO and MÜLLER-GLAUSER 1985) have demonstrated that the connective tissue of rabbit mesentery, following intraperitoneal implantation of V2 carcinoma cells, was markedly increased. Specifically the mesentery increased up to eight fold in thickness and several times in weight. This increase was not due to proliferation of tumor cells but was rather due to the marked increase in hyaluronic acid, chondroitin sulfate and collagen. In addition, the fibroblasts

were also increased in number, suggesting that these mesenchymal cells were stimulated by the tumor cells to proliferate and synthesize matrix constituents. In support of this argument is the fact that intraperitoneal implantation of liver homogenates did not cause any structural change in the rabbit mesentery (IOZZO and MÜLLER-GLAUSER 1985).

From the above studies it appears that, at least in some malignant neoplasms, the cancer cells do modulate their extracellular environment either directly by cell-cell interactions, possibly via cell surface molecules, or indirectly via the elaboration of tumor cytokines that exert a stimulatory activity on the mesenchymal cells. These cytokines, which are often found in the supernatant of cancer cells *in vitro*, may function as local hormones *in vivo*, and exist in small quantities, traveling short distances between host cells. The expression of such cytokines may be the key to the specificity and appearance of desmoplasia in a given neoplasm. Their presence would also explain why only certain types of tumors are capable of inducing this response in host cells, whereas other tumors that either lack or are incapable of expressing these gene products fail to induce it.

7 Conclusions and Perspectives

This chapter has described quantitative and qualitative alterations in proteoglycans occurring in human tumors and has discussed some of the possible regulatory mechanisms. It is apparent from the evidence presented above that proteoglycans do play a role in the growth and evolution of malignant neoplasms. They function as a kind of molecular sieve, and as such regulate the filtration of a variety of "signals" that reach the cells and eventually influence their function. Proteoglycans are responsible for retaining water, can act as growth promoting agents, and favor cell adhesion and migration along defined routes. It is conceivable that, in analogy with developmental systems, the well-hydrated, proteoglycan-rich matrix may favor the evolution of malignant cells by providing a growth-supporting environment. It is also apparent that neoplastic cells with a capacity to invade neighboring structures have the potential to alter the adjacent connective tissue and to modulate the metabolism of host mesenchymal cells. This ability can be manifested by either the abnormal synthesis or degradation of matrix macromolecules or by the release of tumor cytokines that trigger the synthesis or breakdown of matrix constituents. The proposed concept that tumor cells "condition" their pericellular environment offers a novel perspective in investigating this complex phenomenon. If one accepts this hypothesis, then a number of new and provocative questions emerge. For example, are the tumor cells the sole protagonists or does the full expression of the tumor extracellular matrix depend on rather complex host-tumor cell interactions and if this were the case, should we not consider the tumor extracellular matrix as a "specific" response rather than as a non-specific repair as previously regarded? What are the molecular mechanisms that dictate the lack or overproduction of matrix in a given neoplasm? Is the presence of specific tumor cytokines the key to

desmoplasia? Is there any tissue specificity? The point to be stressed is the difficulty in making generalizations about the mechanisms regulating matrix metabolism in cancer. It is probable that one type of tumor favors one mechanism over the others, but even that may exhibit subtle variations at various locations within the neoplasm itself. For the past several years our laboratory has been interested in investigating some of the above questions and the search for their answers is what makes research in this field challenging and worthwhile.

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Basal Membrane Antigens as Tumor Markers*

J. CASELITZ

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1 Introduction

In multicellular organisms the basal membrane, a complex extracellular matrix, plays a leading role in maintenance of the structure and function of the organism

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(VRACKO 1974, 1982; HAY 1978; FURTHMAYR 1982; STANLEY et al. 1982a, b; FOIDART 1984; ABRAHAMSON 1986; INGBER et al. 1986). The basal lamina (basement membrane, basement lamina) is primarily defined by its topography and structure, as revealed by light and electron microscopy. The electron microscopical analysis reveals three different zones: *lamina densa*, *lamina rara (sive lucida)*, *lamina densa*. It is well known that the connective tissue matrix is composed of collagens, proteoglycans and glycoproteins, which influence the differentiation of the cells, their morphogenesis and the tendency to proliferation. MARTINEZ-HERNANDEZ and AMENTA (1983) have divided the substances composing the basal membrane into those which are *intrinsic* (collagen type IV, laminin, heparan sulfate proteoglycan, entactin) and those which are *extrinsic* (fibronectin and type V collagen).

2 Methodology of Morphological Analysis of the Basal Membrane

2.1 Histochemical Methods

For the detection of basal lamina in light microscopy, several histochemical techniques can be applied (VACCA 1985). These techniques are based partly on the classical *PAS method*. Lillie's PAS Allochrome method can be used for the distinction of reticulum fibers and basal lamina. The basal lamina can be visualized by the periodic acid-sodium bisulfite-resorcin fuchsin technique (VACCA 1985). *Reticulum fibers* and the *basal lamina* are decorated by Wilder silver method, whereas Weigert's haematoxylin-picro-sirius red stain labels collagen fibers, reticulum fibers and basement membrane (VACCA 1985). Other techniques have been developed for the analysis of the intercellular matrix.

The advantage of these techniques, especially that of PAS staining, is their application in daily routine work (FLOTTE et al. 1980). They allow detection of the structure of the basal membrane which may be sufficient for practical diagnostic application. For detailed questions, however, other methods have to be applied.

In *electron microscopical histochemistry*, the basal lamina is demonstrated by uranium and lead staining, and by the use of ruthenium red.

2.2 Immunohistochemistry

The principles of *immunohistochemistry* have been reviewed in many good monographs where details of techniques and protocols can be found (e.g. ELIAS 1982; BULLOCK and PETRUCZ 1982, 1983; TAYLOR 1986). Currently the triple layer PAP technique, the avidin-biotin technique and the alkaline phosphatase technique are used, parallel to the still valuable technique of immunofluorescence. The important advantage of immunohistochemical investigation is an exact localization of virtually any substance in the basal membrane, provided there is the availability of appropriate antibodies.

Experimentally (and diagnostically in the analysis of autoaggressive diseases) human antibodies against reticulin fibers and against basal membrane substances can be applied (Goodpasture's syndrome and others) (ELIAS 1982).

In most investigations, experimentally produced antibodies against basal membrane substances, either polyclonal or monoclonal, have been used.

Antibodies against collagen IV (SCHUPPAN et al. 1980; FOIDART and YAAR 1981; KUEHN et al. 1981; WEBER et al. 1984a, b; MARTIN et al. 1985), against laminin (TIMPL et al. 1979; FOIDART et al. 1980; ENGEL et al. 1981; ALBRECHTSEN et al. 1981; TIMPL et al. 1982) and against fibronectin (LINDER et al. 1985; ENGVALL et al. 1978; KUUSELA et al. 1978; NERI et al. 1981; RUOSLAHTI et al. 1980, 1981, 1982; RUOSLAHTI 1984) have been used.

2.3 Immunoelectronmicroscopy

The classical analysis of the basal membrane has been done using conventional electron microscopy (e.g. BRIGGAMAN and WHEELER 1975; VRACKO 1974, 1982). Further information is provided by the combination of electron microscopy and immunohistochemistry. Immunoelectronmicroscopy follows the same principles as light microscopical immunohistochemical methods. In practice, many problems of appropriate preservation and appropriate reaction of the antibodies exist (e.g. TOKUYASU 1980; WILLINGHAM 1980; POLAK and VAN NOORDEN 1983). Since information on electronmicroscopy is provided by differences in electron density, the labelling is generally done by means of electron dense particles, such as ferritin, colloidal gold or others.

SANO et al. (1981) analysed collagen type IV in bovine lung parenchyma by an immunoelectron-microscopical approach. FOIDART et al. (1980) studied the distribution of laminin with immunoelectronmicroscopical methods. FURCHT et al. (1978a) analysed the localization of fibronectin on the surface of myoblasts by lightmicroscopic and electronmicroscopic methods. HEDMAN (1980) presented an immunoelectronmicroscopical study of fibronectin. Some authors have analysed elastin at the ultrastructural level (DAMIANO et al. 1978, 1981, 1984; FUKUDA et al. 1984).

3 Overview of Basal Membrane and Its Associated Substances

3.1 Collagen Types

To date, about 10 different collagens have been identified (BORNSTEIN and SAGE 1980; GAY and MILLER 1983; MARTIN et al. 1985).

The *general structural principle* of the *collagens* is a composition from three polypeptide chains called alpha chains. The different *subtypes* of the collagens are characterized by the different aminosequences of the alpha chains. The chains are alpha helical, are characterized by the amino acid sequence glycine-X-Y and contain hydroxyproline or hydroxylysine as a major component.

Type I, II and III belong to the interstitial collagens (MARTIN et al. 1985). The extracellular collagens are identified as type I and III. Around the mesenchymal cells collagen type V is identified. This type is associated with the pericellular exocytoskeleton and collagen type IV (GAY and MILLER 1983). These collagens are associated with other proteins/glycoproteins, such as *fibronectin*, *chondroitin sulfate*, and *heparan sulfate*, *elastin* (RUOSLAHTI et al. 1981; URRY 1983; IOZZO 1984).

Collagen type III, originally detected in the fetal skin, is present in a variety of tissues like type I collagen. It is thought to comprise the reticulin fibers detected by histochemical methods (MARTIN et al. 1985).

The aggregation of *collagen type IV* forms a stable network for the basement membrane where other components are included (TIMPL et al. 1981). Type IV collagen is regularly and extensively localized in the *basal membranes* (MARTINEZ-HERNANDEZ et al. 1981a, b). *Collagen type IV* has an alpha helical structure which is interrupted by protein sequences which are different from Glycine-X-Y. It has relatively large amounts of 3-hydroxyproline and is richer in carbohydrate side chains than the interstitial collagens. Collagen type IV has, as a special feature, the 7S region. This is the cross-linking site for four collagen type IV molecules. All antibodies against collagen type IV decorate exclusively the basal membrane, the labelling being localized in the lamina rara and densa on the ultrastructural level (ROLL et al. 1980; MARTINEZ-HERNANDEZ et al. 1981a, b; SANO et al. 1981; FARQUHAR et al. 1982).

Collagen type V, a stromal and pericellular collagen, belongs to the *extrinsic components* of the basal membrane (MARTINEZ-HERNANDEZ and AMENTA 1983). It is related to collagen IV due to its amino acid content and chemical characteristics (CHUNG et al. 1976). Collagen V has been found in the basal membrane of the glomerulus, in other basal membranes it was not found (GAY et al. 1981; SANO et al. 1981; MARTINEZ-HERNANDEZ et al. 1982). Collagen type V is now accepted as being present in the basal membrane of some vessels and in the stroma surrounding collagen type I bundles (MARTINEZ-HERNANDEZ and AMENTA 1983). Type V collagen is increased in desmoplastic reactions of carcinomas (BARSKY et al. 1982).

3.2 Laminin

Laminin is one of the best characterized *non-collagenous* glycoproteins of the basal membranes which are associated with collagen type IV (TIMPL et al. 1979, 1982). Laminin has a cross like shape with one long and two short arms (ENGEL et al. 1981; TIMPL et al. 1982). The amino acid sequence of laminin is different from that of fibronectin (TIMPL et al. 1982).

Using antibodies against laminin, one finds a specific staining of the *basal lamina*, the reaction being more intense in the lamina rara (FARQUHAR et al. 1982; FOIDART et al. 1980; MADRI et al. 1980; MARTINEZ-HERNANDEZ et al. 1981a, b, 1982). Laminin is strongly involved in *cell adhesion* and *attachment* (TERRANOVA et al. 1980).

3.3 Fibronectin

Fibronectin (for review of the biochemical aspects: RUOSLAHTI et al. 1981; HYNES and YAMADA 1982) is a *dimeric protein* with two polypeptide chains (molecular weight 220,000 Dalton). The fibronectins from different sources, one of which is *fibroblasts*, are chemically closely related and cannot be distinguished on the immunological level. Fibronectin being highly conserved during evolution, antibodies against this protein display an extensive interspecies cross reactivity. Fibronectins interact with many other macromolecules (collagens, fibrin, actin, heparin, DNA, hyaluronic acid, cell surfaces and bacteria). The interaction with collagens interferes with the basal membrane and fibronectin, although not specifically restricted to the basal membrane, may be regarded as an important factor (*extrinsic factor* according to MARTINEZ-HERNANDEZ and AMENTA 1983; see also COURTOY et al. 1980; D'ARDENNE et al. 1983a, b; D'ARDENNE and MCGEE 1984).

The biological activities of fibronectin are characterized as *mediation* of cell *adhesion* and *spreading*, promoting of cell *motility*, *prevention of fusion* of myoblasts and differentiation of chondrocytes, non-specific *opsonin* in plasma (KESKI-OJA et al. 1980; RUOSLAHTI et al. 1981; TARIN et al. 1982; TAKASHIMA and GRINNELL 1984). Fibronectin is produced by various cell types (*epithelial cells*: liver cells, kidney cells, cells of the gut, cells of the breast and of the amniotic membranes; *mesenchymal cells*: fibroblasts, myoblasts, chondrocytes, Schwann cells, endothelial cells, melanoma cells, macrophages and astroglial cells; see also HEDMAN et al. 1978; RUOSLAHTI 1981; HANTAI et al. 1983). Fibronectin is distributed in three different compartments: intracellular, at the cell surface and in the extracellular matrix (FURCHT et al. 1978a, b).

The *tissue distribution of fibronectin* follows the distribution of loose connective tissue, fibronectin being augmented in the limiting membranes such as basement membranes (MOSHER et al. 1977; MADRI et al. 1980; FOIDART and YAAR 1981; LAURIE et al. 1982). Fibronectin is located at the *interface* of the *basal membrane* and the abutting cells (RUOSLAHTI et al. 1981). As it plays a role in cell attachment and orientation of cells, fibronectin decreases towards the tips of the colonic epithelium, thus allowing a shedding of epithelial cells (QUARONI et al. 1978).

Apart from its importance in *neoplastic development*, fibronectin plays an interesting role in other pathological conditions. It is increased in sclerodermia and other diseases of the *skin* (COOPER et al. 1979; FYRAND 1980), in diseases of the *kidney* (e.g. diabetic nephropathy, glomerulonephritis, transplanted kidneys) and in *diabetes mellitus* (SCHEINMAN et al. 1978; WEISS et al. 1979; RUOSLAHTI 1981).

Lack of fibronectin, commonly reported for transformed cells in vitro and in some neoplastic lesions (see below), is not always due to the fact that malignant cells do not produce fibronectin, but that it seems to be released into the culture medium and is not found on the cell surface (RUOSLAHTI 1981).

The lack of fibronectin may be one of the features that identify cells with a capacity to metastasize.

3.4 Others

3.4.1 *Entactin*

Entactin is a highly sulfated protein with a molecular weight of approximately 150,000 Dalton. It is distinct from laminin. Although it has been localized in the rat kidney by immunoelectronmicroscopy, its definite localization in the basal membrane has still to be elucidated (MARTINEZ-HERNANDEZ and AMENTA 1983). Its distribution seems to be closely related to that of laminin, but on the immunoelectronmicroscopical level, the basal membranes may differ in their content and distribution of laminin and entactin (MCCARTHY et al. 1985).

3.4.2 *Heparan Sulfate*

Heparan sulfate proteoglycan is another component of the basal membrane which is dealt with in another chapter of this volume (IOZZO, Chapter 9). In immunohistochemical studies, the labelling of heparan sulfate seems to follow that of laminin and collagen type IV (BIREMBAUT et al. 1985).

3.4.3 *Bullous Pemphigoid Antigen*

The basal membrane antigen "*bullous pemphigoid antigen*" is characterized as a protein of 220,000 Dalton which is distinct from laminin and collagen IV (STANLEY et al. 1982a, b). It is found in the lamina lucida of the basal membrane zone, and in the basal membrane of squamous epithelia (WEBER et al. 1984a, b).

New antigens in basal membrane (e.g. nidogen) have been reported by TIMPL et al. (1983).

4 Change of Basal Membrane Associated Substances During Neoplastic Disorders

4.1 General Rules

The changes of the structure and synthesis of the basal membrane can be considered on the following levels:

4.1.1 *Synthesis of Basal Membrane Associated Material by Neoplastic Cells*

The substance which has especially been analysed with respect to the production site is *fibronectin*. Fibronectin has been thought to interfere with the *metastatic behaviour* of tumor cells (NERI et al. 1981). Primary carcinomas display a dispersed pattern of fibronectin which is also present in the surrounding tissue (STAMPFER et al. 1981). MARTIN et al. (1984) found a reciprocal expression of laminin and fibronectin receptors in the different tumor cells.

On the level of *collagen*, a difference between the different subtypes and neoplastic growth has not been shown (GAY and GAY 1985), although DAVID and VAN DEN BERGHE (1983) report the production of less sulfated proteoglycans by transformed mammary cells. KAO et al. (1984) showed that the matrix of human breast tumor cells is mitogenic for fibroblasts.

4.1.2 *Synthesis of Basal Membrane Associated Substances by Surrounding Tissues as a Distinct Response of the Organism*

Some human tumors are accompanied by a fibrous response. This reaction, often designated as scirrhous or desmoplastic, is well documented in mammary carcinomas (GAY and RHODES 1986). BARSKY et al. (1982) demonstrated that in the desmoplastic part of human mammary carcinoma collagen type V is augmented. Although former papers suggested tumor cells as the source of the desmoplastic material, it now generally accepted that cells of the surrounding stroma, especially the myofibroblasts (SEEMAYER et al. 1980), are the source (BARSKY et al. 1984). It is collagen type V which is synthesized by fibroblasts (GAY and GAY 1985).

4.1.3 *Degradation of Basal Membrane Substances by Tumor Cells*

In normal tissue and benign lesions, the basal membrane is generally intact as has been shown on the *electron microscopical level* (OZZELO 1959; FISHER 1976; STEGNER 1986) and by *immunohistochemistry* (GAY and GAY 1985). In preneoplastic conditions, disturbances of the basal membrane substances may be observed. In true invasive carcinomas, parts of the basal membrane are destroyed. This may be due to the influence of proteolytic enzymes (LIOTTA 1982; LIOTTA et al. 1983; LIOTTA 1984). Besides this "active" principle, destruction of the basal membrane may follow the increased pressure of proliferating cells (BARSKY et al. 1983; LIOTTA et al. 1983).

4.2 Changes in Tumors

An overview over the distribution of basal membrane substances is given in Table 1.

Table 1. Distribution pattern of basal membrane associated substances in neoplastic diseases. (Modified after BIREMBAUT et al. 1985)

Tumor	Intrinsic components (collagen type IV, laminin, heparansulfate)
Preinvasive carcinomas	Basal membrane labelling with interruptions
Invasive carcinomas	Irregular staining around well-differentiated (glandular) structures and adjacent to the basal cells of solid/epidermoid carcinomas
Sarcomas	
Leiomyosarcoma	Irregular pericellular staining in well differentiated areas
Neurofibrosarcoma	
Liposarcoma	
Malignant fibrous histiocytoma	Vascular basal membrane labelling
Osteosarcoma	No pericellular staining
Malignant hemangiopericytoma	
undifferentiated sarcomas	

Table 1 (continued)

Tumor	Extrinsic components (fibronectin)
Preinvasive carcinomas	Basal membrane labelling with interruptions, present in the <i>stroma</i>
Invasive carcinomas	present in the <i>stroma</i> intensely in <i>desmoplastic reaction</i>
Sarcomas	
Leiomyosarcoma	irregular pericellular labelling
Neurofibrosarcoma	
Liposarcoma	
Malignant fibrous histiocytoma	irregular peri- and intercellular labelling
Osteosarcoma	and vascular basal membrane labelling
Malignant hemangiopericytoma	
undifferentiated sarcomas	

4.2.1 Skin and Mucosa (Figs. 1, 2)

The changes in the multilayered epithelium of skin and mucosa are similar with respect to the basal membrane. In invasive and metastatic squamous carcinomas of the head and neck, collagen type IV displayed focal thickening, reduplication, aggregation, attenuation and segmental defects (CAM et al. 1984; CARTER et al. 1985; VISSER et al. 1986). Focal interruptions of laminin and collagen type IV in the basal membranes are found in *dysplasias of the laryngeal mucosa* (VISSER et al. 1986). However, intact basal membrane and reduplications may be found in some squamous cell carcinomas of the skin by the analysis of collagen type IV (GUSTERSON et al. 1984; GUSTERSON et al. 1986). A production of collagen type IV is found in tumors with a higher degree of differentiation (GUSTERSON et al. 1986).

In *solid basal cell carcinoma* the cells still appear to preserve their characteristic production of basal membrane material, but seem to have lost the ability of polar distribution of this material (WEBER et al. 1982).

In *cylindromas* of the skin, collagen type IV, laminin and bullous pemphigoid antigen were localized in the PAS positive hyaline zone, the staining was confined to a zone encircling the tumor nests (WICK and TIMPL 1980; WEBER et al. 1984a, b; KALLIOINEN et al. 1984; KALLIOINEN 1985). Immunoelectronmicroscopic observations revealed a positive staining for collagen type IV and laminin throughout the basal membrane material of the tumor (KALLIOINEN 1985). Fibronectin was uniformly distributed over the tumor stroma and at the periphery of the cell islands (WEBER et al. 1984a, b). Eccrine spiradenomas produce an excessive amount of basal membrane associated material, whereas the basal membrane is absent in many areas of trichofolliculomas and related tumors (KALLIOINEN et al. 1984). Malignant tumors of the skin (hidradenocarcinomas, sebaceous carcinomas) display narrow strips between infiltrating tumor cell clusters (KALLIOINEN et al. 1984).

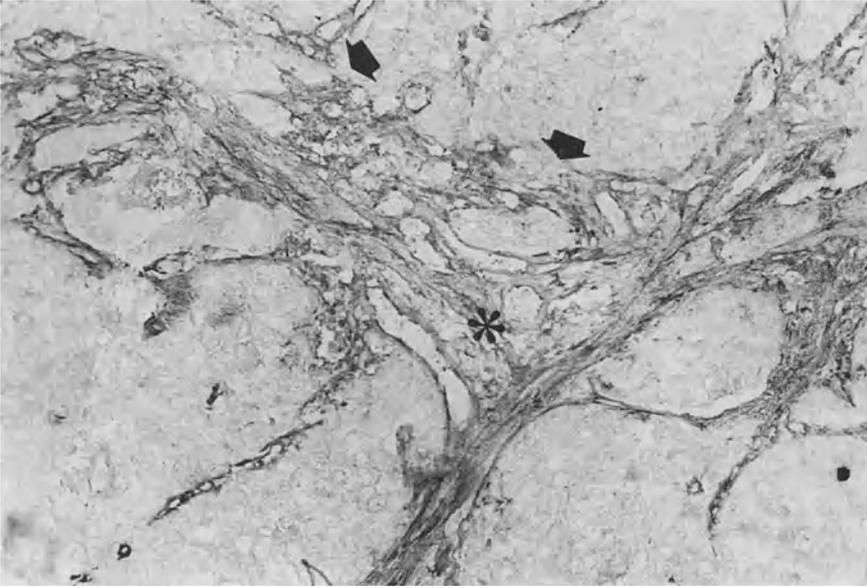


Fig. 1. Fibronectin in stroma of invasive squamous cell carcinoma. Intense staining in the stromal part (*asterisk*). Disrupted fibronectin staining in the part of the basal membrane (*arrows*). Cryostate section. $\times 300$

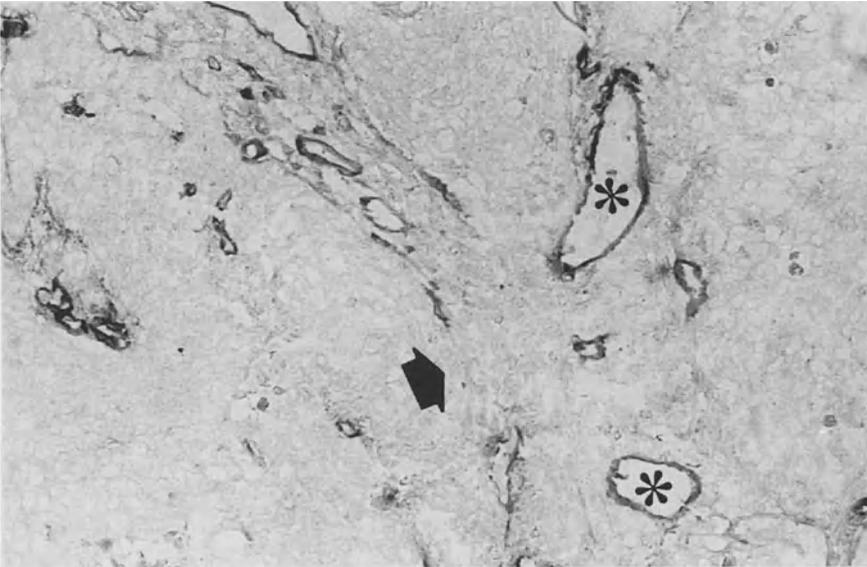


Fig. 2. Collagen type IV staining in invasive squamous cell carcinoma (identical cases as in Fig. 1). Intact basal membrane around the blood vessels (*asterisk*). Disrupted basal membrane in the infiltrating part of the tumor (*arrow*). Cryostate section. $\times 300$

4.2.2 Mammary Carcinoma

In agreement with ultrastructural observations (OZZELLO 1959; FISHER 1976) laminin and collagen IV in *mammary carcinomas* are generally reduced (PITELKA et al. 1980; ALBRECHTSEN et al. 1981; GAY and GAY 1985; REMBERGER and NERLICH 1985; SCHAUER et al. 1985). The stages of neoplastic transformation of human breast tissue can be monitored by dissolution of basement membrane components (SIEGAL et al. 1981). Mouse mammary epithelial cells synthesize an undersulfated basement membrane proteoglycan (DAVID and VAN DEN BERGHE 1983). The synthesis of basement proteins is enhanced during the differentiation of rat mammary tumor epithelial cells (WARBURTON et al. 1982a, b).

The source of the substances appearing during the desmoplastic reaction in mammary carcinomas is the (myo)fibroblast (BARSKY et al. 1984). Collagen type V (BARSKY et al. 1982) and fibronectin (STAMPFER et al. 1981) are augmented in the desmoplastic stromal reaction of mammary carcinomas (CASELITZ et al. 1985a, b) and is found in the stromal part of medullary carcinomas in an irregular pattern (LABAT-ROBERT et al. 1981). In culture, fibronectin is reduced in malignant human mammary epithelial cells (YANG et al. 1980).

Myoepithelial cells are closely related to the basal membrane. The disturbances of the basal membrane during neoplastic invasion is accompanied by both an interruption of the myoepithelial cell layer and the staining for laminin and collagen IV (GUSTERSON et al. 1982; WARBURTON et al. 1982a, b). Myoepithelial cells have a close relationship to basal membrane associated substances, collagen type IV, laminin and, in an indirect way, to fibronectin (GUSTERSON et al. 1982).

4.2.3 Salivary Gland Tumors (Figs. 3, 4, 5)

In *pleomorphic adenomas* (Figs. 3, 4, 5) a considerable amount of basal membrane associated material, collagen type IV and laminin, is localized in the stroma and around the tumor cell groups (D'ARDENNE et al. 1983a, b; CASELITZ 1984; TOTO and HSU 1985). The labelling for fibronectin is especially intense at the border of cell groups and duct-like structures (CASELITZ 1984). At the morphological level it cannot be decided whether fibronectin is "epithelial" or "mesenchymal" by origin (D'ARDENNE et al. 1983a, b). A possible source of part of the basal membrane associated substances is the *myoepithelial cell* (for observations in Sjögren's disease: PALMER 1986). The staining of the basal membrane substances seems to be parallel to, but not always identical with, the staining for elastic fibers. In *adenoid cystic carcinomas* laminin and collagen were found in the stromal trabeculae and pseudocysts, fibronectin displayed a similar, but sometimes more diffuse, staining pattern (D'ARDENNE et al. 1983a, b; CASELITZ 1984; CASELITZ et al. 1986; TOIDA et al. 1984; for mammary adenoid cystic carcinoma: WELLS et al. 1986). Elastic fibers were found in the neighbourhood (AZZOPARDI and ZAYID 1971; ADKINS and DALEY 1974; DAVID and BUCHNER 1980).

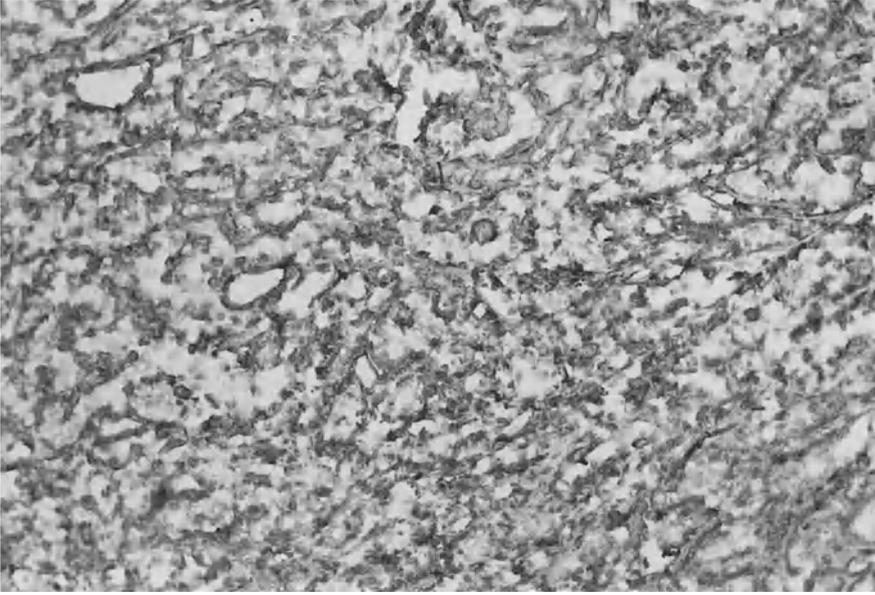


Fig. 3. Collagen type IV in pleomorphic adenoma. Strong labelling in stromal (mucoid) part of pleomorphic adenoma. Cryostate section. $\times 300$

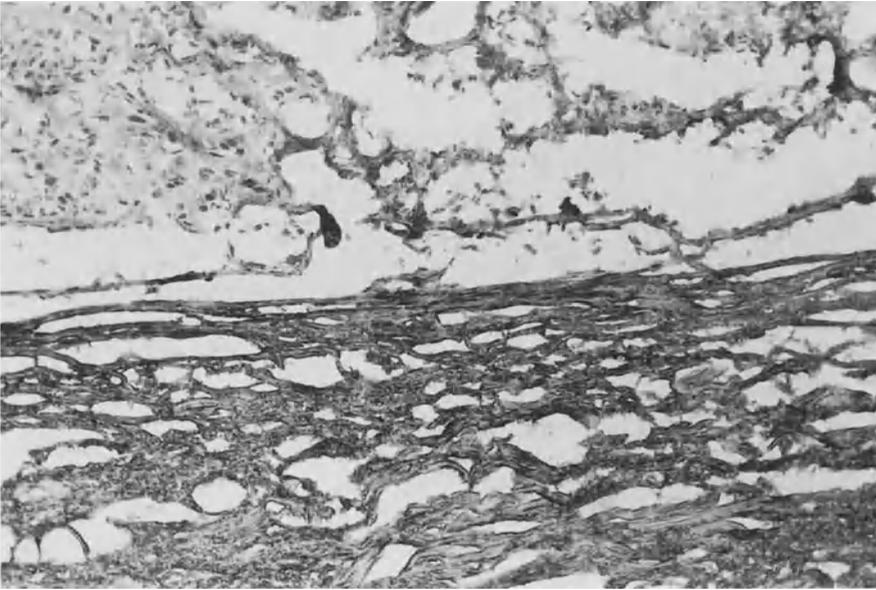


Fig. 4. Collagen type III in capsule of pleomorphic adenoma. Only weak staining in the tumor stroma. Cryostate section. $\times 300$. (We gratefully acknowledge the kind gift of collagen type III antibody from Dr. SCHUPPAN, Berlin)

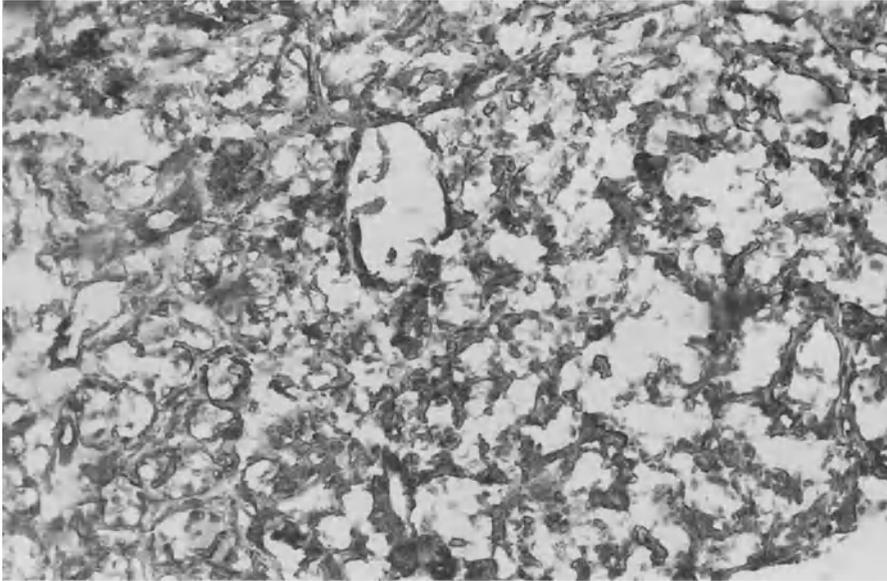


Fig. 5. Fibronectin in pleomorphic adenoma. Strong labelling in stromal (mucoid) part of pleomorphic adenoma. Cryostate section. $\times 300$

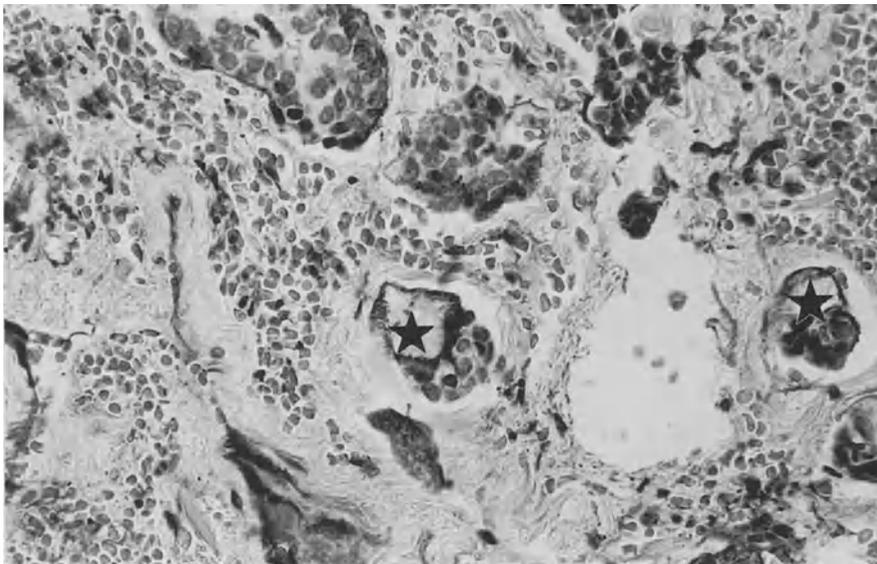


Fig. 6. Collagen type IV in adenoid cystic carcinoma. Strong staining of the material (*asterisks*) in the pseudocysts (especially at the border of the lining myoepithelial-like cells). Cryostate section. $\times 300$

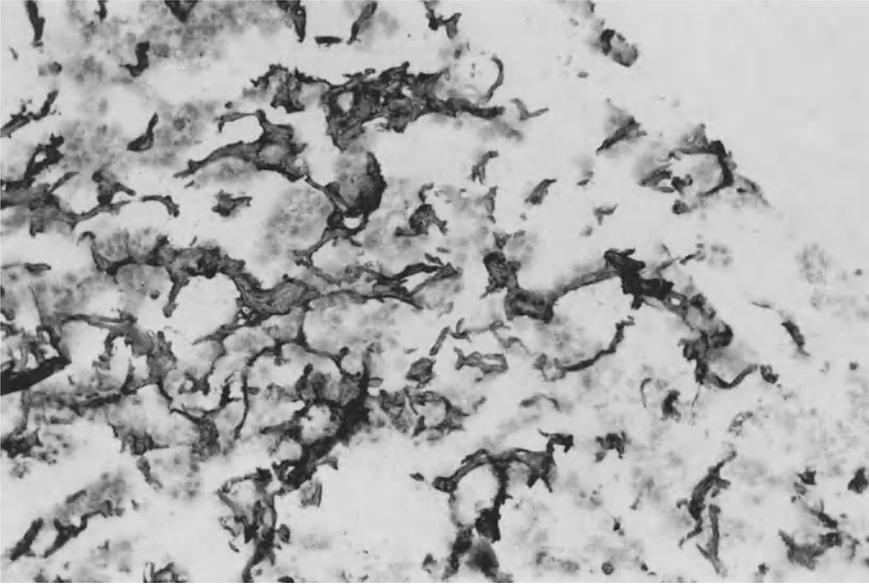


Fig. 7. Fibronectin in acinic cell tumor. Although the tumor is highly differentiated, there is a clear interruption of the fibronectin staining in the region of the basal membrane. Cryostate section. $\times 300$. (We gratefully acknowledge the help of Dr. WUSTROW, Kiel, in providing fresh material)

In other types of malignant carcinomas of the salivary glands (Fig. 7), the staining for laminin, collagen type IV, and for fibronectin is generally interrupted in the region of the basal membrane (CASELITZ 1984; D'ARDENNE et al. 1983 a, b).

4.2.4 Pancreas

The staining for *laminin* and *collagen type IV* is intact in pancreatitis. There is a loss of these substances in pancreatic carcinoma (BARKSKY et al. 1983; HAGLUND et al. 1984).

4.2.5 Stomach

The so-called "linitis plastica" is accompanied by an intense reaction for *fibronectin* and *collagen type III* (D'ARDENNE et al. 1983 a, b). These observations are similar to that of the desmoplastic carcinoma in mammary carcinoma.

4.2.6 Colon

Laminin was present in an *uninterrupted* linear staining in the basal membranes of normal, hyperplastic and adenomatous colorectal mucosa (KELLOKUMPU et al. 1985). The *early invasion* of colonic carcinomas was generally accompanied by a desmoplastic reaction with intense, but somewhat irregular staining for fibronectin and collagen IV (D'ARDENNE et al. 1983a, b). The basal lamina and the staining for *laminin* was disrupted in poorly differentiated colonic adenocarcinomas (BURTIN et al. 1982), whereas slight staining was still observed in the better differentiated tumors (KELLOKUMPU et al. 1985).

4.2.7 Lung and Bronchial Tree

In intraepithelial lesions, the basal membrane shows a disrupted staining for *collagen type IV* and *laminin*. These substances, although irregularly distributed in true invasive lesions, may be found around some well-differentiated solid tumor groups. *Fibronectin* is augmented in the stroma and displays no special augmentation in the region of the basal membrane (LABAT-ROBERT et al. 1981; BIREMBAUT et al. 1985).

4.2.8 Urinary Tract

Premalignant lesions display an intact basal membrane. Staining for *collagen type IV* and *laminin* is interrupted in invasive urothelial carcinomas. *Fibronectin* is still preserved in the stroma of the urinary bladder, but it is lost around infiltrating cells (LABAT-ROBERT et al. 1981).

4.2.9 Prostate Gland

Fibronectin is found pericellularly on the surface of smooth muscle cells in normal and hyperplastic prostate glands (LABAT-ROBERT et al. 1981). Although still present at the surfaces of the muscle cells, fibronectin is lost around infiltrating malignant cells (LABAT-ROBERT et al. 1981).

4.2.10 Female Genital Tract

In normal exocervix and most dysplastic and malignant preinvasive lesions, the staining for *laminin* and *collagen type IV* is linear (BIREMBAUT et al. 1985). *Fibronectin* is visualized in the basal membrane of uterine glands and around the cells of the endometrial stroma (LABAT-ROBERT et al. 1981). It is lost at the basal membrane of invasive carcinomas, but may be present in the stroma in large quantities (LABAT-ROBERT et al. 1981).

4.2.11 Endocrine Tissue

BIREMBAUT et al. (1985) and LABAT-ROBERT et al. (1981) report a loss of basal membrane substances in follicular carcinomas of the thyroid gland.

4.2.12 *Connective Tissue*

In *benign proliferations*, laminin, collagen type IV and fibronectin are found in all vascular basal membranes and around all muscular and Schwann cells and adipocytes in a linear pericellular layer.

Staining for laminin and collagen type IV is found around the vessels in malignant mesenchymal tumors. However, there is a decrease in the pericellular staining of muscle cells, Schwann cells and adipocytes (LABAT-ROBERT et al. 1981; BIREMBAUT et al. 1985). Fibronectin, however, persists as intercellular and pericellular material in most sarcomas, especially in cases with fibroblastic differentiation (BIREMBAUT et al. 1985). STENMAN and VAHERI (1981) found single cells in sarcomas which were surrounded by fibronectin. It was abundant in the connective tissue (STENMAN and VAHERI 1981) but LABAT-ROBERT et al. (1981), found a loss of the pericellular labelling and a decreased staining of fibronectin in the extracellular matrix. Biphasic synovial cell sarcoma is reported to exhibit extracellular linear collagen type IV and laminin (BARSKY et al. 1983). In the basal membrane of neurofibromas, collagen type IV and laminin were localized (WEBER et al. 1984a).

5 Conclusion and Outlook

From the viewpoint of diagnostic pathology, *basal membranes* can be regarded as *structural barriers* to the invasion and metastatic procedure of malignant cells. Separating epithelial and other cells from the surrounding stroma, they are obstacles to invasion from tumor cells. For many substances which are directly and indirectly associated with the basal membrane, there is some evidence that the invasive progression of a tumor is accompanied by a loss of these substances.

In *surgical pathology*, antibodies against laminin and collagen type IV turn out to be very specific and valuable if one is interested in studying the structure and integrity of the basal membrane. Consequently, most studies agree that the immunohistochemical labelling for laminin and collagen type IV is intact and linear in normal tissues (LABAT-ROBERT et al. 1981; BARSKY et al. 1983; CARTER et al. 1985; GUSTERSON et al. 1986). As far as can be demonstrated at the light microscopic level, there is a co-distribution of collagen type IV and laminin. In contrast, there is *interruption* of the basal membrane in many invasive tumors, immunohistologically visualized by the destruction of the decoration for collagen type IV and laminin (ALBRECHTSEN et al. 1981; LABAT-ROBERT et al. 1981; BIREMBAUT et al. 1985; GAY and GAY 1985). This destruction has been shown in invasive carcinomas of the skin (GUSTERSON et al. 1986), of the breast (ALBRECHTSEN et al. 1981; GAY and GAY 1985), of the lung (LABAT-ROBERT et al. 1981), of the pancreas (BARSKY et al. 1983; HAGLUND et al. 1984) and of other tissues (BIREMBAUT et al. 1985). Thus, staining for laminin and collagen can be used to analyse the process of invasion.

GUSTERSON et al. (1986), however, observed that in some invasive carcinomas the immunohistological lining for laminin and collagen type IV is intact. This is in particular found in *well differentiated carcinomas*, where some tumor cells seem to preserve the capacity of forming a basal membrane. Therefore, there may be some exceptions to the general rule of interruption of the basal membrane in malignant tumors.

In *pre-malignant lesions*, many observations show disturbances of the basal membrane. This phenomenon has been interpreted as being due to the onset

of infiltration. Other authors report an intact basal membrane in premalignant lesions. The differences may be explained by the different phases of a "pre-malignant" lesion and by the different sensitivity of immunohistochemistry due to technical details like fixation, different antibodies and others.

Fibronectin is generally reduced on the surface of neoplastic cells in comparison with their normal counterparts. Since the reduction at the surface of the cells is not always accompanied by a reduction of the production of fibronectin, its decrease may be due to the influence of *proteases* (HYNES 1981; HYNES and YAMADA 1982; SALO et al. 1982). MCCARTHY et al. (1985) review the literature indicating that there is a general association between the loss in cell surface fibronectin and transformation. The details of this relationship may be more complicated, since many experimental studies have not always demonstrated a clear cut correlation between the metastatic potential of a cell and the lack of cell surface fibronectin. Different mechanisms may lead to the decrease in fibronectin (MCCARTHY et al. 1985). Transformation may initiate a decrease of cell surface associated fibronectin due to the inhibition of production. Alternatively, the cell may degrade or alter this protein and there may be a change in the distribution of the cell surface receptors for fibronectin. Other authors offer the model of an inappropriate secretion of fibronectin to the surface (RUOSLAHTI 1984). From the experimental data, a choice between the different models cannot be made at the moment. Given the function of fibronectin in cell movement, its absence would perhaps explain the increased tendency to movement and invasion by malignant cells. For cell lines a *correlation* between the *decrease* of the cell surface fibronectin and their *tumorigenicity* has been found (KAHN and SHIN 1979).

Tumor cells display a *receptor* for fibronectin and can interact with fibronectin coated surfaces (RUOSLAHTI 1984). The future study of this receptor and its interaction with fibronectin will be of special interest for certain aspects of tumor biology.

In *metastatic processes* (SCHIRRMACHER 1984), attachment of tumor cells to *laminin* may play an important role. Tumor cells with a high affinity for laminin attachment display a higher pulmonary metastatic potential than those tumor cells with a low affinity for laminin (TERRANOVA et al. 1982). Antibodies against laminin can prevent the metastatic behaviour (MCCARTHY et al. 1985). A specific receptor for laminin has been isolated in malignant cells (TERRANOVA et al. 1983). There are some indications of an association between the laminin molecule and the interaction with the contractile cytoskeleton (MCCARTHY et al. 1985).

Future investigations will deal with a number of additional *attachment proteins*, such as gp 140, entactin, epinectin (MCCARTHY et al. 1985) and others.

An interesting aspect is the *interaction* of the *basal membrane* associated substances with the *cytoskeleton* of the cell. Immunohistological and immunoelectronmicroscopic data point to the fact that fibronectin has an influence on binding to the orientation of actin filaments (D'ARDENNE and MCGEE 1984). These studies would be interesting for the interpretation of a number of physiological and pathological aspects of the cell.

Basal membrane associated substances have been analysed in the *serum* of patients with neoplastic diseases (D'ARDENNE and MCGEE 1984). A number

of authors did not find differences in the plasma levels of fibronectin of normal and cancer patients. Intravascular coagulation produced a marked reduction in plasma fibronectin; liver metastasis were accompanied by low levels of fibronectin (D'ARDENNE and MCGEE 1984).

Another point is the differences in the distribution pattern of the various basal membrane substances. It is necessary to establish the precise distribution pattern at the *ultrastructural level*. This would give new answers to several questions. Differences at the conventional immunohistological level should be analysed on the basis of standardized techniques.

Basement membrane loss, however, is not always accompanied with malignancy. Some invasive tumors, especially the well-differentiated ones, exhibit focal extracellular reaction for both collagen type IV and laminin (GUSTERSON et al. 1986). However, basal membrane loss may be observed in some *reparative and inflammatory processes* (e.g. dermatitis herpetiformis).

LIOTTA (1984) stressed not only the importance of the composition of the basal membrane in neoplastic disease, but also the role of certain receptor proteins against the basal membrane substances. Experimental data suggest that there are receptors against this basal membrane component which may be altered in number or degree of occupancy in human neoplastic disease. Breast carcinoma tissue for example, contains a higher number of receptors and unoccupied receptors compared with benign breast tissue. The laminin receptors of normal breast epithelium seems to be polarized at the basal surface. In contrast, in invasive carcinoma cells this receptor may be distributed over the entire surface of the cell. The laminin receptor can stimulate hematogenous metastases.

There is evidence, however, that metastatic cells adhere specifically to basal membranes, essentially through an increase in plasma membrane receptors for laminin (ABRAHAMSON 1986). When attached to basal membranes, these may be destroyed following the influence of proteolytic enzymes. This would permit the neoplastic cells to transmigrate across the altered basal membrane and metastasize. In contrast to malignant tumors, little apparent degradation takes place in benign tumors.

A particular point has to be made about certain special differentiated tumors like *mixed salivary gland tumors* which exhibit a focal augmentation of basal membrane associated material (e.g. laminin and fibronectin as "extrinsic" factors). Perhaps this increase in basal-membrane-like material may be related to the appearance of *myoepithelial cells* (and also to the clinical behavior). Other tumors with myoepithelial like cells display an augmentation of basal membrane material, although arranged in quite a different manner (e.g. pseudocysts in adenoid cystic carcinomas). This special arrangement may be a possible marker for the insidious behaviour of the adenoid cystic carcinoma in the head and neck region.

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Lectins and Blood Group Substances as "Tumor Markers"*

J. CASELITZ

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List of abbreviations

Abbreviation	Name	Abbreviation	Name
BSA I, II	Bandeira (Griffonia simplicifolia I, II)	PHA	Phaseolus vulgaris (red kidney bean)
Con A	Concanavalia A (Canavalia ensiformis)	PNA	Arachis hypogaea (pea nut)
DBA	Dolichos biflorus (horse gram)	PWA	Phytolacca americana (Pokeweed)
HPA	Helix pomatia	PSA	Pisum sativum
LCA	Lens culinaris	RCA	Ricinus communis
LPA	Limulus polyphemus	SBA	Glycine max (Soy bean)
LTA	Lotus tetragonolobus	STA	Solanum tuberosum
MPA	Maclura pomifera	TGP	Tetragonolobus purpurea
PLA	Phaseolus lunatus (lima bean)	UEA	Ulex europaeus
PHA	Phaseolus vulgaris (black kidney bean)	VVA	Vicia villosa
		WGA	Triticum vulgaris (Wheat germ)

1 Introduction

In recent decades, research efforts have been focused on demonstrating changes in cell surface membrane during neoplastic transformation. This emphasis stems from the observation that the cell surface membrane may determine several characteristics of neoplastic cells such as decreased adhesion, loss of contact inhibition, increased growth rate, prolonged survival, increased invasiveness and motility, expression of "new" antigens and escape from immunological surveillance (review for glycolipids and glycoproteins in cancer: EMMELOT 1973; KÖTTGEN et al. 1979; YOGESWARAN 1983; HAKOMORI 1984; BAENZINGER 1986).

A recent advance in the histochemistry of complex carbohydrates resulted from the use of lectins which bind to specific carbohydrates and localize them in tissue sections (RAPIN and BURGER 1974). AB isoantigens are widely distributed in human tissues and loss of AB isoantigen has been shown to be an early marker for neoplastic transformation in some tissues. The relation of ABH blood groups and development has been reviewed by SZULMAN (1980).

Lectins are defined as proteins or glycoproteins of non-immune origin which bind specifically to carbohydrate residues. The term "lectin" is derived from the latin "legere" and refers the specificity to "pick out" special carbohydrate chains (GOLDSTEIN and HAYES 1978; ROTH 1978). Lectins are often, but not exclusively, derived from plants and from animals (invertebrates and in lower vertebrates) (GOLDSTEIN 1981; LEATHEM and ATKINS 1983). The ubiquitous existence of lectins has now been established (LIS and SHARON 1986). More than 100 lectins have been purified. All possess at least two binding sites for carbohydrate chains. Through their specific interaction, they exhibit many important biological functions such as distinguishing human blood groups, preferential agglutination of immature or malignant cells and mitogenic stimulation of lymphocytes.

The extensive literature on lectins has been summarized in several reviews (UHLENBRUCK 1971, 1980, 1981; NICOLSON 1974, 1976, 1978; SHARON and LIS 1975; SCHREVEL et al. 1981; BOG-HANSEN 1982; SCHAUER 1982a, b; BOG-HANSEN and SPENGLER 1983; LEATHEM et al. 1983; BOG-HANSEN and VAN DRIESSCHE 1986; LIS and SHARON 1986).

The lectins may be classified on the basis of their carbohydrate binding specificity (usually agglutination of erythrocytes or precipitation of carbohydrate containing polymers) (GOLDSTEIN 1981; LIS and SHARON 1986).

1. *D-Mannose (D-Glucose) binding lectins:*

Canavalia ensiformis, *Lens culinaris*, *Pisum sativum*, *Vicia faba*.

This group is mitogenic for lymphocytes. According to their amino acid sequences, these lectins seem to be closely related.

2. *N-Acetyl-D-Galactosamine binding lectins:*

Dolichos biflorus, *Phaseolus vulgaris*, Soy bean agglutinin, *Helix pomatia*, *Saphora japonica*.

These lectins bind and agglutinate type A red blood cells. *Phaseolus vulgaris* (lima bean) is a good mitogen.

3. *N-Acetyl-D-Glucosamine binding lectins:*

Wheat germ, *Ulex europaeus* I, *Solanum tuberosum*, *Phytolacca americana* (Poke weed), *Datura stramonium*, *Griffonia* (sive *Bandeiraea*) *simplicifolia* I.

These lectins bind to chitin and are best inhibited by oligomers of N acetyl D glucosamine.

Pokeweed is a potent mitogen for T and B lymphocytes, whereas Wheat germ agglutinin and *Datura stramonium* agglutinin are only weakly mitogenic towards T cells.

4. *D-Galactose binding lectins:*

Abrus precatorius, *Arachis hypogaea* (Pea nut), *Griffonia* (sive *Bandeiraea*) *simplicifolia* I, *Maclura pomifera*, *Ricinus communis* (Castor bean).

The GSA (BSA) and *maclura pomifera* lectins agglutinate type B erythrocytes.

5. *L-fucose binding lectins:*

Lotus tetragonolobus agglutinin, *Ulex europaeus* I, *Anguilla anguilla*.

These non-mitogenic lectins are useful reagents for typing of human O erythrocytes.

6. *Sialic acid binding lectins:*

Sialic acid binding lectins have been isolated from the horseshoe crab (*Limulus polyphemus*) and other types of crabs.

The importance of sialic acid containing glycoconjugates on cell surfaces should make these lectins most interesting for biology and other related scientific disciplines.

7. *Lectins with complex carbohydrate binding sites:*

Phaseolus vulgaris (red kidney bean)

Agaricus bisporus (mushroom)

The lectin of the red kidney bean has a complex binding site.

Neoplastic cell transformation is associated with an *altered carbohydrate composition* of plasma membranes (NICOLSON 1976; WARREN et al. 1978). Demonstration of altered lectin binding by tumor cells has been used to predict the likelihood of recurrence, the invasive potential of the neoplasm and to distinguish benign from malignant tumors. Lectin binding by tumor cells in tissue sections has been used for the study of various normal, dysplastic, and neoplastic tissues. The aim of this presentation is to elucidate the use of lectins as *morphological tumor markers*, and the literature reviewed is influenced by this purpose. Other aspects of lectins are dealt with in some excellent reviews (UHLENBRUCK 1971; NICOLSON 1974; SHARON and LIS 1975; ROTH 1978; SCHREVEL et al. 1981; SCHAUER 1982a, b; LEATHEM et al. 1983; LIS and SHARON 1986).

Among the large number of lectins, only some of these substances have been analysed on human materials. Those which are commonly used are: Peanut agglutinin (*Arachis hypogaea*), Wheatgerm agglutinin (*Tritium vulgaris*), *Ulex europaeus*, *Helix pomatia*, *Dolichos biflorus*, *Ricinus communis*, *Concanavalia ensiformis* (COOPER 1984). The relation of some lectins to the blood group substances is given in Table 1 (for details: NICOLSON 1974; ROTH 1978; SCHREVEL et al. 1981; LEATHEM et al. 1983; LIS and SHARON 1986).

Table 1. Lectins and their relation to blood group substances

Blood group agglutinated	Origin of lectin
A	Phaseolus limensis Vicia cracca Dolichos biflorus Crotalaria aegyptiaca
B	Bandeiraea simplicifolia I
A + B	Sophora japonica Calpurinea aurea
H	Cytisus sessiliformis Laburnum alpinum Lotus tetragonolobus Ulex europaeus
M	Iris amara
N	Vivia graminea Bauhinia purpurea
Disaccharide of the Thomsen-Friedenreich antigen	Arachis hypopeae

2 Methodology of Detection of Carbohydrate Residues in the Cellular Membrane

With regard to application in cytochemistry (SCHREVEL et al. 1979), the methods for the detection of cell glycoconjugates are summarized by SCHREVEL et al. (1981) and CULLING and REID (1982). Four main methods can be distinguished:

1. Detection of *anionic sites*
 - a) Use of cationic dyes (Alcian red, toluidine blue, ruthenium red)
 - b) Use of cationic colloid (colloidal iron)
 - c) Use of cationized ferritin or proteins.
2. Detection of *alcohol residues*
3. Detection of *carbohydrate groups by use of lectins*
4. Application of *antibodies* (esp. monoclonal antibodies).

Methods 1 and 2 have to be regarded as techniques of broad specificity, whereas 3 and 4 are techniques of rather narrow specificity.

This paper concentrates on the application of lectins.

2.1 Affinity Histochemistry by Lectins

Lectins have been used in connection with various techniques for the identification of specific carbohydrate residues in tissue sections (ROTH and BINDER 1978; SCHULTE and SPICER 1983a, b, c, 1984; ALROY et al. 1984; HORISBERGER 1984; ROTH and TAATJES 1985). Although for some purposes frozen sections may be preferred (study of glycolipid receptors), formaldehyde fixed and paraffin embedded material is suitable for the investigation of many lectin binding sites.

At the *light microscopic level*, lectins have been applied with the following modifications (LEATHEM and ATKINS 1983):

1. *conjugated lectins* (coupled to fluorochromes or chromagens) have been used directly on tissue sections, e.g. lectin-fluorescein isothiocyanate (FITC) and lectin-horseradish peroxidase,
2. in *indirect immunological techniques*, lectins can be used in modifications like lectin-antilectin-FITC (or horseradish peroxidase).
3. lectins can be used as the primary step in the *peroxidase-antiperoxidase technique* (PAP technique)
4. after being coupled to *biotin*, lectins can be used in the avidin-biotin-peroxidase technique.

Although most papers present data for lectin use in light microscopy, elegant studies are being presented on the *ultrastructural level*, by using lectins coupled to gold (ROTH et al. 1983; ROTH and TAATJES 1985; ROTH 1986). The principles of the reactions are analogous to those in light microscopy.

Other applications are the use of lectin binding in *radioautography*, which may be quantified (SAVERIANO et al. 1981).

2.2 Methods for the Detection of Blood Group Antigens

Several methods can be applied to detect carbohydrate residues and blood group antigens on the cellular membrane (DENK et al. 1974; WEINSTEIN et al. 1981). The red cell adherence technique has been applied on histological sections for some time (WEINSTEIN et al. 1981). Immunohistochemical techniques may be used as well: PAP technique (WEINSTEIN et al. 1981) and the avidin-biotin-system on the basis of polyclonal and monoclonal antibodies. An overview of the distribution of blood group substances is given in Tables 2 and 3.

Table 2. ABH antigens in human tumors. (According to WEINSTEIN et al. 1981)

Organ	Tumor
Blood vessels	Angioma Angiosarcoma
Breast	Adenocarcinoma
Colon	Adenocarcinoma
Fallopian tube	Adenocarcinoma
Larynx	Squamous carcinoma
Lung	Squamous carcinoma adenocarcinoma
Oral epithelium	Squamous carcinoma
Ovary	Cystadenoma Cystadenocarcinoma
Pancreas	Adenocarcinoma
Prostate	Adenocarcinoma
Skin	Epidermal appendage tumours
Stomach	Adenocarcinoma
Urinary bladder	Transitional carcinoma Inverted papilloma
Uterine cervix	Squamous carcinoma

Table 3. Presence of Isoantigens A, B, H in normal adult human tissues. (According to WEINSTEIN et al. 1981)

Location	Positive	Negative
Adrenal		Cortex, medulla
Blood	Erythrocytes	White blood cells
Blood vessels	Endothelial cells	
Breast	Glands Ducts	
Bronchus	Pseudostratified columnar epithelia Mucous glands	Serous glands
Central nervous system		Nerve cells Glial cells
Esophagus	Squamous epithelium	Basal cell layer
Duodenum	Epithelial cells Brunner's glands	
Colon	Proximal colon	Distal colon
Gallbladder	Columnar epithelia	
Endometrium	Epithelia (proliferative and secretory)	
Exocervix	Squamous epithelia	
Fallopian tube	Columnar epithelia	
Kidney	Glomeruli collecting tubes convoluted tubes	
Larynx	Squamous epithelia mucous glands	Serous glands
Liver	Kupffer cells	Hepatocytes
Lung	Mucous glands	Serous glands, Hepatocytes
Muscles		smooth and striated muscle cells
Ovary	Cyst fluid	
Pancreas	exocrine glands	islets of Langerhans
Pituitary	colloid pars intermedia	
Placenta	Trophoblast (partly)	Trophoblast Cytotrophoblast
Prostate	Glands	
Epidermis	Horny layer of squamous epithelium Sweat glands	Cells of basal and Malphigian layers Sebaceous glands
Small intestine	Columnar epithelia Brunner's glands Paneth cells	
Stomach	Columnar epithelia	
Testis		Tubules
Tongue	Squamous epithelia Mucous glands	Basal layer
Trachea	Pseudostratified Columnar epithelia Mucous glands	serous glands
Urinary system	Urinary bladder, ureter	
Vagina	Squamous epithelia mucous glands	basal layer
Connective tissue		Collagen, bone, cartilage

3 Distribution Pattern in Human Organs and Tumors

3.1 Epidermis and Mucosa

The human epidermis (and related structures, like oral mucosa) represents a model of differentiation, the expression of which may be analysed on fundamental and applied levels (ELIAS et al. 1983; RITTMAN and MACKENZIE 1983). REANO et al. (1982) have divided the lectins into 3 groups:

Group 1 (*BSA I, DBA, Limulus polyphemus, TGP, UEA, WGA*) did not stain the normal epidermis. *Group 2* (*Canavalia ensiformis, Maclura pomifera, Phaseolus vulgaris, RCA 1*) exhibited a "pemphigus-like" intercellular labelling of the whole epidermis with exception of the stratum corneum. *Group 3* (*PNA, Glycine max, Helix pomatia, Sophora japonica*) displayed a selective intercellular labelling of the stratum spinosum and the stratum granulosum. In psoriatic epidermis, not only the basal layer, but also cells from the adjacent lower stratum spinosum were found to be negative for the lectins in group 3 (REANO et al. 1982).

PNA (Peanut Agglutinin) (Fig. 1)

PNA binding sites in human epidermis have been analysed by REANO et al. (1982), HYUN et al. (1984a–d) and LÖNING (1984). Its expression in squamous cell carcinomas is variable. Most Merkel cell tumors were positive for PNA (PAJOR et al. 1986), this lectin being considered as a valuable marker for this tumor.

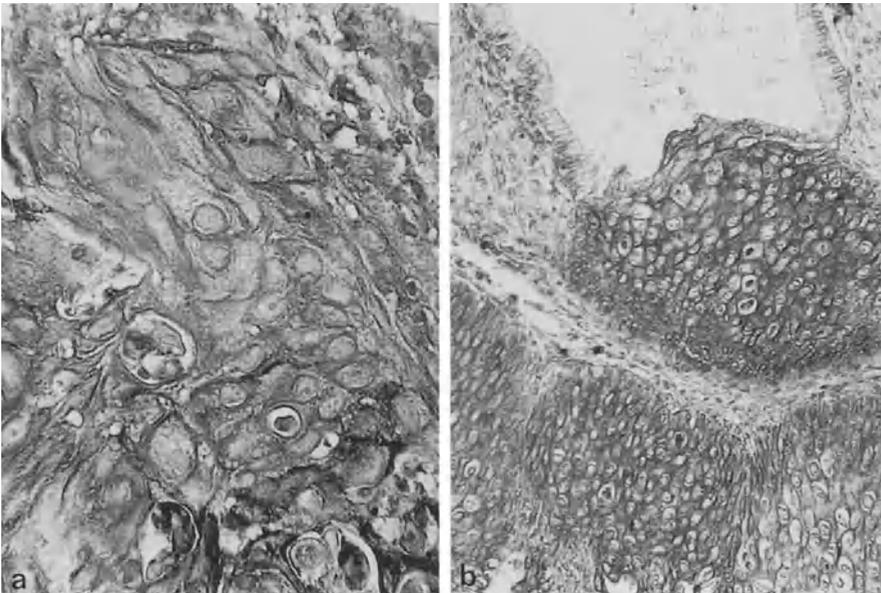


Fig. 1. a PNA binding sites in squamous cell carcinoma of the skin. Intense staining of tumor cells after sialidase (neuraminidase) digestion. $\times 300$. **b** PNA binding sites in carcinoma in situ of the cervix. Intense staining of tumor cells after sialidase (neuraminidase) digestion. $\times 300$

WGA (Wheat Germ Agglutinin)

WGA receptors do not play an important role in carcinomas of the epidermis and the mucosa (REANO et al. 1982, HYUN et al. 1984a–d). WGA receptors display a zonal arrangement in the oral mucosa (HOSAKA et al. 1985).

UEA (Ulex Europaeus Agglutinin)

UEA1 receptors do not play an important role in carcinomas of the epidermis and oral mucosa (REANO et al. 1982, HOSAKA et al. 1985).

HPA (Helix Pomatia Agglutinin)

The binding of HPA to squamous carcinomas of the epidermis and the mucosa is variable (LÖNING 1984).

Basal cell carcinomas are negative for DBA, whereas normal basal cells are in about 50% positive (COGGI et al. 1983). RCA 1 binding was slightly decreased in *actinic keratoses* and cases of *Bowen's disease*. In epidermal outgrowths from the edges of healing wound and in squamous and basal cell carcinomas, the binding of RCA 1 was diminished (GRAEM 1982). Receptors in oral carcinomas could not be demonstrated in invading islands of the tumors (DABELSTEEN and MACKENZIE 1978; DABELSTEEN et al. 1978). There is a shift in carbohydrate composition in *inflammatory* and, more important, in *neoplastic* lesions (DABELSTEEN and MACKENZIE 1978).

Other lectins (Con A, SBA, BSA, SJA) have been analysed in the human epidermis and oral mucosa and its neoplastic counterparts (REANO et al. 1982; GRAEM and DABELSTEEN 1984; HOSAKA et al. 1985).

Blood Group Antigens

Loss of epithelial blood group substance A in oral premalignant lesions and oral carcinomas have been described by DABELSTEEN and PINDBORG (1973), by DABELSTEEN et al. (1975) and by DABELSTEEN and DANIELS (1983). Similar data are reported by LIU et al. (1974) using the red cell adherence technique, and by PRENDERGAST et al. (1968). LIN et al. (1977) compared isoantigens A, B and H in normal mucosa and in carcinoma of the larynx. DABELSTEEN et al. (1974) analysed blood group substance A in carcinoma of the larynx.

ENGLAND et al. (1979) analysed blood group antigens A, B, H in normal skin and in tumors of the epidermal appendages.

3.2 Salivary Glands*PNA (Peanut Agglutinin)* (Fig. 2)

PNA receptors are found in many salivary gland tumours, especially in the malignant ones like adenoid cystic carcinomas (CASELITZ et al. 1986), but also in pleomorphic adenomas (CASELITZ and SEIFERT 1981; CASELITZ 1987). DALEY et al. (1985) report a weak PNA staining in various salivary gland tumours, mostly apically localized, in the ductal cells or in the cystic areas.

WGA (Wheat Germ Agglutinin) (Fig. 3)

Corresponding to the normal tissue, WGA generally decorates the luminal membrane of the duct cells in the tubular parts of pleomorphic adenomas and ade-

noid cystic carcinomas (CASELITZ et al. 1986; CASELITZ 1987). DALEY et al. (1985) report a staining of the stromal part of these tumours. Mucous cells and cystic contents in mucoepidermoid tumours stained intensely, whereas intermediate and squamous cells were negative (DALEY et al. 1985).

Con A binding was present in the cytoplasm of many non-ductal cells of *pleomorphic adenomas* and in most of the basaloid cells of *adenoid cystic carcinoma* (DALEY et al. 1985). Some myoepithelial-like cells were positive. Most mucous cells in mucoepidermoid tumours and most cells in acinic cell carcinomas displayed a moderate to intense binding (DALEY et al. 1985). DALEY et al. (1985) report a weak, erratic binding of *SBA* in the apical glycocalyx of ductal cells, mucous cells and cystic contents.

TGP (*Tetragonolobus purpurea*) binding features were similar to those of WGA (DALEY et al. 1985).

Blood Group Antigens

Blood group substances are found in the saliva. They can be identified in salivary gland tissue and salivary gland tumours (HAMPER et al. 1986). In normal tissue the expression of blood group substances corresponded to the serological blood group. Blood group substance H was found in almost every gland. In some glands, Le-b was rather selective for mucous acini. In salivary gland tumours, blood group substances were expressed in benign and highly differentiated tumors (HAMPER et al. 1986). WOLTERING et al. (1983) analysed parotid neoplasms by the specific red cell adherence technique. There was no reaction with red cell agglutination with the ductal epithelium. In malignant tumors there seems to be a loss of ABH substances as shown by this technique.

3.3 Mammary Gland and Mammary Carcinoma

PNA (Peanut Agglutinin) (Fig. 4)

Various lectins have been analysed in the mammary gland. The most important one is the *Peanut Agglutinin (PNA)* which detects the disaccharide Gal-GalNac which plays a leading role in the Thomsen-Friedenreich-phenomenon. It is related to the MN blood group system (SPRINGER et al. 1974, 1975; KLEIN et al. 1979; FRANKLIN 1983; HAGEMAN et al. 1983).

Despite the fact that PNA may be helpful in detecting carcinoma cells in lymph node metastases (STEGNER et al. 1981) its primary importance lies in the observation that the expression of lectin binding sites are demonstrated in those carcinomas which are positive for *estrogen receptors* (LEATHEM et al. 1983; BÖCKER et al. 1984, 1985; KLEIN and WÜRZ 1985; TOTOVIC et al. 1985; VIERBUCHEN et al. 1985; REMMELE et al. 1986). Although these studies confirm the general correlation of PNA binding sites and estrogen receptors, the practical problem often lies in finding the right "cut off level" for a given method and collective.

The general association of high differentiation and the expression of PNA receptors has been demonstrated by many groups (HOWARD and BATSAKIS 1980; HOWARD et al. 1981). Well differentiated mammary carcinomas will localize

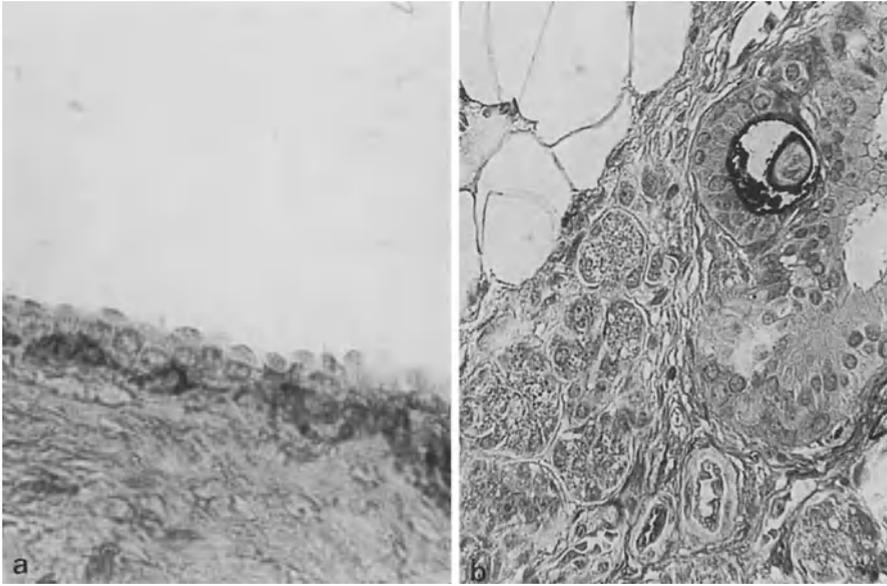


Fig. 2. a PNA binding sites in excretory duct of human parotid gland. Staining of basal cell fraction after sialidase digestion. $\times 300$. **b** PNA binding sites in duct cells of human parotid gland. Staining of the apical part of the cells. $\times 300$

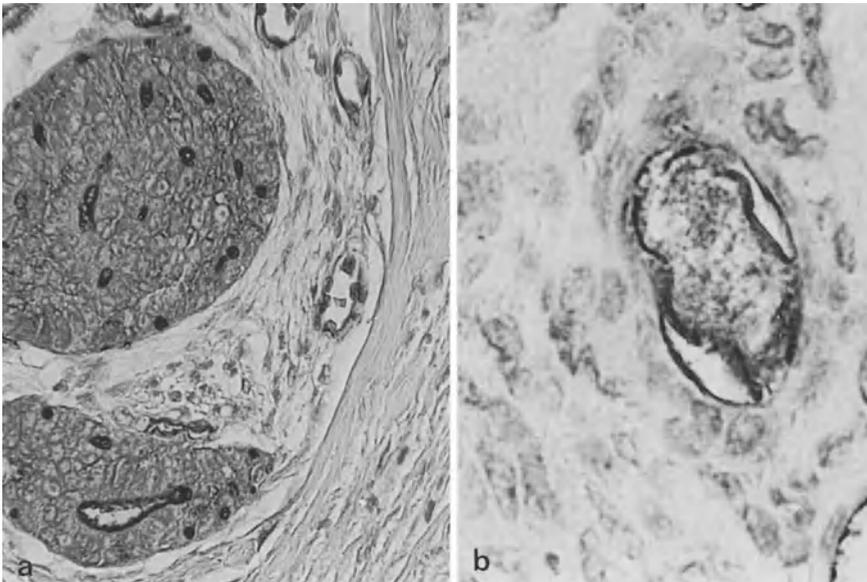


Fig. 3. a PNA binding sites in tubular part of adenoid cystic carcinoma. Staining of the luminal surface of the tumor cells. $\times 300$. **b** WGA binding sites in pleomorphic adenoma. Strong staining of the apical part in the tubules. $\times 300$

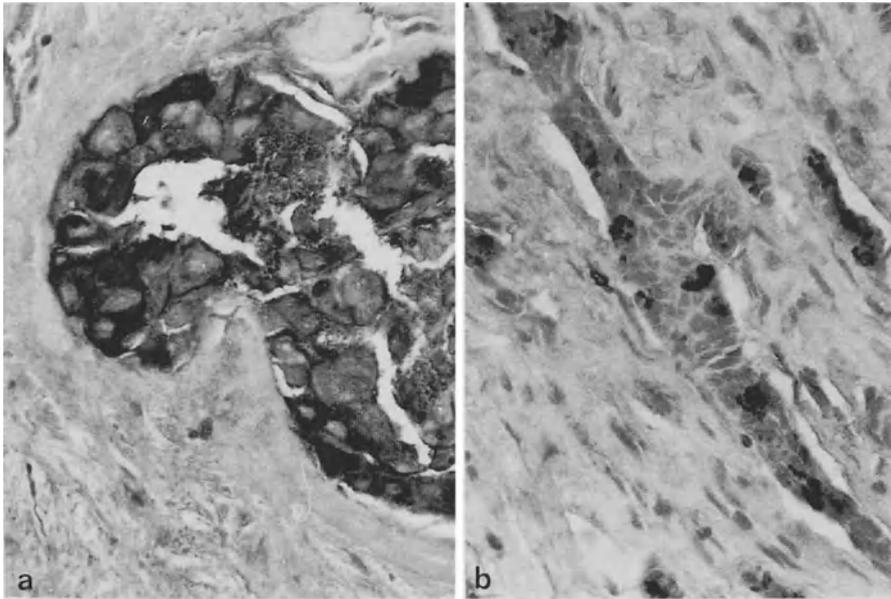


Fig. 4. **a** PNA binding sites (after sialidase digestion) in solid mammary carcinoma (NOS). Strong cytoplasmic staining of the tumor cells. $\times 300$. **b** PNA binding sites (after sialidase digestion) in infiltrating lobular carcinoma of the mammary gland. Vacuolar and globular staining of the cytoplasm of the tumor cells. $\times 300$

PNA to the apical portion of epithelial cells. Solid or poorly differentiated carcinomas of the breast will either localize PNA intracytoplasmically or not at all. KAHN and BAUMAL (1985) studied the differences in lectin binding in tissue sections of human and murine malignant tumors and their metastases. There was a weaker staining for PNA (without sialidase (neuraminidase) digestion) in those primary breast carcinomas which had no metastases. The staining was strong in metastatic tumours of breast carcinomas.

New aspects are introduced by the combination of affinity histochemistry with PNA and cytophotometry, and by the use of cell culture systems (DAXENBICHLER et al. 1985; ZIPPEL et al. 1985).

WGA (Wheat Germ Agglutinin) (Fig. 5)

In cancer cells there is generally a shift from luminal towards cytoplasmic staining (LEATHEM et al. 1983; FRANKLIN 1983; WALKER 1984).

Primary and metastatic breast carcinoma cells are equally positive for WGA (KAHN and BAUMAL 1985). WGA binding to tumour cells suggests that a large proportion of the exposed carbohydrates in mammary cancers consists of *N-acetyl-D-glucosamine (GlucNAc) and/or sialic acid*. There are some data suggesting that the carbohydrate stained in the current study may include CEA, which can be extracted from breast carcinomas by Sepharose columns coupled to WGA (SANTEN et al. 1980).

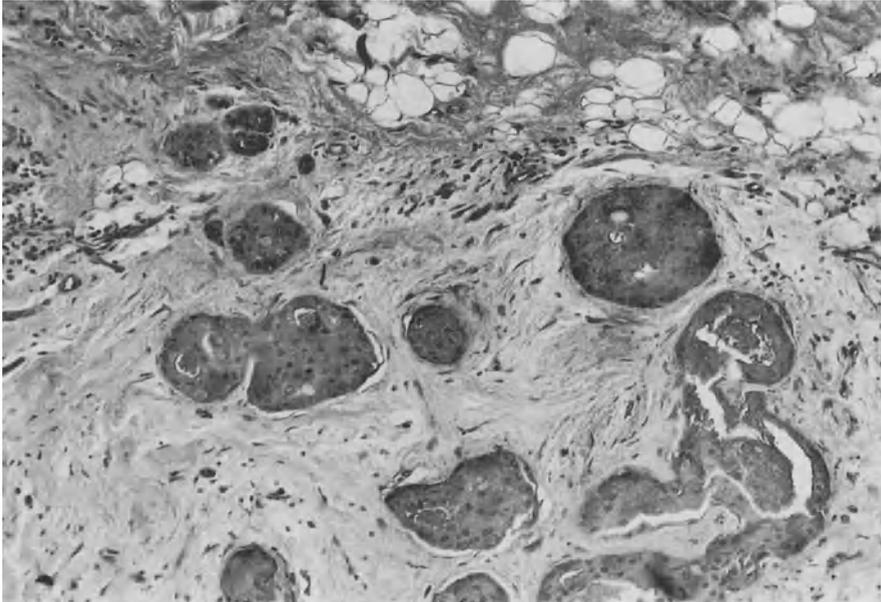


Fig. 5. WGA binding sites (after sialidase digestion) in solid carcinoma of the mammary gland. Strong cytoplasmic staining of the tumor cells. $\times 150$

HPA (Helix Pomatia Agglutinin)

Helix pomatia agglutinin was observed in the luminal surface and the secretion of normal cell in the mammary gland (LEATHEM and ATKINS 1983). It is found in a subpopulation of cancer cells (LEATHEM et al. 1983).

DBA, RCA, Con A, PWA, LTA and SBA receptors have been studied in human mammary cancer cells (FRANKLIN 1983; LEATHEM and ATKINS 1983; LEATHEM et al. 1983; WALKER 1985b).

Blood Group Antigens

AB isoantigen expression in normal breast tissue was found to be largely confined to the mammary duct system, whereas loss of AB isoantigen expression was a feature of intraductal carcinoma (STRAUCHEN et al. 1980). Varying degrees of isoantigen loss were observed in *proliferative lesions* associated with fibrocystic disease. In contrast to other tissues, loss of AB isoantigen expression in proliferative breast lesions is not necessarily evidence of malignancy. GUPTA and SCHUSTER (1973) compared isoantigens A, B, H in benign and malignant lesions of the breast.

3.4 Lung and Bronchial Tree

PNA receptors have been analysed in *cell lines* of small cell lung cancer and squamous cell carcinoma (RAEDLER et al. 1985). 80 to 100% of the cells in

the cell line were positive for PNA (RAEDLER et al. 1985). MIYAUCHI et al. (1982a) have identified PNA receptors in human lung carcinomas. RAEDLER et al. (1985) analysed the distribution of WGA, UEA, HPA, and RCA1 in cell cultures of human lung cancers (squamous cell carcinoma and oat cell carcinoma). 80 to 100% of the tumor cells were positive for RCA1. Cell cultures from *oat cell carcinomas* were negative for *Con A* (RAEDLER et al. 1985). *LCA* and *SBA* receptors were present in cell lines of human oat cell carcinoma and squamous cell carcinoma of the lung (RAEDLER et al. 1985). The same group analysed various other lectins in human lung carcinomas.

Blood Group Antigens

DAVIDSOHN and NI (1969) found a loss of AB isoantigens in human lung carcinomas.

3.5 Gastrointestinal Tract

3.5.1 Stomach

PNA (Peanut Agglutinin)

There was some change in the PNA binding pattern during metaplasia of the gastric mucosa (COGGI et al. 1983; KUHLMANN et al. 1983; BUR and FRANKLIN 1985). *Carcinomas* of the intestine and diffuse types contained both *PNA-positive* and *PNA-negative* cells of which PNA-positive cells occurred preferentially in the tumour periphery adjacent to normal mucosa (KUHLMANN et al. 1983). Extracellular mucus and cell borders are intensely stained by *PNA* after sialidase (neuraminidase) treatment, 50% of the cases being positive even without enzyme digestion (COGGI et al. 1983).

UEA (Ulex Europaeus Agglutinin)

Gastric carcinomas revealed a heterogenous picture for UEA with positive and negative tumor cells (KUHLMANN et al. 1983; BUR and FRANKLIN 1985).

Some normal cells of the gastric mucosa and some tumors were positive for *WGA* (FISCHER et al. 1983, 1984). About half of carcinomas of the intestinal type were positive for *DBA* (KUHLMANN et al. 1983). Tumour cells were sometimes strongly stained for *BSA* (BUR and FRANKLIN 1985), and occasionally for *SBA* receptors (COGGI et al. 1983; BUR and FRANKLIN 1985).

Blood Group Antigens

Generally, there is a loss of blood group substances in human gastric cancer (COWAN 1962; DAVIDSOHN et al. 1971a; DENK et al. 1974; PICARD et al. 1978; FINAN et al. 1983), but not in the surrounding normal mucosa (FINAN et al. 1983). SLOCOMBE et al. (1980) studied the distribution of blood group antigens in gastric carcinoma by the immunoperoxidase technique. No clear relationship between the tumor differentiation and preservation of blood group substances was found. In some, even well differentiated, tumours there was a loss of blood

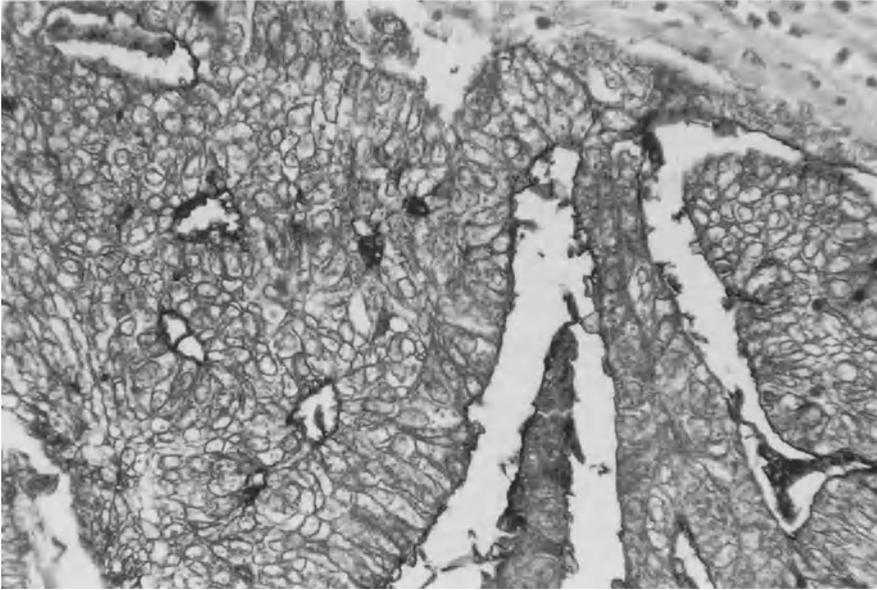


Fig. 6. WGA binding sites in adenocarcinoma of the stomach (intestinal type). $\times 300$

group substances (A, B, H), obviously due to the fact that in these cells there is a blocking of the normal elongation of the carbohydrate portion of blood group substances (SLOCOMBE et al. 1980). Gastric carcinomas presented a loss of ABH antigens and an augmentation of Lewis a and I (Ma) antigens, the neoplastic tissue showing a marked heterogeneity of expression. Ca 19-9, corresponding to sialylated Lewis a antigen, was detected in 62% of gastric carcinomas (HIROHASHI et al. 1984). ERNST et al. (1984) detected Lewis a antigen in gastric carcinoma (80%) more often than in normal gastric mucosa (40%).

3.5.2 Colon

PNA (Peanut Agglutinin) (Fig. 7)

In adenomas, PNA reacts with the supranuclear region of adenoma cells that produces mucin goblets. On contrast, neoplastic non-mucinous columnar cells react with PNA in the apical and glycocalyx region (COOPER and REUTER 1983). PNA receptors are found in the apical and glycocalyx region of the colon *cancer cells*, indicating that the synthesis of MN blood group related glycoproteins is disturbed in these cells (BOLAND et al. 1982; COOPER 1982; HSU and RAINE 1982; COGGI et al. 1983). There was a strong labelling of the mucin in the lumen (BOLAND et al. 1982), especially in colloid carcinoma.

UEA (Ulex Europaeus Agglutinin)

The distribution pattern of UEA receptors in the normal human gastrointestinal tract, including the colon, and in adenomas and carcinomas of the colon have

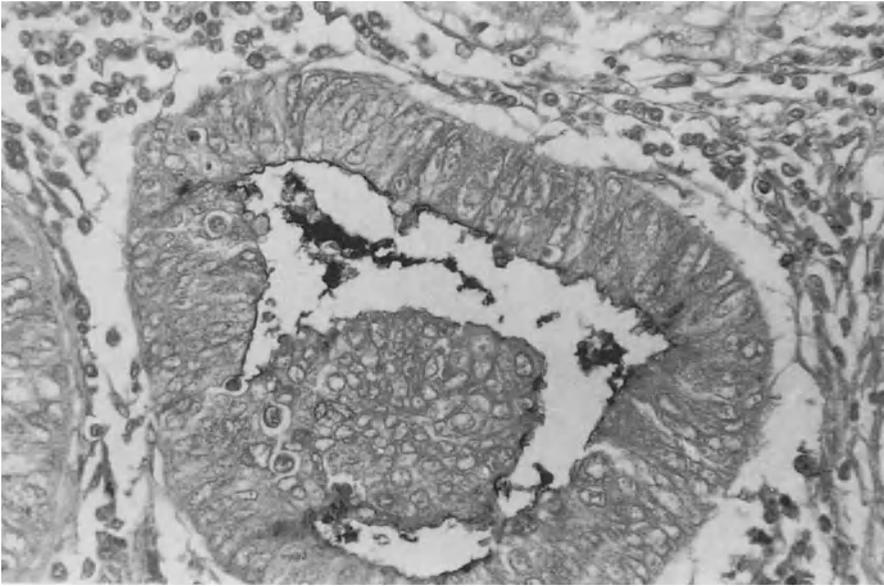


Fig. 7. PNA binding sites (after sialidase digestion) in adenocarcinoma of the colon. $\times 300$

been analysed by YONEZAWA et al. (1982) and by biochemical methods (MATSUSHITA et al. 1985).

In contrast to the finding in the normal mucosa (COGGI et al. 1983), adenomas of the colonic mucosa are strongly positive for *DBA* (COGGI et al. 1983). Although dysplastic epithelium is positive for *DBA* 1, this pattern seems to be lost in parts of the true carcinoma. *RCA* (*RCA* 1) receptors are found in epithelial cells of the large bowel (FISCHER et al. 1983). In contrast to the findings with *HPA*, *Con A* receptors are strongly expressed in epithelial cells of the large bowel (FISCHER et al. 1983). According to NAKAYAMA et al. (1985) the *Bandeiraea (Groffonia) simplicifolia agglutinin II* displayed a strong affinity to colonic carcinoma cells. The staining was enhanced after prior digestion with neuraminidase.

Blood Group Substances

DAVIDSOHN et al. (1966) studied the distribution of A, B, H substances in gastrointestinal carcinoma. ABDEFATTAH-GAD and DENK (1980) analysed epithelial blood group antigens in human carcinoma of the distal colon. Other groups analysed the glycoproteins metabolism in inflammatory and neoplastic diseases of the human colon (KIM et al. 1974; KIM and ISAACS 1975). DENK et al. (1975) found epithelial blood group antigens in the secretory part of goblet cells with slight-to-moderate atypia and in the whole cell in cases of pronounced dedifferentiation.

3.6 Liver

Lectin receptors have been especially analysed in rodent tissue. CHERQUI et al. (1982) report the use of lectins for the characterization of the insulin receptor

glycosidic moiety in rat adipocytes and hepatocytes. VIRTANEN et al. (1978) analysed the distribution of various lectins (Con A, WGA, RCA, SBA, Lotus tetragonolus agglutinin) in rat liver cell surface fractions.

3.7 Pancreas

PNA (Peanut Agglutinin)

Binding of *PNA* is only inconsistently found in normal tissue. It is obvious in hyperplastic conditions and ductal carcinoma (KLÖPPEL et al. 1984; KLÖPPEL and FITZGERALD 1986).

Hyperplastic lesions are strongly positive and duct carcinomas are inconsistently positive for *WGA* (KLÖPPEL and FITZGERALD 1986). *UEA* receptors are prominent in hyperplastic lesions of the pancreatic gland (KLÖPPEL and FITZGERALD 1986). *HPA* receptors are present in the normal pancreatic gland and in pancreatic carcinomas (KLÖPPEL et al. 1984; KLÖPPEL and FITZGERALD 1986), they are augmented in hyperplastic conditions of the pancreatic tissue (KLÖPPEL and FITZGERALD 1986).

Other Lectins

DBA receptors present a similar distribution pattern as *WGA* receptors (KLÖPPEL et al. 1984). Normal pancreatic tissue is negative, hyperplastic lesions are strongly positive, pancreatic carcinomas are positive (KLÖPPEL and FITZGERALD 1986).

Blood Group Antigens

DAVIDSOHN et al. (1971b) analysed the distribution of isoantigens A, B, H in carcinoma of the pancreas.

3.8 Urogenital Tract

3.8.1 *Kidney*

PNA receptors were localized in nephrogenic adenomas (DEVINE et al. 1984). The presence of *PNA* in *renal carcinoma* is analysed by LEATHEM and ATKINS (1983). Nephroblastomas are positive for *PNA* (WICK et al. 1986).

There is a granular staining with renal oncocytomas with *WGA* (HOLTHÖFER 1986). Epithelial parts of nephroblastomas are positive for *WGA* (WICK et al. 1986). *UEA* only binds to vascular endothelium (ULRICH et al. 1985). In some tumors, the degree of vascularization can be evaluated by use of this lectin. *HPA* is present in *renal carcinomas* (RAEDLER et al. 1982).

Other Lectins

Renal oncocytomas are reported to have a high affinity for *DBA* (ULRICH et al. 1985). In renal oncocytomas, there is a granular staining of the cytoplasm (HOLTHÖFER 1986). Nephroblastomas are positive for *SBA* (WICK et al. 1986).

Blood Group Antigens

PEDAL and BAEDEKER (1985) analysed the distribution of A, B, H and secretor substances in kidneys. They found a reliable staining with the PAP technique, by using monoclonal antibodies.

3.8.2 Urinary Bladder

The expression and the loss of blood group antigens in bladder antigens have been analysed by several groups (for review: WEINSTEIN et al. 1981; ALROY et al. 1982a, b; LIMAS and LANGE 1982). Expression of the ABH blood group antigens has been related to tumor behaviour and patient's prognosis in transitional-cell carcinoma of the urinary bladder.

PNA (*Peanut Agglutinin*)

COON et al. (1982) found that the Thomsen-Friedenreich Antigen as detected by the PNA affinity histochemistry is absent in *normal bladder mucosa* and can only be detected after digestion with sialidase (neuraminidase). Three types of PNA binding in urothelial carcinomas were established (COOPER 1984, see also Table 4):

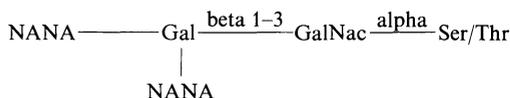
1. *cryptic (+) neoplasms* with PNA binding sites only after neuraminidase digestion
2. *Thomsen-Friedenreich antigen positive carcinoma cells* with PNA binding sites prior to neuraminidase digestion
3. *cryptic (-) neoplasms* that were negative for PNA binding sites even after neuraminidase treatment.

The worst prognosis is found in the group which were cryptic T antigen negative (COON et al. 1982). In this context, the expression of the Thomsen-Friedenreich antigen (without and after neuraminidase treatment) may be a prognostically useful immunohistochemical marker, together with the expression of ABH substances.

A new approach by dual parameter flow cytometric measurements of DNA content and PNA binding is presented by ØRNTOFT et al. (1986). The cellular lectin binding seems to be correlated to the degree of aneuploidy.

Table 4. Diagrammatic presentation of probable structures corresponding to different T-antigen reactivities of bladder carcinomas (similar findings in other tumours)

Cryptic T antigen



T Antigen



Cryptic antigen undetectable



WGA (Wheat Germ Agglutinin) and UEA (Ulex Europaeus Agglutinin)

WGA receptors which are present in normal urothelial epithelium are lost in urinary bladder carcinomas (NIELSEN et al. 1986). A positive staining with *UEA* was observed in most blood group 0 patients with a urothelial carcinoma (79%) and in most tumours of patients with blood group A or B (90%). In blood group A patients, NIELSEN et al. (1986) found staining of the cytoplasm of the luminal urothelial cells. In situ dedifferentiation was associated with staining of cytoplasm of all layers, the truly invasive cells being negative for *UEA* (NIELSEN et al. 1986).

Blood Group Antigens

The behaviour of blood group substances in carcinomas of the urothelium is well documented in many papers (among others: KAY and WALLACE 1961; KATO 1977; JOHNSON and LAMM 1980; LIMAS and LANGE 1982; for review: WEINSTEIN et al. 1981). There is a loss of blood group substances in poorly differentiated carcinomas.

3.8.3 Male Genital Tract

3.8.3.1 Prostate Gland

PNA (Peanut Agglutinin)

A strong expression of PNA receptors is observed in many prostatic carcinomas. The pattern is not changed after antiandrogenic treatment (WERNERT and DHOM 1986).

Other Lectins

UCCI et al. (1983) and LEATHEM and ATKINS (1983) analysed the distribution of lectins in the normal prostate gland and in carcinomas of this organ.

Blood Group Antigens

GUPTA et al. (1973) report a loss of AB isoantigens in prostate cancer.

3.8.3.2 Testis

In *human germ cell tumors*, most embryonal carcinomas in adult testis were positive, all yolk sac tumours were positive for *PNA*. Most seminomas and choriocarcinomas were negative (TESHIMA et al. 1984). Squamous epithelium in teratomas were positive for *PNA* (TESHIMA et al. 1984). *WGA* receptors were found in *spermatozoa* of man (DE CEREZO et al. 1982). In seminomas, all tumor cells were positive for this lectin (LEE et al. 1985). All yolk sac tumours and 7 of 16 embryonal carcinomas were positive for *UEA* whereas seminomas and choriocarcinomas were negative (TESHIMA et al. 1984). Squamous epithelium in teratomas was positive for *UEA* (TESHIMA et al. 1984).

Other Lectins

LEE et al. (1985) and TESHIMA et al. (1984) report the different binding patterns of lectins in the various tumors of the testis.

3.9 Female Genital Tract

3.9.1 Endometrium

More than 70% of endometrial carcinomas displayed a binding for PNA with different structural patterns (ZIPPEL et al. 1986). *WGA receptors* were localized at the cell luminal border of endometrial carcinoma (KLUSKENS et al. 1984). *Con A* binding is increased in cells in endometrial carcinoma (KLUSKENS et al. 1984). LEE and DAMJANOV (1985) analysed different lectin binding pattern with respect to the functional status of the endometrium.

3.9.2 Ovary

There are few reports on the distribution of lectin binding sites in ovarian tissue. DIETEL et al. (1986) found that the staining pattern of various lectins (*PNA, WGA, UEA I, RCA, Con A, SBA*) was not correlated with the cellular differentiation of normal tissue and various neoplastic disorders.

Concerning the *blood group substances*, blood group substances of the ABO system and the Lewis system were found in normal tissues and benign tumors. In borderline lesions the over all expression of blood group substances was reduced by 15%. The reduction of blood group substance expression was even more distinct malignant ovarian tumors (DIETEL et al. 1986).

3.9.3 Placenta

PNA and WGA binding sites are found at the basement membrane of the human placenta (LEE and DAMJANOV 1984). *UEA 1* receptors have not been found in the trophoblast (LEE and DAMJANOV 1984). LEE and DAMJANOV (1984) analysed various lectins and their stage related binding to human placental tissues (*HPA, DBA, Con A, BPA, BSA I, BSA II, HAA, LBA, LCA, MPA PHA E, PHA L, RCA 1, RCA 2, SBA, STA, VVA*).

Blood Group Antigens

LOKE and BALLARD (1973) localized blood group A antigens in human trophoblast cells. STEJSKAL et al. (1973) analysed the distribution of isoantigens in the human fetus.

3.10 Haematopoietic and Lymphoreticular System

3.10.1 Haematopoietic and Related System

The different blood group substances can be identified on the surface of red cells, either by the use of polyclonal and monoclonal antibodies or by appropriate lectins (for review: UHLENBRUCK

1971 and others). STODDART et al. (1980) have analysed lectin staining of carbohydrate of haemic cells. BAYER et al. (1976) localized lectin and antibody receptors on erythrocytes.

Untreated erythrocytes do not bind *PNA*. Owing to the removal of terminal sialic acid from its protein (glycophorin), erythrocytes react with *PNA* after digestion with neuraminidase (POSCHMANN et al. 1976 and others). The removal of neuraminic (sialic) acid exposes the normally cryptic T blood group antigen. *WGA* labelling for leucocytes is generally stronger than for erythrocytes and platelets (ZINSMEISTER and ACKERMAN 1983). *RCA* reacted with the plasmalemma and cytoplasm of normal human bone marrow cells and blood cells. There was no detectable difference between normal and leukaemic cells (STODDART et al. 1980). *Con A* reacted with the plasmalemma and cytoplasm of normal human bone marrow cells and blood cells.

3.10.2 Lymphoid Tissue

PNA (Peanut Agglutinin)

Different groups obtained different results by use of *PNA*, probably due to the various techniques of detecting lectin receptors (COOPER 1984). *PNA* has been shown to react with activated T cells (CHERVENAK and COHEN 1982), it binds to follicular center lymphocytes (STRAUCHEN 1985), to human B lymphocytes (COOPER 1984), and reacts with immature human thymocytes (REISNER et al. 1979). Centroblasts and centrocytes are said to react with *PNA* (COGGI et al. 1983). HOWARD and BATSAKIS (1982) found that *PNA* was a marker for tissue histiocytes. There is an intracytoplasmic binding in histiocytes of reactive germinal centers (REE and HSU 1983). Nodular lymphomas display a diffuse staining with *PNA* (REE and HSU 1983). Cells in Burkitt's lymphoma are positive for *PNA* (MIYAUCHI et al. 1982). REE et al. (1983) demonstrated that stromal macrophage-histiocytes were positive in diffuse large cell lymphomas. MÖLLER (1982) found that Hodgkin cells are positive for *PNA*. This kind of reaction may be useful in paraffin embedded material. Hsu et al. (1985) did not find a constant reaction with Hodgkin and Sternberg-Reed cells (cell line studies in Hodgkin's disease: UHLENBRUCK et al. 1986).

WGA binding sites found in macrophages and tumor cells of diffuse large cell lymphoma (REE et al. 1983). STRAUCHEN (1985) has analysed *UEA* receptors in lymphomas. Lymphatic vessels, in contrast to blood vessels, are negative for *UEA* (WALKER 1985a).

Con A binding sites have been identified in lymphoma cells (RAPIN and BURGER 1974) and in stromal macrophages-histiocytes of diffuse large cell lymphoma (REE 1983). STRAUCHEN (1985) reports binding of *Con A* to the follicular center macrophages and a positive population of dendritic cells in follicular lymphomas. *Con A* labelling to stromal histiocytes in paraffin sections may indicate a high risk of relapse in Hodgkin's disease (REE 1986). *SBA* binding sites have been found in lymphoma cells by RAPIN and BURGER (1974). RAEDLER et al. (1986a, b) found that the majority of *SBA* positive cells were T 11 positive and had to be regarded as T cells. The *SBA* positive cells were interpreted as immature.

3.10.3 Blood Vessels and Lymphatic Vessels *UEA (Ulex Europaeus Agglutinin)*

The value of *UEA* as a marker for vascular epithelium has been demonstrated by various papers (YONEZAWA et al. 1982; HOLTHÖFER et al. 1982; BORISCH et al. 1983; MIETTINEN et al. 1983; MÖLLER and LENNERT 1984; WALKER 1985a). Kaposi sarcoma, haemangioma and angiosarcoma are *UEA* 1 and factor VIII positive (ANGERVALL et al. 1985; BENDELAC et al. 1985). Another lectin with fucose

binding, Lotus agglutinin, failed to react with endothelium, suggesting a rather specific type of fucose expression in human endothelial cells (HOLTHÖFER et al. 1982).

Blood Group Antigens

FEIGL et al. (1976) analysed blood group isoantigens in benign and malignant vascular tumors.

3.11 Central Nervous System and Pituitary Gland

PNA (Peanut Agglutinin)

The main targets of PNA binding in the normal central nervous system and pituitary gland are the luminal membrane of the *ependymal cells*, the brush border of the *chorioid plexus* after neuraminidase digestion and a subpopulation of cells in the *anterior pituitary gland*. *Astrocytes* are positive for PNA, as well (SCHWECHHEIMER et al. 1984). MÜLLER et al. 1980 demonstrated receptors for PNA in normal choroid plexus and of plexus papillomas, and draw some implications for the role of Thomsen Friedenreich antigen in bacterial and viral infections of the brain. Nerve fibers, perineurium and blood vessels are negative for PNA (ESTRUCH and DAMJANOV 1986). *Granular cells* and *granular cell tumors* are strongly positive for PNA (SCHWECHHEIMER et al. 1983).

UEA (Ulex Europaeus Agglutinin)

UEA binding sites were only found in *vascular endothelial cells* and in some cells of the pituitary gland (SCHWECHHEIMER et al. 1984). Nerve fibers and perineurium are negative (ESTRUCH and DAMJANOV 1986).

Blood Group Antigens

JELLINGER and DENK (1974) analysed blood group isoantigens in angioblastic meningiomas and haemangioblastomas of the central nervous system. They found that the prominent cell type in haemangioblastomas is the endothelial cell.

3.12 Other Tissues

Lectin-binding sites have been analysed in *melanin-producing* cells by UNO et al. (1985). Melanoma cells, dermal melanocytes, epidermal melanocytes, and pigmented nevus cells were studied on the electron microscopical level by use of horseradish peroxidase coupled lectins (*PNA*, *WGA*, *RCA*, *CON A*). *Melanoma cells* had a higher number of WGA binding sites and a lower number of PNA binding sites (UNO et al. 1985).

In *ameloblastomas* (VEDTOFTE and DABELSTEEN 1981), cell membrane bound WGA receptors were shown in the epithelial cells. The distribution of RCA 1 and SBA receptors was related to the morphology of peripheral cells in ameloblastomas.

SOBRINHO-SIMÕES and DAMJANOV (1986) present data on the lectin binding of papillary and follicular carcinoma of the thyroid gland. *PNA* receptors are not found in normal thyroid tissue (follicular cells, colloid, stroma). Only one poorly differentiated carcinoma and one papillary carcinoma were positive for this lectin (SOBRINHO-SIMÕES and DAMJANOV 1986). *WGA* reacted strongly with follicular cells, colloid and stroma. Receptors for this lectin were positive in all thyroid carcinomas (SOBRINHO-SIMÕES and DAMJANOV 1986). *UEA* only reacted with blood vessels in normal and neoplastic thyroid cancers (SOBRINHO-SIMÕES and DAMJANOV 1986).

HPA receptors were found in stromal blood vessels of tissue from blood group A donor patients. *HPA* receptors were localized in all 7 papillary carcinomas of the study (SOBRINHO-SIMÕES and DAMJANOV 1986). Benign adenomatous lesions and follicular carcinomas were negative for this receptor. *DBA* reacted with stroma of normal thyroid tissue. Only one poorly differentiated follicular

carcinoma was positive (SOBRINHO-SIMÕES and DAMJANOV 1986). RCA I and II receptors are found in follicular cells and colloid to a varying degree. They are regularly present in the stroma. RCA I and II receptors were detected in benign adenomatous lesions, as well as in follicular and papillary carcinomas (SOBRINHO-SIMÕES and DAMJANOV 1986). *Succinyl Con A* binding sites were demonstrated in follicular cells, colloid and stroma. They are present in all carcinomas (SOBRINHO-SIMÕES and DAMJANOV 1986).

4 Discussion

Carbohydrate residues of membrane glycoproteins and glycolipids have a major influence on the chemical and physical properties of the cell membrane and can serve to regulate cell metabolism, membrane transport, growth and various functional activities of the cell. Surface carbohydrates are known to be involved in cell recognition, cell interaction and serve as receptor sites for a wide variety of substances (KÖTTGEN et al. 1979; HAKOMORI 1984; BAENZINGER 1986). The type and the spatial orientation of these carbohydrates are variable and form branching chains on the external surface of the cell.

The use of the multifaceted properties of lectins has led to their application in the investigation of various cellular phenomena. The primary event in these applications involves the interaction of the lectin with specific carbohydrate residues on the surface of the cell membrane. Lectins coupled to fluorescent tags, ferritin and radiotracers have been used to study the nature, number, and distribution of sugars on cells and tissues (UHLENBRUCK 1971; NICOLSON 1974; SHARON and LIS 1975; SCHREVEL et al. 1981; SCHAUER 1982a, b; YOGESWARAN 1983; LEATHEM et al. 1983; LIS and SHARON 1986). A large number of biologically important glycoproteins (e.g. membrane components, plasma components, enzymes and antigens) have been isolated by means of immobilized lectin columns. An effective use of lectins has been the development of techniques for the fractionation of animal cells. Lectins have been employed as carriers for the delivery of chemotherapeutic agents.

The distribution of lectin-binding carbohydrates is highly ordered in benign tissues and the disorganization of the carbohydrates in neoplasms may be exploited for diagnostic and therapeutic purposes. From morphological studies of the application of lectins, the following general observations can be made:

1. During neoplastic dedifferentiation there is a *change of membrane sugars*.
2. There are some lectins which are associated with *malignant differentiation* (PNA).
3. In tumors, there is often a *loss of "higher" blood group substances*, when these substances belong to the normal "background" of the patient.

A good example for the type of changes occurring in tumours is given by a *model of PNA related change* (Table 4).

The T antigen is believed to be the precursor carbohydrate of a variety of glycoproteins and glycolipids, including M and N blood group determinants. Being normally masked by *N-acetylneuraminic (sialic) acid residues*, this antigen is only accessible for binding to PNA after neuraminidase digestion. It has been postulated that T antigen expression in malignancy is a result of

accelerated degradation or incomplete synthesis of normal cell surface glycoproteins (SPRINGER et al. 1975; see Table 4). Different patterns of distribution are found by PNA affinity histochemistry: *granular globular pattern*, *vacuolar pattern*, *diffuse pattern* (BÖCKER et al. 1985). The data show that the lectin binding sites are the expression of functional differentiation. Obviously, the disaccharid Gal-Gal-Nac which is detected by the peanut agglutinin is not bound to the same protein components as in erythrocytes (HAMPER et al. 1984; BÖCKER et al. 1985). The expression of PNA receptors is correlated with the appearance of estrogen receptors in mammary carcinomas (BÖCKER et al. 1984; KLEIN and WÜRZ 1985; TOTOVIC et al. 1985).

PNA receptors are found in various types of salivary tumors. It seems to be expressed more intensely in malignant salivary gland tumors (e.g. carcinomas in pleomorphic adenomas) than in the benign counterparts (CASELITZ 1987). The expression of PNA receptors alone cannot be taken as a sign of malignancy, but as a marker displaying a certain tendency of differentiation. The PNA-receptor specific disaccharide is not bound to the same protein component as in erythrocytes (HAMPER et al. 1984).

The presence of PNA receptors in *colonic* epithelium has been identified bound to the Golgi apparatus in normal cells (SATO and SPICER 1982a, b; COOPER 1984). Taking the normal route of glycoprotein synthesis, the supranuclear distribution corresponds to the detection of PNA of nascent oligosaccharides prior to the addition of terminal neuraminic acid (COOPER 1984). This orderly arrangement is distributed during neoplastic development (COOPER 1984). 50% of the adenocarcinomas are positive for PNA (COGGI et al. 1983). Other authors found a labelling of mucin in all carcinomas of the large bowel. The divergence of the results may be due to technical reasons (fixation, embedding procedure, different types of affinity histochemistry).

In urothelial carcinomas, the presence of TF antigen is generally detected in the low differentiated tumors (COON et al. 1982). Interestingly, there are neoplasms with direct (without digestion) PNA receptors, neoplasms with cryptic PNA receptors and those without any PNA receptors. The worst prognosis was found in those carcinomas which were directly positive for Thomsen Friedenreich receptors and the cryptic Thomsen Friedenreich negative tumors.

In lymphoid tissue, PNA receptors have been found in activated T cells (CHERVENAK and COHEN 1982), follicular center cells (STRAUCHEN 1985), in B cells and in histiocytes (REE et al. 1983). MÖLLER (1982) claimed that Hodgkin cells are positive for PNA. Thus, PNA may be used in identifying several cell types in a given tissue. SCHWECHHEIMER et al. (1983) showed that this lectin was positive for granular cells. In this context, it can be used as an histochemical marker.

A lectin which is valuable in differential diagnosis is the UEA lectin. Many authors (HOLTHÖFER et al. 1982; BORISCH et al. 1983; MIETTINEN et al. 1983; MÖLLER and LENNERT 1984) have shown that this fucose specific lectin binds to endothelial cells under normal and neoplastic conditions. It may be used to analyse the normal architecture in different tissues (e.g. MÖLLER and LENNERT 1984), and to identify the vascular nature of some tumors (ANGERVALL et al. 1985; BENDELAC et al. 1985).

The AB isoantigens, in addition to their presence on erythrocytes and in secretions of certain individuals, are expressed by a variety of body tissues. AB isoantigens are demonstrable on the cell surface of epithelial cells of the skin, oral cavity, bronchopulmonary tract, gastrointestinal tract, genitourinary tract, and vascular endothelium. The expression of AB isoantigens by epithelial lining type cells and general absence of these antigens in mesenchymal cells may imply that these antigens are related to epithelial differentiation. In stratified epithelia, AB isoantigen expression is absent from the least differentiated cell type, namely the basal layer. AB antigens become present in the more differentiated superficial layers. This gives support to the idea that AB isoantigen expression is a marker of differentiated epithelial cell function.

The disappearance of AB isoantigens in malignant epithelial tumours has been demonstrated for carcinomas of the lung, the gastrointestinal tract, bladder, cervix uteri, and prostate. The loss of blood group A and B determinants has been documented in a large variety of human tumours.

On the basis of large studies HAKOMORI (1984, 1986) found the following changes:

1. Tumor tissues *delete A and B* determinants due to blocked activity of A and B transferases.
2. The *type 2 chain* is fucosylated by enhanced transferases and *accumulates Le x (or X) hapten* in some tumors.
3. The *type 1 chain* is fucosylated by enhanced transferases, Le a and Le b hapten being accumulated regardless of the Lewis status of the host (HAKOMORI 1984).

Many monoclonal antibodies with a distinctive reactivity towards human lung, gastric and colonic cancer and towards leukaemias, are identified as being directed against Le x structure (HAKOMORI and KANNAGI 1983). Le x structure is present in many normal human tissues (gastrointestinal epithelial cells of kidney tubules) and its accumulation in human neoplasms (e.g. colonic cancer) is noteworthy (FUKUSHI et al. 1984; HAKOMORI et al. 1983).

Some monoclonal antibodies reacting with tumour cells were specific for Le b or sialosyl Le a (KOPROWSKI et al. 1979, 1981). Accumulation of both Le a and Le b antigens in human adenocarcinoma may be the basis for an association of sialosyl Le a or Le b.

Blocked synthesis of ABH antigens may go together with the accumulation of precursor carbohydrates type 1 or type 2 chain (HAKOMORI 1984). A-like antigens may be expressed in tumours of O or B blood group. Other antigens playing a role in human tumours are P-like and P1-like antigens, P K antigen in Burkitt's lymphoma and the Forssman and related antigens (HAKOMORI 1984).

The following conclusions may be drawn from the studies with human blood groups in tumours (HAKOMORI 1984).

1. Human blood group ABH determinants in erythrocytes are carried by unbranched and branched type 2 chains. Fetal and newborn erythrocytes contain mainly unbranched type 2 chain and adult erythrocytes contained branched type 2 chain.
2. In the majority of human carcinomas, blood group ABH determinants are lost due to incomplete synthesis, consequently the precursor substances increase, either type 1 chain (Le c type) or type 2 chain (li type).

3. Various types of human adenocarcinoma (lung, gastric, colonic, liver, pancreatic) accumulate a large quantity of fucolipids with Le a, X (Le x) and their sialylated derivatives. Various monoclonal antibodies have been identified as being directed to these structures.

4. Some tumours derived from gastrointestinal tissue of blood group O or B individuals present a neosynthesis of the incompatible A-like antigen, different from the Forssman antigen. P-like structures may play a special role in individual cases (HAKOMORI 1984).

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1 Neuroendocrine Tumors

1.1 Definition and Markers

Neuroendocrine tumors arise from organs known to secrete neuroendocrine messengers, e.g. hormones or substances with paracrine activity, and/or to contain cells displaying neuroendocrine characteristics or activity. The term "neuroendocrine" is used here to define the secretory products of the cells and tumors rather than their nature and embryological derivation (see SOLCIA et al. 1984). Although individual tumor types do not occur frequently, neuroendocrine tumors as a group are common.

There are several markers characteristic of neuroendocrine cells, some of which have been used for tumor typing (see below). One group of markers occurs independently of production of regulatory peptides by normal or tumorous neuroendocrine cells. This group of *broad-spectrum markers* (Sect. 1.3) includes 1) the presence of voltage-dependent Na^+ and/or Ca^{++} channels in the cell membrane, 2) receptors for specific ligands, e.g. nerve growth factor,

3) cytoskeletal proteins, e.g. intermediate filaments, neurofilaments (Sect. 1.3.2), 4) neuron-specific enolase (Sect. 1.3.3), 5) granule matrix constituents, e.g. chromogranins and Leu-7 (Sects. 1.3.4 and 1.3.5), 6) granule membrane constituents, e.g. cytochrome B 561 or synaptophysin (Sect. 1.3.5), and 7) amine biosynthetic enzymes.

The second group of markers represents *specific markers*, including regulatory peptides (Sect. 1.4).

1.2 Role of Tumor Markers in the Diagnosis and Differential Diagnosis

The purpose of the use of tumor markers depends on the investigated tumor types. In general, the following questions can often be answered, at least partially:

- localization of the tumor,
- precise functional diagnosis,
- differential diagnosis,
- grade of differentiation,
- assessment of biological behavior,
- effects of therapy,
- morphologico-functional correlations.

Conventional histology using tinctorial stains is still a prerequisite for the diagnosis of tumors. However, it is not possible to reach a precise, functionally significant diagnosis of neuroendocrine tumors using conventional histology. These tumors often cause characteristic symptoms, or well defined syndromes caused by inappropriate hormone secretion. The concentration in the patient's serum of many secretory products can be determined. It is necessary that the pathologist provide a specific and functionally significant diagnosis and determine the morphological-functional correlations. The final evidence for the production and secretion of a substance can be provided only by the combined effort of the clinician, the biochemist, and the morphologist.

In order to define posttranslational products or to localize mRNA at the cellular and/or subcellular levels, the morphologist must use immunocytochemical methods or in-situ hybridization techniques. The use of these techniques is of particular interest in the differentiation of metastases of neuroendocrine tumors. Some types of tumors, e.g., pancreatic endocrine tumors, endocrine tumors of the gastrointestinal tract (carcinoids) are of low grade malignancy, and can be treated, using drugs with a specific action directed against specific types of tumor cells. The precise definition of a metastasis and/or the localization of the primary is therefore important for the patient.

1.3 Broad-Spectrum Markers (see Fig. 12)

1.3.1 Significance

The importance of broad-spectrum markers is to provide the pathologist with a clue to the neuroendocrine differentiation of a tumor, if conventional histology

does not reveal it. In addition, these markers are independent of hormone production by tumor cells, e.g. they are valuable in the diagnosis of tumors producing messengers, as well as in the diagnosis of inactive or poorly differentiated tumors.

1.3.2 *Cytokeratins*

With a few exceptions, cells of neuroendocrine tumors contain cytokeratins, in particular cytokeratins 8 and 18 (HOEFLER et al. 1986). Using a monoclonal antibody directed against an epitope of a sequence common to all presently known cytokeratins (FRANKE et al. 1987), and reacting with the cytoskeleton of all epithelial and mesothelial cells (monoclonal antibody lu-5; VON OVERBECK et al. 1985), virtually all cells of neuroendocrine tumors yield a reaction. The reaction pattern may be pancytoplasmic, "dot-like" paranuclear, or cell membrane-associated (Fig. 1a, 2a, 5a). Exceptions, e.g. tumor cells not containing cytokeratins, are cells of tumors of the adrenal medulla and paragangliomas, steroid-hormone-producing tumors of the adrenal cortex, testis, and ovary, and melanocytes. An immunocytochemical reaction for cytokeratins used as the first step in a diagnostic algorithm (Fig. 12) may therefore give an important clue to the differential diagnosis of neuroendocrine tumors.

1.3.3 *Neuron-Specific Enolase*

The soluble, cytoplasmic metallo-enzyme enolase (E.C. 4.2.1.11) catalyzes the interconversion of 2-phospho-D-glycerate and phosphoenolpyruvate (PEP) in the glycolytic pathway, and is present in every cell. Enolase is a dimer with a molecular weight of 87,000 daltons. The following dimers occur in various cell types: dimer alpha-alpha in the hepatocyte and other non-neural tissues, dimer beta-beta in muscle cells, and dimer gamma-gamma in neurones and neuroendocrine cells ("neuron-specific enolase"). A heterodimer alpha-gamma has also been found to occur in neurons and neuroendocrine cells (SCHMECHEL et al. 1978a, b, 1980).

The occurrence of various dimers in a given cell is not mutually exclusive, but there is a markedly higher concentration of enolase gamma-gamma than of other dimers in neurons and neuroendocrine cells. The term "neuron-specific enolase" is therefore too restrictive because this dimer is not restricted to neuroendocrine cells. Nevertheless, using antisera of high specificity for dimer gamma-gamma, it is possible to localize it at the light and electron microscopic level in the cytoplasm of neurons and neuroendocrine cells (Fig. 1b, 2b), but only to a very limited extent in non-neuroendocrine cells. This reaction has been used extensively and successfully in morphological analysis (TAPIA et al. 1981; DHILLON et al. 1982; OSKAM et al. 1985; IWASE et al. 1986; OSBORN et al. 1986), and as a serum marker of neuroendocrine tumors (CARNEY et al. 1982; PRINZ and MARANGOS 1982; ZELTZER et al. 1983).

In our experience, neuron-specific enolase is a useful marker for neuroendocrine tumors in the second step of a diagnostic flow-chart (Fig. 12), provided that the tissue was rapidly and thoroughly fixed. A non-specific reaction often occurs in necrotic or autolytic areas. It is rare that all cells of a given tumor display a clear-cut reaction. There is no detectable reaction for neuron-specific enolase in tumors of the parathyroid glands, tumors of the thyroid with follicular differentiation, and in tumors producing steroid hormones.

1.3.4 *Chromogranins*

Chromogranins are a group of acidic monomeric proteins of various sizes, which form a major part of the soluble proteins in the secretory granules of the adrenal medulla (WILSON and LLOYD 1984). The largest of the polypeptides is chromogranin A (MW 68,000 daltons), which makes up approx. 40 to 50% of the total soluble granule proteins of the adrenal medulla. A monoclonal antibody to human chromogranin A has been shown to react with normal (endocrine) cells and

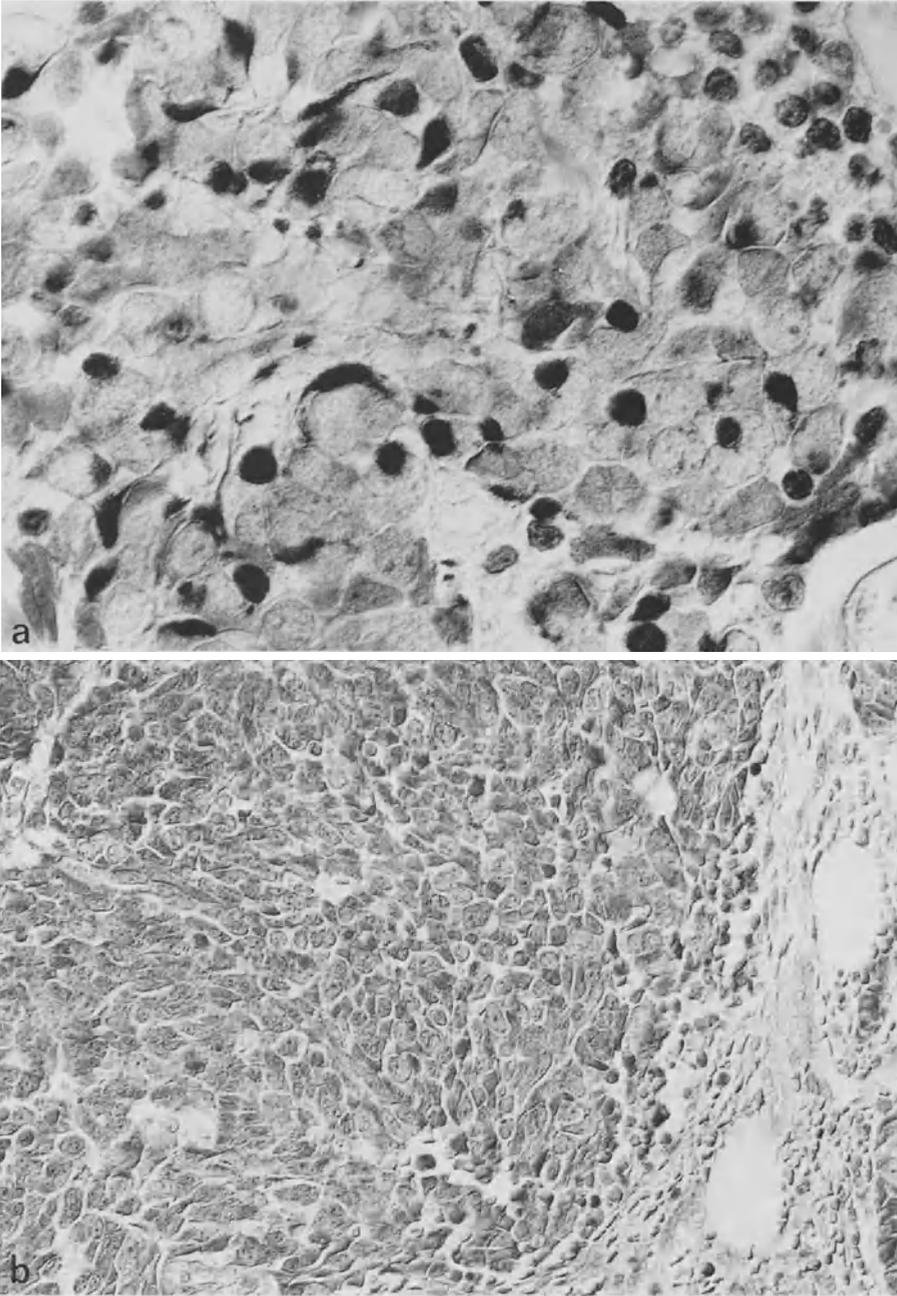


Fig. 1 a, b. Merkel cell tumor (neuroendocrine carcinoma of the skin). Avidin-biotin complex technique, differential interference contrast optics. **a** "Dot-like" reaction for cytokeratins in many tumor cells. Monoclonal antibody to a panepithelial antigen, $\times 1000$. **b** Neuron-specific enolase in the cytoplasm of the majority of tumor cells, $\times 200$

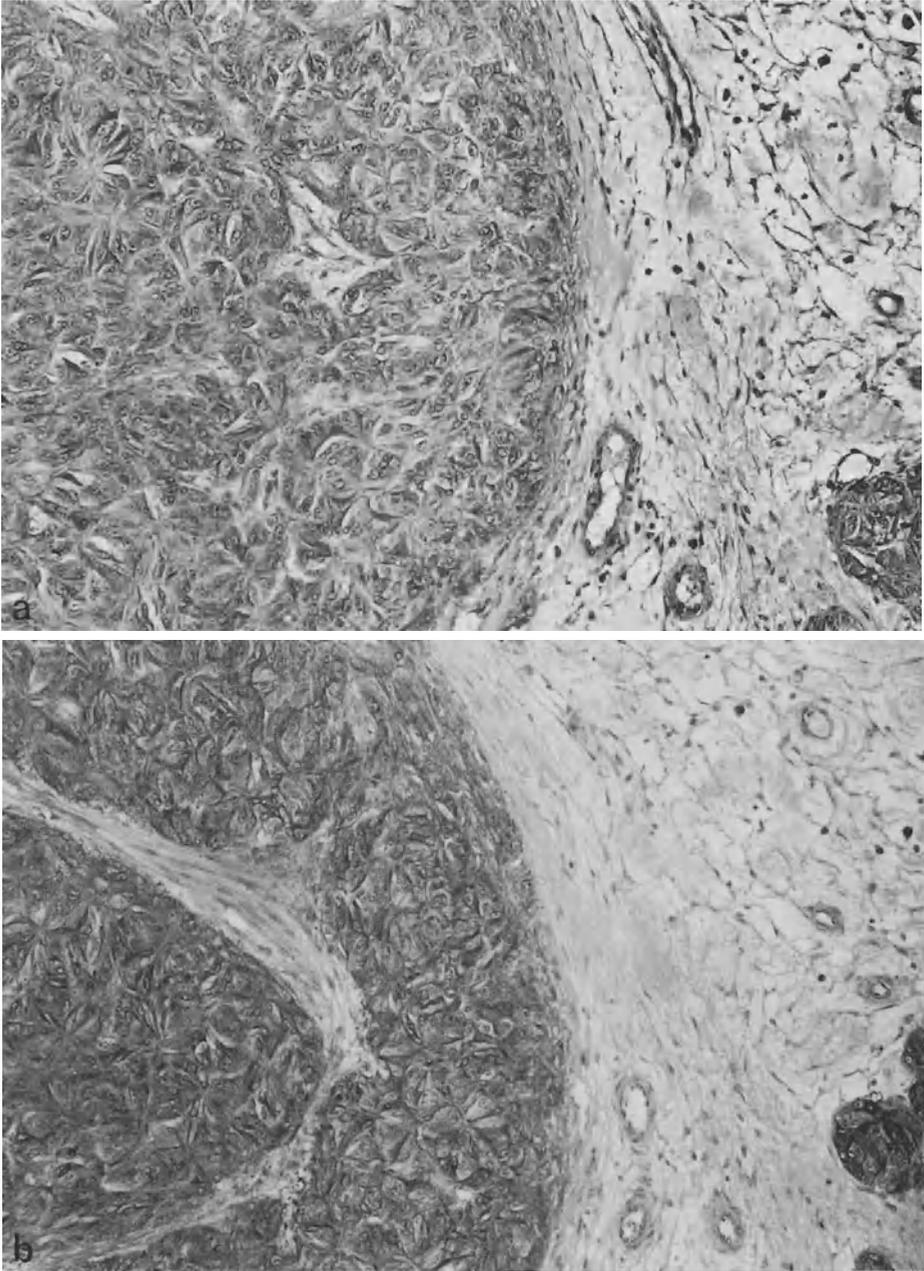


Fig. 2a, b. Malignant vipoma of the pancreas. Avidin-biotin complex technique. **a** Diffuse cytoplasmic reaction for cytokeratins in virtually all tumor cells, $\times 100$. **b** Neuron-specific enolase present in the cytoplasm of almost all tumor cells, $\times 100$

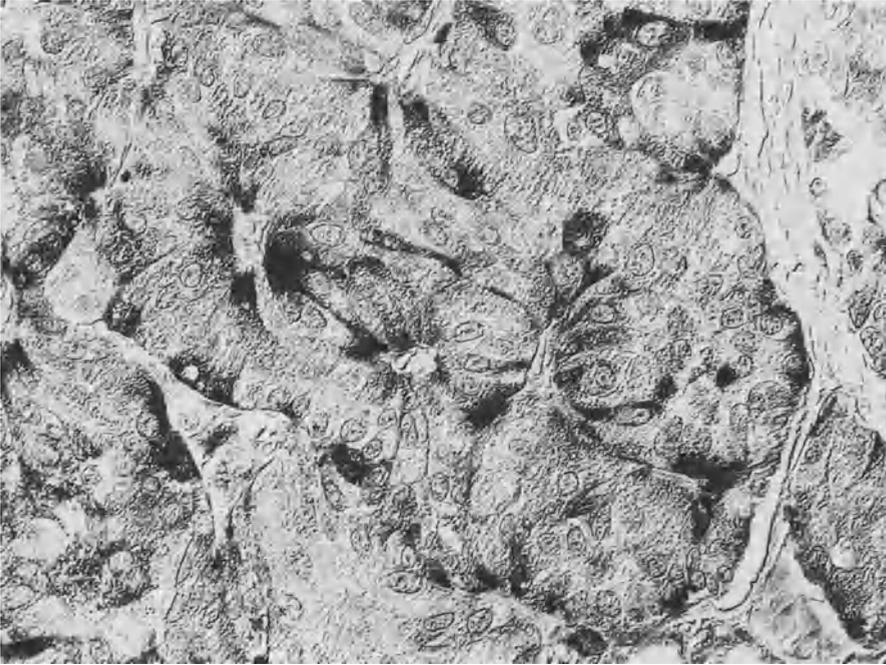


Fig. 3. Malignant insulinoma of the pancreas. Strong reaction for chromogranin A in a large number of tumor cells. Avidin-biotin complex technique, $\times 400$, differential interference contrast optics

tumors arising in the adrenal medulla, endocrine pancreas (Fig. 3), parathyroid glands, anterior pituitary gland, the thyroid (C-cells), and medullary carcinomas. In addition, a focal staining has been observed in Merkel cell tumors, neuroblastoma, and small-cell carcinoma of the lung. The antibody did not react with melanomas, naevi, the posterior pituitary gland, and neurones (LLOYD and WILSON 1983; EHRHART et al. 1986). Chromogranin A could be localized in cytoplasmic secretory granules of paragangliomas by immunoelectron microscopy (WILSON and LLOYD 1984). In addition, chromogranin A was shown to be secreted by the aforementioned tumors. The measurement of plasma chromogranin A may therefore be a useful diagnostic procedure in patients presumed to be suffering from neuroendocrine tumors (O'CONNOR and DEFTOS 1986). Recently, the sequence of bovine chromogranin A has been described, using its complementary DNA. In addition, the presence of the mRNA of prechromogranin A in adrenal medulla, brain, anterior pituitary and parathyroid glands has been demonstrated. It is conceivable that chromogranin A is a precursor for smaller peptides or that it may be important in the binding process of intragranular calcium (IACANGELO et al. 1986).

In our experience, using the aforementioned monoclonal antibody the specific reaction for chromogranin A is a useful adjunct in the diagnosis of neuroendocrine tumors, yielding good results on formaldehyde-fixed and paraffin-embedded tissue. A large number, but not all, cells react in the tumors mentioned above. However, we found the following exceptions: 1) We were unable to obtain a reaction of chromogranin A in small cell carcinoma of the bronchus, and 2) in our series the reaction was not reliable in endocrine tumors of the gastrointestinal tract (carcinoids), and it was often negative in carcinoids of the colon and rectum. In contrast to tumors a large number of cells were regu-

larly visualized in the epithelium of the entire gastrointestinal tract. In contrast to neuron-specific enolase, the reaction for chromogranin A is useful to differentiate melanocytic tumors from other neuroendocrine neoplasms. It can be used as a second-step marker (Fig. 12).

1.3.5 Other Broad-Spectrum Markers

A new monoclonal antibody (Phe-5) has recently been commercialized. It can be used on formaldehyde-fixed paraffin-embedded tissue. In our test series, the reactions were almost identical to the antibody directed against chromogranin A. A reaction was obtained in some carcinoids of the colon, but there was no reaction in small cell carcinomas of the lung. At present, no literature concerning this antibody and the recognized antigen is yet available.

One potentially useful marker for neuroendocrine cells is synaptophysin. Synaptophysin is a specific component isolated from the membranes of presynaptic vesicles of bovine neurons. It is a glycosylated polypeptide (MW 38,000 daltons), and it has been reported to be visualized regularly in almost all neurons and in a majority of neuroendocrine tumors. A prerequisite for its demonstration is good preservation of conventionally fixed and paraffin-embedded tissue or incubation of freshly frozen tissue (GOULD et al. 1986; WIEDENMANN et al. 1986; GOULD et al. 1987).

Recently, it has been reported that anti-lymphocyte antibody Leu-7 (HNK), directed against an epitope present on the cell surface of natural killer (NK) lymphocytes, and with myelin-associated glycoprotein (MAG), yields an intense reaction with normal and neoplastic adrenal medullary cells. Immunocytochemical studies and immunoblot analyses showed that this antibody apparently reacts with the carbohydrate moiety of a protein (molecular weight 75,000 daltons) localized in the matrix of chromaffin granules. The antibody was shown to react with a small percentage of normal pancreatic islet cells, cells of the anterior pituitary, and endocrine cells of the gastrointestinal tract. We found a reaction in tumor cells of formaldehyde-fixed, paraffin-embedded tissue of medullary thyroid carcinomas, pheochromocytomas, paragangliomas, endocrine pancreatic tumors, and carcinoids of the stomach, duodenum, ileum, and rectum. No reaction was obtained in carcinoids of the colon. It was suggested that Leu-7 immunoreactivity might be a marker of specific subsets of secretory granules. It is also important to realize that antibody Leu-7 reacts with the surface of cells of small-cell carcinoma of the lung (TISCHLER et al. 1986).

1.4 Specific Markers (see Fig. 12)

Peptides, glycoproteins, biogenic amines, catecholamines, and, to a limited extent, steroid hormones may be used as markers in a second or third step of an algorithm designed for the precise typing of neuroendocrine tumors. The immunocytochemical localization of secretory products of neurons and of endocrine cells producing peptides, proteins, glycoproteins and/or amines, and of chromogranins is based on the fact that the intracellular pathway of these substances is regulated as well as polarized. In a steady state of the cell metabolism, a post-Golgi pool of secretory products, precursors thereof, and substances contained in the matrix of secretory granules exists. The secretory products can be stored for several hours in granules (KELLY 1985; LAUFFER et al. 1985; WICKNER and LODISH 1985). In this post-Golgi compartment, a high concentration of secretory products and other substances contained in the granular matrix

is present. The granules can be analyzed morphologically by electron microscopy. Secretory products as well as other substances in the granular matrix can be identified and localized at light and electron microscopic levels by immunological reactions.

The extensive use of these markers has provided the cell biologist and the pathologist with new insights into the process of hormone biosynthesis and the secretion and the regulation thereof, and into the phenotypic heterogeneity of tumor cells.

2 Tumors of the Pituitary Gland

Tumors of the adenohypophysis leading to symptoms account for 6–10% of all intracranial neoplasms; microadenomas can be detected in 8–22.5% of all autopsied patients. Their prevalence increases with age. By using tinctorial stains (HE and PAS-orange G stains) the following variants of pituitary tumors can be differentiated: acidophil, mucoid, chromophobe, and oncocyctic tumors (SAEGER 1981; for the complete classification of the World Health Organization see WILLIAMS et al. 1980). By far the most frequent tumors are chromophobe adenomas, followed by acidophil and mucoid tumors. The delimitation of these tumors from the adjacent pituitary parenchyma is often difficult because a tumor capsule is most often lacking. In addition, it is unfortunately impossible to correlate reliably the properties of tumor cells based on tinctorial stains, with the production of hormones (Fig. 4a and b).

The precise diagnosis of tumors of the pituitary gland was based for a long time on electron microscopy (LANDOLT 1975; EZRIN et al. 1982; KOVACS and HORVATH 1986). At present, the diagnosis is most often made by using immunocytochemistry, which is more reliable and faster than electron microscopy (HEITZ and OBERHOLZER 1984). Electron microscopy and immunoelectron microscopy are important tools for research.

The purpose of localizing hormonal tumor markers in the pituitary gland is to provide a precise diagnosis, to establish morphologico-functional correlations, and to observe the effects of therapy. Assessment of the biological behaviour of pituitary tumors can still be based on conventional histological observation because metastasizing carcinomas are extremely uncommon, and the invasion of bony structures surrounding the sella can be readily seen at conventional histology. The localization of the tumor is possible using tinctorial stains because the alveolar structure is in general much coarser or may be lacking in the tumor, in contrast to the adjacent pituitary parenchyma.

The most important immunocytochemical reactions are those for prolactin, growth hormone (Fig. 4b), and corticotropin. In addition, it is very useful to localize the alpha-chain, which is virtually identical in all glycoprotein hormones (LANDOLT and HEITZ 1986), the hormone specific beta-chains of follicle stimulating hormone (FSH), luteinizing hormone (LH), and thyrotropin (TSH).

The ratio of chromophobe tumors is 39.1–79.5%, of acidophil tumors 15–59%, and of mucoid tumors 0–19% based on conventional histology (BAKAY

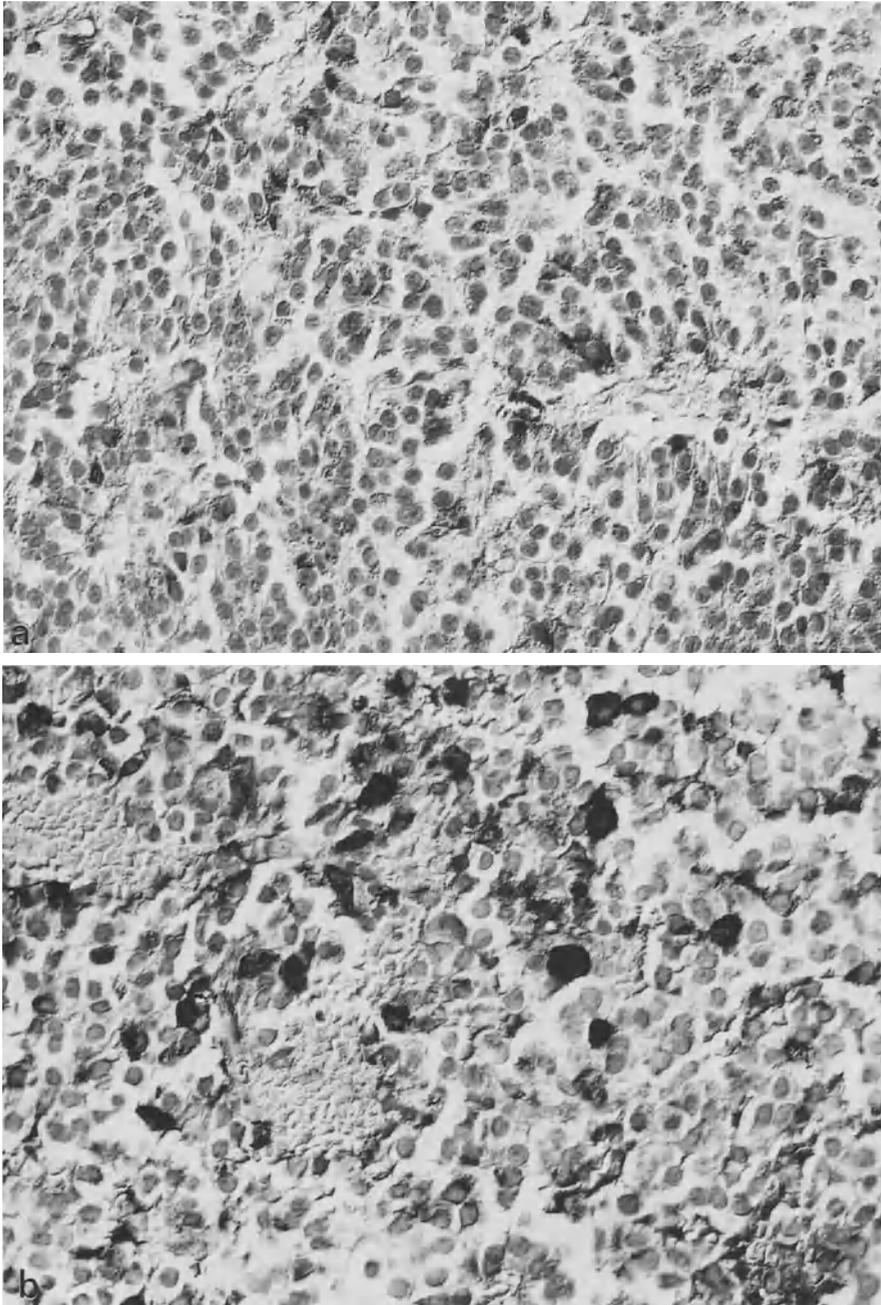


Fig. 4 a, b. Pituitary adenoma causing acromegaly. Differential interference contrast optics. **a** Absence of staining: chromophobe adenoma, PAS-orange G, $\times 200$. **b** Presence of growth hormone in the cytoplasm of many tumor cells: growth hormone secreting adenoma. Avidin-biotin complex technique, $\times 400$

Table 1. Type of tumors of the pituitary gland

Prolactinomas	194	51 %
Growth hormone-producing adenomas	93	24.1%
Corticotropin-producing adenomas	26	7 %
FSH-producing adenomas	3	0.8%
LH-producing adenomas	5	1.3%
TSH-producing adenomas	4	1 %
“Alpha-only” adenomas	13	3.4%
Multihormonal adenomas	13	3.4%
Adenomas without detectable secretory products	30	8 %
	381	100 %

FSH, follicle-stimulating hormone; *LH*, luteinizing hormone; *TSH*, thyroid-stimulating hormone

Table 2. Alpha-subunit of glycoprotein hormones in 92 pituitary adenomas

Alpha-only	13
GH and alpha	6
PRL and alpha	1
ACTH and alpha	1
GH and PRL and alpha	10
Beta-subunits of FSH, LH, and alpha	12
Beta-subunits and GH, and alpha	3
	46

GH, growth hormone; *PRL*, prolactin; *ACTH*, corticotropin; *FSH*, follicle-stimulating hormone; *LH*, luteinizing hormone

1950; GIROD et al. 1980; SAEGER 1981). The systematic use of immunocytochemistry has led to a considerably more precise diagnosis (Table 1).

The progress in comparison with conventional histology is obvious because in only 8% of the tumors no secretory product was identified. This progress allowed the establishment of a modern, functionally-oriented classification of pituitary tumors (Table 1). It is striking that the prolactinoma is by far the most common tumor of the pituitary, followed by growth hormone and corticotropin producing adenomas. FSH-, LH- and TSH-producing adenomas are rare. One of the important findings was the common occurrence of phenotypically heterogeneous tumors producing two or more hormones, most often prolactin combined with growth hormone (HEITZ 1979). Another striking result was a combined production of growth hormone and of the alpha-chain (common to the pituitary glycoprotein hormones and human chorionic gonadotropin; Table 2). An additional finding was the occurrence of “alpha-only adenomas”, which was found by radioimmunoassay of the patient’s sera (MACFARLANE et al. 1980; RIDGWAY et al. 1981; KLIBANSKY et al. 1983), and by immunocytochemistry (LANDOLT and HEITZ 1986). These tumors do not cause hormonally-induced symptoms.

The results obtained with immunocytochemical techniques on tumor tissue correlate well with clinical and biochemical findings. In addition, the immunocytochemical localization of hormones can be correlated with the localization of receptors for hypothalamic hormones on tumor cells, which opens new avenues for future research (REUBI et al. 1987).

3 Tumors of the Thyroid Gland

Diagnosis and treatment of tumors of the thyroid gland are good examples of the use of functional criteria in oncology, e.g. determination of iodine-trapping by tumor tissue, or of the production of thyroglobulin. The two most important problems in the diagnosis of these tumors are 1) the assessment of the biological behaviour of highly differentiated follicular adenomas and carcinomas respectively, and 2) the classification of poorly differentiated tumors. At present, the first problem can be solved only by a very precise and thorough histological inspection of the tumor, carefully excluding or confirming penetration and perforation of the tumor capsule, and the invasion of blood vessels. Immunocytochemistry and electron microscopy are of no help in solving this problem.

Significant progress has been made in the differentiation of poorly differentiated tumors. Information on the cellular characteristics of these tumors is essential because therapy and prognosis of the various tumor types are very different (MEISSNER 1984). Tumor cells with epithelial differentiation can be defined by immunocytochemical localization of cytokeratins. There is a coexpression of cytokeratins and vimentin in approx. 50% of follicular, and in virtually all papillary carcinomas (SCHROEDER 1986). The analysis of large series of poorly differentiated tumors has yielded the following information: 1) The presence of cytokeratins (Fig. 5a), often combined with vimentin (Fig. 5b) can be observed in most anaplastic carcinomas (CARCANGIU et al. 1985; HEITZ 1986). 2) "Undifferentiated carcinomas" of the small cell type according to the WHO classification (HEDINGER and SOBIN 1974) are often malignant non-Hodgkin lymphomas (TOBLER et al. 1984; ANSCOMBE and WRIGHT 1985; RALFKIAER et al. 1985). They are most often negative for cytokeratins, but give reactions for various markers of lymphocytes. 3) Tumors with characteristics of malignant hemangioendotheliomas are uncommon but they apparently exist (EGLOFF 1983; PFALTZ et al. 1983; RUCHTI et al. 1984). Their reaction pattern can be summarized as follows: factor VIII-related antigen positive, *Ulex europaeus* agglutinin 1 positive, cytokeratins negative. 4) The histological aspect of medullary carcinomas can be solid and poorly differentiated. These carcinomas may be identified by a positive reaction for calcitonin, calcitonin-gene-related peptide and carcinoembryonic antigen.

The differential diagnosis of tumors with follicular or C-cell differentiation is most often possible by visualizing markers such as triiodothyronin (T3), thyroxin (T4), and thyroglobulin (Fig. 6) in tumors with follicular differentiation. Calcitonin, neuron-specific enolase, chromogranin A, and carcinoembryonic anti-

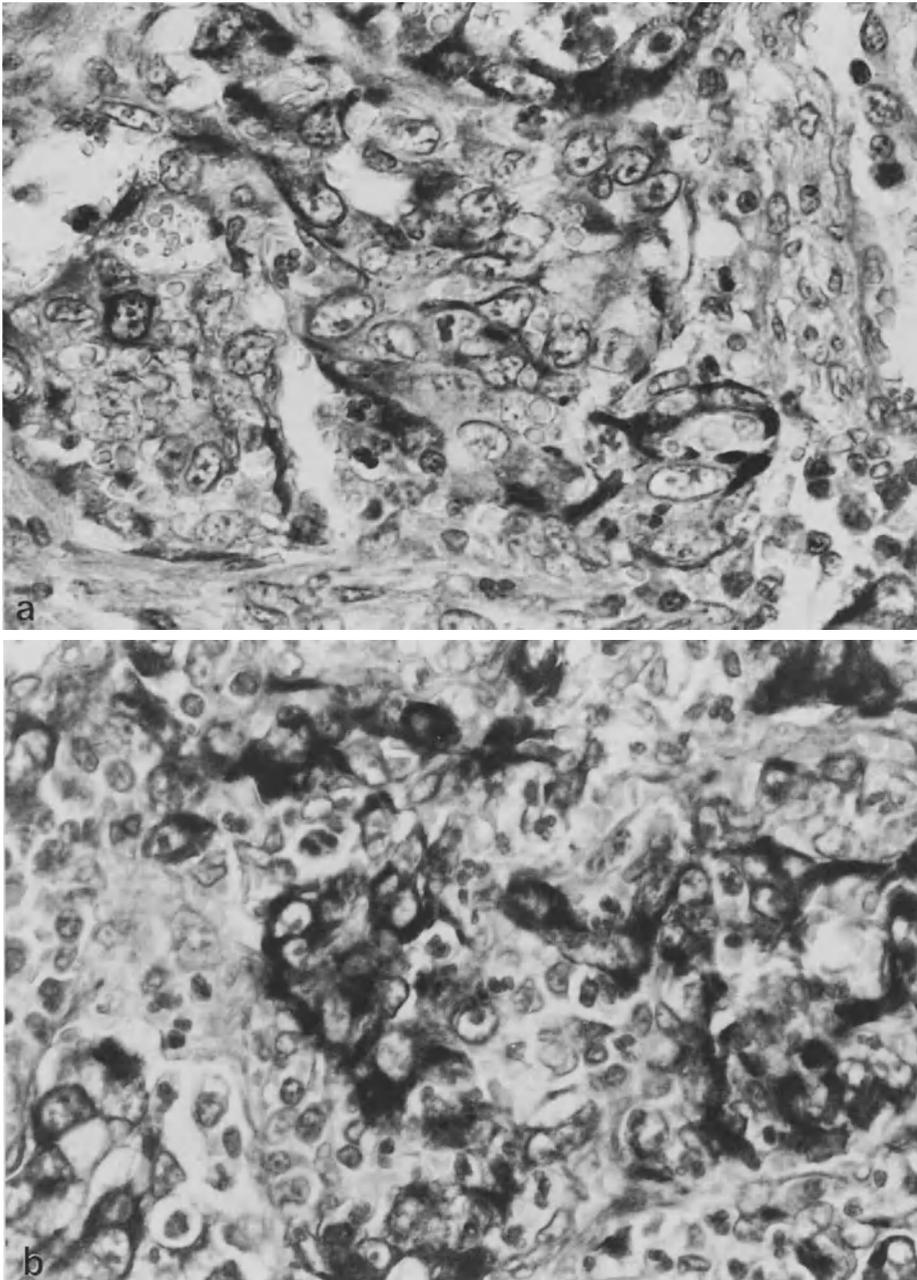


Fig. 5a, b. Undifferentiated (anaplastic) carcinoma of the thyroid. Avidin-biotin complex technique. **a** Presence of cyokeratins in the cytoplasm of many tumor cells, $\times 500$. **b** Co-expression of vimentin with cyokeratin, $\times 500$

4 Tumors of the Parathyroid Gland

Conventional histology of an adenoma or a hyperplasia of the parathyroid glands provides the most important clues to the diagnosis (AKERSTROEM 1980; ALTENAEHR 1981). It is possible to localize parathyroid hormone by immunocytochemical techniques (DIETEL et al. 1980). In addition, adenomas of the parathyroid glands have been reported to contain chromogranin A. The localization of these markers is rarely necessary for the diagnosis of these tumors, but they have played an important role in the research into the pathways of intracellular transport of hormones.

5 Endocrine Tumors of the Pancreas

The classification of pancreatic endocrine tumors has been revolutionized by the systematic use of radioimmunoassay for measuring hormones in the patient's plasma, and by electron microscopy and immunocytochemistry for visualizing the site of hormone production in the cells. Prior to the use of these techniques the classification was simple, as only B- and non-B cell tumors could be differentiated by conventional histology with special tinctorial stains. No reliable criterion for typing cells, or for hormone production or secretion is possible by means of histological observation. The growth pattern of a tumor, e.g. trabecular, glandular, or solid, cannot be reliably correlated with the various types of tumors (HEITZ 1984).

In our opinion, immunocytochemical and/or electron microscopical analysis of these tumors is a must at present for functional classification (HEITZ et al. 1982).

Virtually all endocrine tumors of the pancreas contain broad-spectrum markers, e.g. cytokeratins, neuron-specific enolase (HEITZ and KLOEPPPEL 1984), and chromogranins (Fig. 2b, 3), and yield positive reactions to the antibody Phe-5. By these reactions they can be differentiated reliably from exocrine tumors, which sometimes resemble endocrine tumors, e.g. the solid cystic (papillary-cystic) tumor (MOROHOSHI et al. 1987). The markers of the second or third steps in the diagnostic algorithm are eutopic pancreatic hormones, e.g. insulin, glucagon, somatostatin, and pancreatic polypeptide. In addition, hormones "ectopic" to the adult human pancreas can be visualized, e.g. gastrin, vasoactive intestinal polypeptide (VIP), or, if appropriate, corticotropin, calcitonin and others. The occurrence of growth hormone-releasing hormone immunoreactivity has recently been reported in approx. 25% of pancreatic endocrine tumors, even in the absence of associated acromegaly (DAYAL et al. 1986).

The problem of the definition of the biological behavior in pancreatic endocrine tumors is particularly difficult to solve. Many authors agree that histological observation does not allow a prediction of the course of the disease in the absence of massive invasion of vessels or of an adjacent organ, which are rare findings. The only reliable criterion is metastases in the regional lymph



Fig. 6. Papillary thyroid carcinoma producing thyroglobulin. Unlabeled antibody enzyme technique, $\times 500$

gen are typical markers of medullary carcinomas. Recently the production of several other peptides by medullary thyroid carcinomas has been reported. These findings offer an additional possibility of recognizing tumors with C-cell characteristics (KRISCH et al. 1985; SIKRI et al. 1985). Lately, some authors have described tumors with combined follicular and C-cell differentiation in a limited number of tumors (LJUNGBERG et al. 1984).

An important aid in the diagnosis and therapeutic management of the patient is the determination of production of thyroglobulin by thyroid tumors with follicular or papillary differentiation. Most authors agree that the majority of tumors with histologically recognizable follicular or papillary differentiation produce thyroglobulin (92–98%). Production of thyroglobulin can be shown by immunocytochemistry on formaldehyde-fixed and paraffin-embedded tissue (BOECKER et al. 1980; RYFF-DE LECHE et al. 1986). After total thyroidectomy the determination of the serum concentration of thyroglobulin can serve as a guide in the search for metastases without carrying out a total body scan. Levels of serum thyroglobulin rising above the normal range provide an indication of recurrency or metastases. On the other hand, tumors lacking follicular differentiation (solid tumors), and poorly differentiated carcinomas do not produce significant amounts of thyroglobulin. The immunohistochemical findings in the tumor tissue correlate well with the findings of serum levels of thyroglobulin (RYFF-DE LECHE et al. 1986).

Table 3. Pancreatic endocrine tumors

Tumor	Approx. ratio of all secreting pancreatic endocrine tumors	Biological behavior
Insulinoma	70–75%	90% benign
Glucagonoma	1%	50% malignant
Somatostatinoma	rare	?
PP-oma	rare	50% benign
Gastrinoma	20–25%	60% malignant
VIP-oma	3–5%	60% malignant
Non-secreting tumors	20–25% ^a	50% malignant

^a of the total of pancreatic endocrine tumors

PP, pancreatic polypeptide; *VIP*, vasoactive intestinal polypeptide

nodes or the liver. It is therefore of interest that the production, and sometimes the secretion, of the alpha-subunit of glycoprotein hormones (and possibly the rare production of the beta-subunit of human chorionic gonadotropin) could be found in up to 75% of tumors with proven malignancy (KAHN et al. 1977; BLACKMAN et al. 1980; OEBERG and WIDE 1981; HEITZ et al. 1983). These findings mean that the tumor producing the alpha-subunit should be considered malignant, while the absence of alpha by no means proves the benign behavior of the tumor. In some patients the course of the illness, and the presence of metastases could be monitored by measuring serum levels of pancreatic hormones and of the alpha-subunit over several years (KOELZ et al. 1987).

The present widely accepted classification of pancreatic endocrine tumors, their prevalence, and their biological behavior are given in Table 3.

6 Endocrine Tumors (Carcinoids) of the Gastrointestinal Tract and Bronchus

The histological growth pattern of carcinoid tumors is quite uniform. It is therefore impossible to draw a conclusion concerning the site of the primary by analyzing a metastasis by histological observation. In addition, it is often impossible to differentiate carcinoids from pancreatic endocrine tumors, and it may be difficult to distinguish an atypical carcinoid of the bronchus from a small cell carcinoma. Reactions, which visualize markers specific to particular localizations of carcinoids, and which differentiate atypical bronchus carcinoids from small-cell carcinomas, are therefore useful.

Carcinoids consistently react to antibodies directed against “broad-spectrum” markers, with the exception of chromogranin A and Phe-5 (Sections 1.3.4, 1.3.5).

Several series of carcinoid tumors of the gastrointestinal tract have been analyzed by immunocytochemistry using antibodies to serotonin and a large

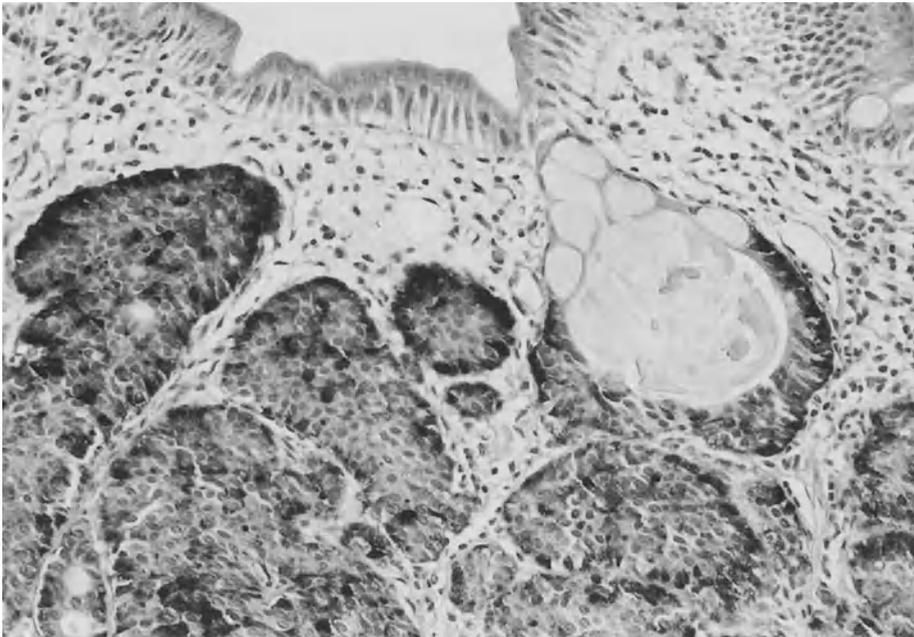


Fig. 7. Endocrine tumor of the ileum (carcinoid). Presence of substance P in many tumor cells. Unlabeled antibody enzyme method, $\times 200$

series of peptides, including tachykinins (ALUMETS et al. 1977, 1980, 1981, 1983; YANG et al. 1983; DAYAL and WOLFE 1984; CONLON et al. 1985; MÄRTENSSON et al. 1985; IWAFUCHI et al. 1986; KOMMINOTH 1986; STAMM et al. 1986). The most important findings in gastrointestinal carcinoids can be summarized as follows: serotonin is the most important marker for the entire group of tumors, while tachykinins, especially substance P, occur almost exclusively in endocrine tumors of the ileum (Fig. 7). These two markers also allow the differentiation of carcinoids of the gastrointestinal tract from pancreatic endocrine tumors, in which they never occur. Gastrin, somatostatin, and pancreatic polypeptide can be localized predominantly in carcinoids of the duodenum. The other peptides investigated, including neurotensin, motilin, glicentin, peptide YY (TATEMOTO et al. 1982), calcitonin, corticotropin, and growth hormone-releasing hormone (DAYAL et al. 1986) occur much less frequently, and are not specific for any localization of the primary. In addition, the alpha-chain of glycoprotein hormones was found in carcinoids of the stomach, duodenum, and rectum, but not in tumors of the ileum. This marker is therefore of only limited use for the localization of the primary, or for the differentiation of a metastasis from a pancreatic endocrine tumor (HEITZ et al. 1987). A striking new finding is the occurrence of a substance identical to prostatic acid phosphatase, or cross-reacting with antisera and monoclonal antibodies raised against prostatic acid phosphatase (SOBIN et al. 1986). We can confirm this finding. It is also

important to realize that there is a reaction for protein S-100-beta (VAN ELDIK et al. 1986) in the majority of tumors. This reaction is not localized in epithelial tumor cells, but in Schwann-cells included in the tumors (HOEFLER and AUBOECK 1984; KOMMINOTH 1986).

The substances localized in bronchial carcinoids, including atypical bronchial carcinoids, and in small-cell cancer include some of the broad-spectrum markers (Section 1.3). In addition, the alpha-chain of glycoprotein hormones was found in 27 out of 55 bronchial carcinoids, but not in small-cell cancer. Serotonin and other peptides could be localized less frequently in carcinoids, but not in 12 small-cell carcinomas. The immunocytochemical reactions for these substances may therefore be useful for the differentiation of atypical carcinoids from small-cell carcinomas (KOMMINOTH 1986).

7 Tumors of the Adrenal Medulla and the Chemoreceptor System

The diagnosis of these tumors often relies on histology. Cytokeratins are lacking in the tumor cells (VON OVERBECK et al. 1985) while virtually all tumors contain significant amounts of neuron-specific enolase (TAPIA et al. 1981; KOMMINOTH 1986; OSBORN et al. 1986), chromogranin A (WILSON and LLOYD 1984) and the neurofilament polypeptide with a molecular weight of 68 kD (OSBORN et al. 1986). Catecholamines may be visualized by using formaldehyde-induced fluorescence (FALCK et al. 1962) or by condensation with glyoxylic acid (DE LA TORRE and SURGEON 1976; LINDVALL et al. 1980). These reactions cannot be carried out in formaldehyde-fixed and paraffin-embedded tissue. Sometimes the production of neuropeptides, e.g. vasoactive intestinal polypeptide, somatostatin and others can be demonstrated.

Protein S-100 can be visualized in satellite, or sustentacular cells (LAURIOLA et al. 1985; PELC et al. 1986). This reaction yields sometimes very striking patterns (Fig. 8).

8 Steroid Hormone-Producing Tumors of the Adrenal Cortex, the Testis, and the Ovary

Steroid hormone-producing cells lack broad spectrum markers, such as cytokeratins, neuron-specific enolase, or chromogranin. It is difficult to localize steroid hormones specifically by immunocytochemistry because their intracellular concentration is low. This is due to the absence of a post-Golgi pool in steroid hormone-producing cells. Enzyme deficiencies or the activity of enzymes can sometimes be demonstrated by enzyme histochemistry, e.g. 3-beta-OH-steroid-dehydrogenase (Fig. 9). By this means, cells producing steroids or lacking this enzyme, e.g. virilizing tumors, can be localized. The diagnosis of this group of tumors can in general be made by conventional histology, and electron microscopy is often valuable for a precise diagnosis.

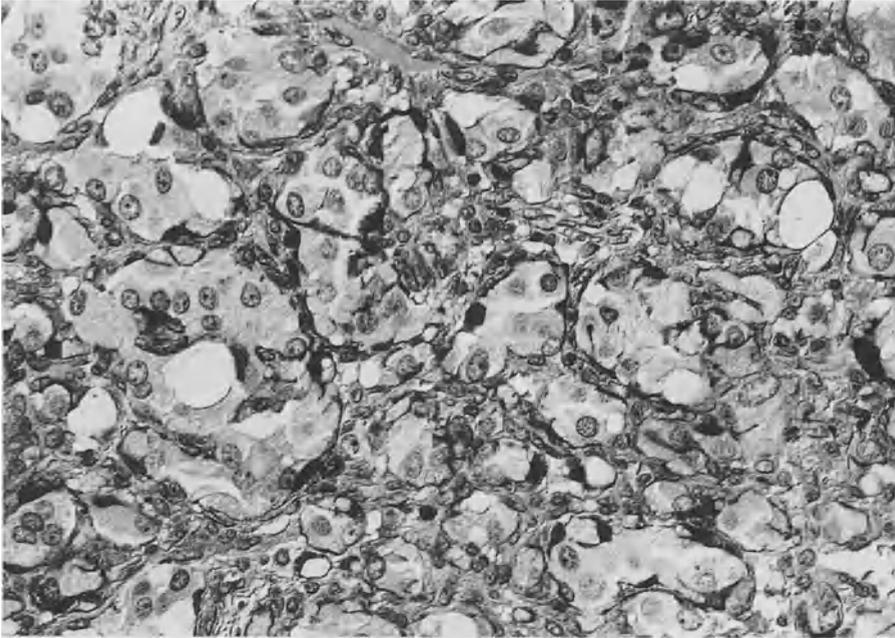


Fig. 8. Lymph node metastasis of a malignant paraganglioma. Localization of protein S-100 in satellite, but not in tumor cells. Avidin-biotin complex technique, $\times 400$



Fig. 9. Virilizing adenoma of the adrenal cortex. Absence of 3-beta OH-steroid dehydrogenase in the tumor, presence of reaction product in non-tumorous adrenal cortical cells (right). Enzyme histochemical reaction for 3-beta OH-steroid dehydrogenase, $\times 200$, differential interference contrast optics

9 Melanoma

The diagnosis of metastases of melanoma is a difficult problem to solve histologically if small amounts of melanin are present, or if pigment is lacking. The precise diagnosis can often be made by electron microscopy based on the presence of cytoplasmic (pre-)melanosomes. Immunocytochemistry can also be helpful: Tumorous melanocytes and their normal counterparts fail to react to antibodies directed against cytokeratins and chromogranin A, but contain vimentin, neuron-specific enolase, and protein S-100 (LOEFFEL et al. 1985; KOMMINOTH 1986; VANSTAPEL et al. 1986). Using one or more of these markers, melanocytic cells can be identified reliably and differentiated from other tumor cells. The reactions for vimentin and S-100 (Fig. 10a and b) are in general intense and present in virtually all tumor cells, while the reaction for neuron-specific enolase is often only focally positive and of lesser intensity. CHERESH et al. (1984) have reported an alkali-labile O-acetylated product of the neurectoderm-associated disialoganglioside GD3 to be expressed specifically by melanoma cells. It will perhaps be possible in the near future to diagnose melanocytic lesions specifically by using single markers. At present, the electron microscopic diagnosis and/or the findings of the aforementioned combination of markers is the most reliable way to diagnose melanocytic tumors.

It is sometimes very difficult to differentiate benign from malignant melanocytic lesions in the skin. A reliable marker should be found for malignancy expressed exclusively by melanocytic tumor cells displaying malignant biological behavior. It has been shown that the expression of neuron-specific enolase increases progressively from benign to malignant (DHILLON and RODE 1982), but this quantitative change has not proved to be a useful criterion in our hands, because differences in staining intensity of neuron-specific enolase may be caused by a large number of technical factors, not related to the biological behavior of the tumor cells. Recently the expression of a high molecular weight chondroitin sulphate proteoglycan consisting of a 250 kD core glycoprotein and a number of glycosaminoglycan side chains by human malignant, but not by normal melanocytes, has been reported (RETIG et al. 1986). It is therefore conceivable that specific markers for malignancy may be used for melanocytic lesions.

10 Merkel Cell Tumors (Neuroendocrine Carcinoma of the Skin)

TOKER described 5 tumors of the skin with a trabecular growth pattern in 1972, and subsequently TANG and TOKER (1978) reported the ultrastructural features of these tumors, including cytoplasmic granules. On the basis of the structural resemblance of the tumor cells to cells of the epidermis first described by MERKEL (1875) the tumors are now known as Merkel cell tumors (neuroendocrine carcinomas) of the skin.

The differentiation of this tumor type from skin metastases of malignant non-Hodgkin lymphomas, melanomas, or of other tumors is very important

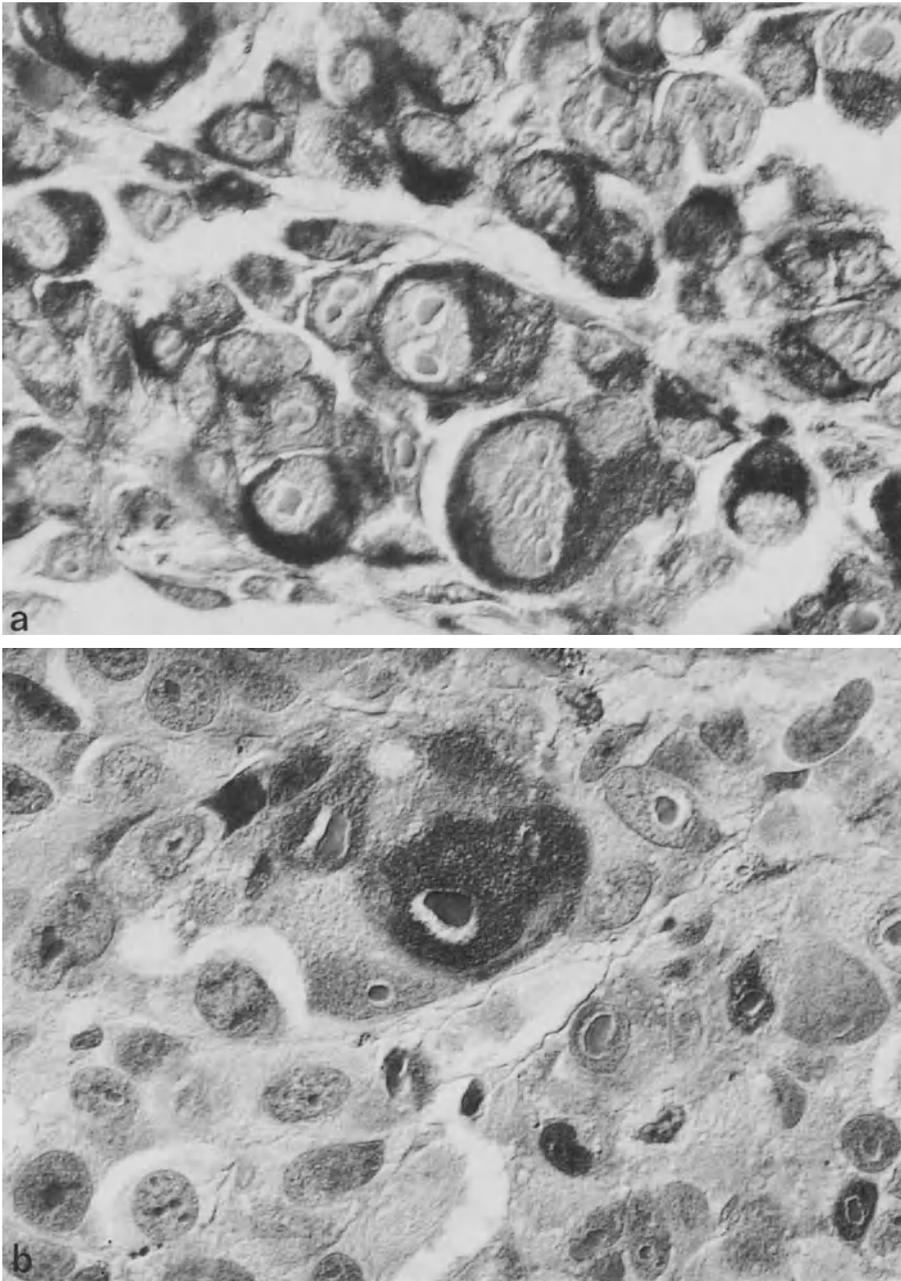


Fig. 10 a, b. Malignant melanoma. Avidin-biotin complex technique, differential interference contrast optics. **a** Localization of vimentin in the cytoplasm of the majority of tumor cells, $\times 1000$. **b** Localization of protein S-100 in several tumor cells, $\times 1000$

in view of its biological behavior of low grade malignancy and, therefore, of the therapeutic implications.

Most authors agree that Merkel cell tumors display characteristics of epithelial and neuroendocrine cells. They yield a strong reaction to cytokeratins 8 and 18, often a dot-like paranuclear reaction is present (Fig. 1 a). By this reaction, a metastasis of a malignant lymphoma, of melanoma, and of all types of sarcomas can be excluded. In addition, a generally conspicuous reaction for neuron-specific enolase (Fig. 1 b) in at least some of the tumor cells, and for chromogranin A in the large majority of the tumor cells and neurofilaments, permit the differentiation of Merkel cell tumors from all non-neuroendocrine neoplasms (GLOOR et al. 1982; WICK et al. 1983; GOULD et al. 1985; BATTIFORA and SILVA 1986; KOMMINOTH 1986). Additional characteristics of a Merkel cell tumor are the presence of a narrow rim of connective tissue between the dermal tumor and the epidermis, and the presence of cytoplasmic granules (diameter 100 to 200 nm; SILVA and MACKAY 1981; PILOTTI et al. 1982; FRIGERIO et al. 1983) and bundles of intermediate filaments (thickness approx. 10 nm). In a small number of tumors the production and secretion of peptides has been reported (GOULD et al. 1980; IWASAKI et al. 1981; DRIJKONINGEN et al. 1986), but this is apparently uncommon. In a recent series the absence of an immunocytochemical reaction for bombesin, leu-enkephalin, met-enkephalin, and beta-endorphin has been reported (WICK et al. 1985). In one report the presence of protein S-100 in Merkel cell tumors has been described (VOIGT et al. 1985). This finding is at variance with those of other authors.

11 Tumors Producing “Ectopic Hormones”

Rapidly accumulating evidence indicates that many tumors produce a variety of substances, including molecules with hormonal or hormone-like biological activity. Ectopic hormone production is one particular form of paraneoplasia. The term ectopic hormone production refers to production of tumor cells causing remote effects resembling known biological actions of hormones (LIDDLE et al. 1969). By definition, ectopic hormones are produced by tumors arising in an organ not secreting the substance under normal physiological conditions. This definition implies that hormone production by metastases of a tumor arising in an endocrine gland is eutopic rather than ectopic. However, it becomes increasingly difficult to apply the given definitions strictly, since new substances are currently found to be produced by hitherto unexpected tissues (HEITZ and STAUB 1981; HEITZ and OBERHOLZER 1984). The proof of ectopic hormone production is difficult. It includes, among other techniques, histology, electron microscopy, immunocytochemistry and immunoelectron microscopy (HEITZ et al. 1981; HEITZ 1986). It is impossible to correlate morphology as analyzed by conventional histology with the production of any hormones by a tumor.

A large number of symptoms induced by ectopic hormone production have been described. The most common tumors associated with ectopic hormone production are carcinomas of the bronchus and the pancreas, carcinoids in

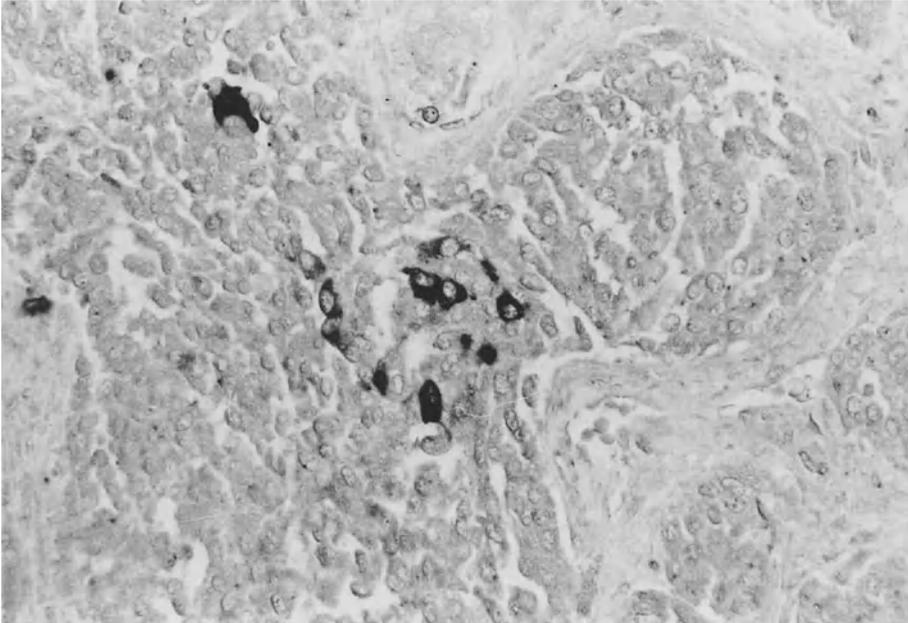


Fig. 11. Malignant paraganglioma of the mediastinum associated with Cushing's disease: production of opiocortins (lipotropin) in a group of tumor cells. Unlabeled antibody enzyme method, $\times 100$

various organs, and thymomas. In many organs the tumors are of the so-called small-cell type. The substances most frequently secreted by these tumors exert effects identical or similar to those of opiocortins (lipotropin-corticotropin-endorphin; Fig. 11), vasopressin (antidiuretic hormone), and gastrin. In addition, elevated serum concentrations of calcitonin and the alpha-chain of the glycoprotein hormones can often be found. The hormones can also be localized in the tumor by immunocytochemistry. Immunocytochemical evidence for ectopic hormone production by tumors is important, because it cannot be provided by conventional histology.

12 Conclusions

The discovery of tumor markers for neuroendocrine tumor cells has permitted far more precise characterizations of many tumors than conventional histology. However, a careful histological observation of a given tumor is a prerequisite for a rational and precise immunocytochemical analysis. In some cases, electron microscopy may be more appropriate than immunocytochemistry for the typing of a tumor, or both techniques might be used in combination to solve such a problem. In the near future *in-situ* hybridization techniques visualizing mRNA of markers may become an important and sensitive tool in typing tumor cells.

The definition of marker production is at present feasible for many tumors on formaldehyde-fixed and paraffin-embedded tissue. The correlation with clini-

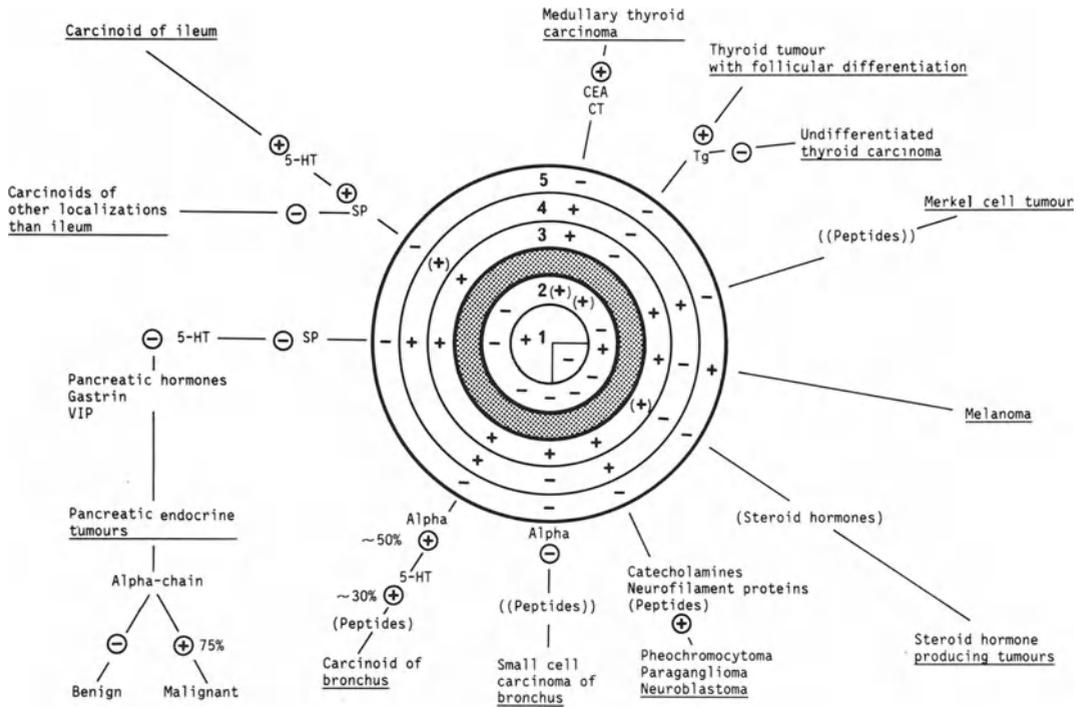


Fig. 12. Proposed systematic diagnostic flow-chart for neuroendocrine tumors. In general, only selected reactions are necessary to reach a final diagnosis. The indicated results of reactions refer exclusively to tumor cells. For details see text. First step of the diagnostic algorithm: Reactions 1 and 2, second step: reactions 3 to 5.

Key: First-step: 1, cytokeratins; 2, vimentin. Second step: 3, neuron-specific enolase; 4, chromogranin A; 5, protein S-100. *CT*, calcitonin; *CEA*, carcino-embryonic antigen; *Tg*, thyroglobulin; *Alpha-chain*, alpha subunit of glycoprotein hormones; *5-HT*, 5-hydroxytryptamine (serotonin); *VIP*, vasoactive intestinal polypeptide; *SP*, substance P. (): Reactions only in part of the tumors, (()): reactions uncommon

cal and biochemical findings can often be established. This represents great progress over conventional histology.

The extensive use of immunocytochemistry has revolutionized the morphological classification of some groups of neuroendocrine tumors. In order to make a classification useful it must be as simple as possible, and it must be meaningful to everybody working in the field. We therefore classify the tumors according to the leading clinical symptoms or to the predominantly secreted hormone. In addition, the probable biological behavior of the tumor (e.g. benign versus malignant), and additional secretory products are mentioned in the diagnosis.

It is furthermore important to realize that most tumors are not characterized by a single marker specific for the given type of tumor cells, but by a group of markers. A precise diagnosis can therefore often be reached by using a diagnostic flow-chart, using combinations of markers (Fig. 12). One important finding made by immunocytochemical observation of tumors was the frequently

occurring phenotypic cell heterogeneity of neuroendocrine tumors (and probably of many other tumors), exemplified by the simultaneous or asynchronous production of more than one secretory product by various cells of the same tumor. With a few exceptions, reliable markers of malignancy are not known at present. The search for this type of tumor marker must be one of the goals of future research.

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Morphological Markers in Neuro-Oncology

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1 Introduction

Like many other morphological disciplines, surgical neuropathology has been greatly advanced by the introduction of immunohistochemical methods. The assessment of antigenic marker proteins in nervous system tumors has generally led to a higher level of diagnostic accuracy. Although the spectrum of available antibodies with proven diagnostic usefulness is still limited, some previously difficult differential diagnoses have become less troublesome and ambiguous (BONNIN and RUBINSTEIN 1984). This is particularly true for the distinction of gliomas and embryonal CNS tumors from metastatic lesions of epithelial and mesenchymal origin, as well as from malignant lymphomas. In addition, immunocytochemistry has expanded our knowledge of the origin of some human brain tumors with a re-evaluation of several entities, the histogenesis and classification of which had been disputed for decades (ZÜLCH 1979) due to the lack of reliable histomorphological criteria. Thus, the identification of abundant glial fibrillary acidic protein (GFAP) in most giant cells of the 'monstrocellular sarcoma' has led to its re-classification as giant cell glioblastoma with a sarcoma-

tous component, i.e., a variant of the glioblastoma. The presence of numerous GFAP positive cells in superficially located cerebral neoplasms of young adults previously classified as malignant mesenchymal tumors, has allowed the identification of a new and now widely acknowledged tumor type, the pleomorphic xanthoastrocytoma (KEPES et al. 1979; GRANT and GALLAGHER 1986). The frequent lack of GFAP expression and the presence of NSE (STEFANSSON and WOLLMANN 1980, 1981) in the subependymal giant cell astrocytoma associated with tuberous sclerosis have caused speculations that these slowly growing, benign lesions may be partially of neuronal origin (NAKAMURA and BECKER 1983; BONNIN et al. 1984). It should, however, be emphasized that the presence within tumor cells of the astrocytic intermediate filament protein GFAP is not necessarily proof of their astrocytic origin. In contrast to their normal counterparts, neoplastic cells have a tendency towards unpredictable changes in gene expression, which may lead to the suppression of a gene product typically present, or the synthesis of marker proteins normally absent, in the cell line of origin. In embryonal tumors, the focal expression of glial or neuronal markers may be indicative of a tendency towards a distinct line of differentiation. In some instances, the possibility cannot be dismissed that a marker protein such as GFAP has been taken up from neighbouring reactive astrocytes. This is exemplified by the dilemma of how to interpret the occasional presence of GFAP in stromal cells of the cerebellar hemangioblastoma (DECK and RUBINSTEIN 1981; McCOMB et al. 1986). Future studies employing *in situ* hybridization techniques for the identification of specific mRNA sequences (LEWIS and COWAN 1985) may aid in overcoming such ambiguities.

2 Biochemically Defined Neural Marker Proteins

2.1 Glial Fibrillary Acidic Protein (GFAP)

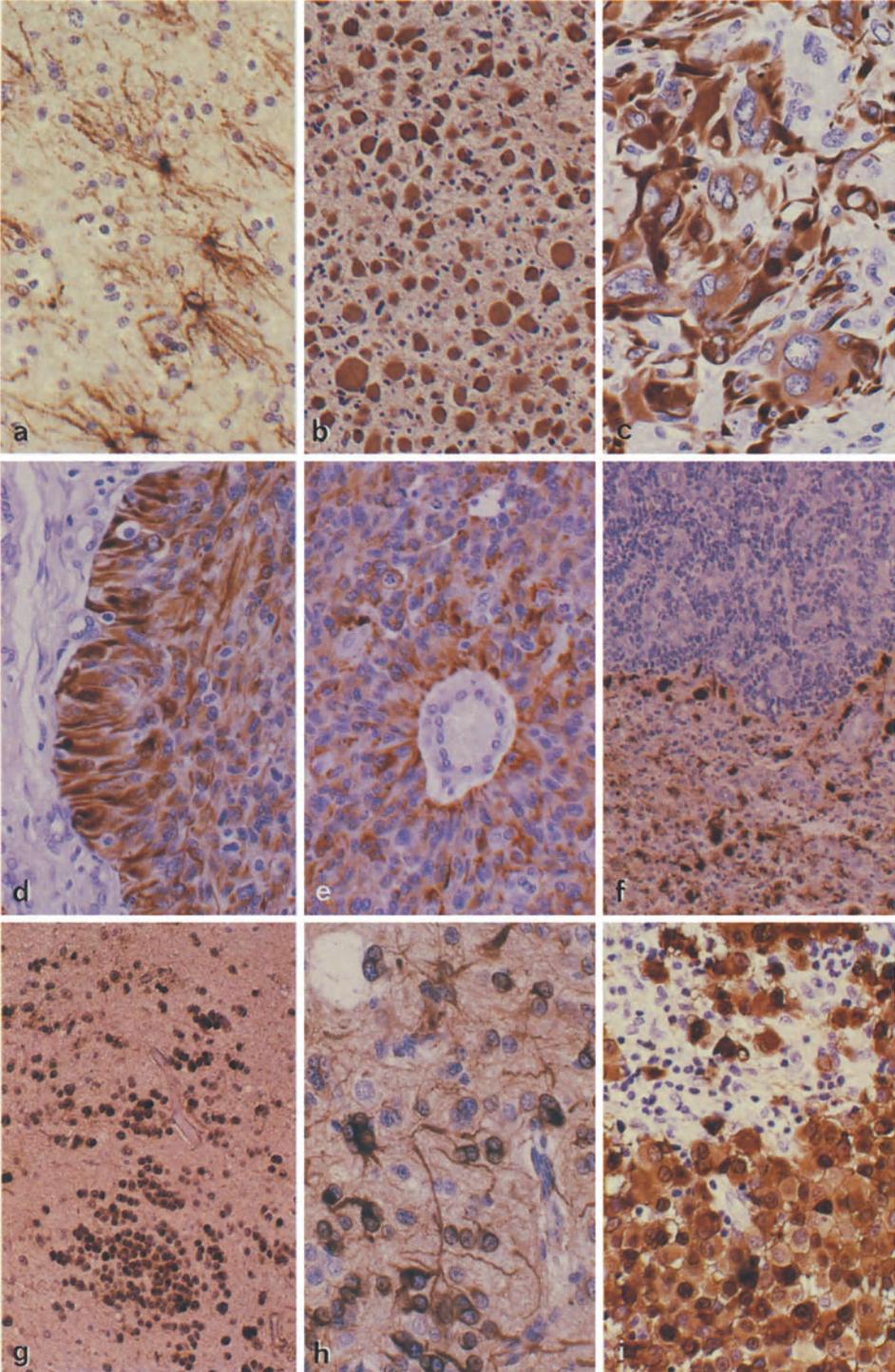
Since its discovery by ENG et al. in 1971, GFAP has been extensively investigated and is presently regarded as the most useful marker antigen for astroglial cells in health and disease. Its impact on surgical neuropathology is unrivalled by any other tumor marker. This is mainly due to the availability of a large panel of reliable antibodies, a high immunocytochemical "signal to noise" ratio and a low chance of cross reactivity with GFAP-like antigens in extraneural neoplasms.

Biochemical and immunological studies have revealed that GFAP is a cytoskeletal protein of 55 kD which was shown to polymerize *in vitro* to form smooth, curvilinear or rectilinear 10 nm intermediate filaments. When the *in vitro* assembly is carried out in the absence of reducing agents, approximately 50% of the total protein migrates as a dimer, indicating that the polymerization is accompanied by oxidation of SH-groups (RUEGER et al. 1979). There is recent evidence that subtypes of GFAP exist which can be distinguished by specific monoclonal antibodies (GHEUENS et al. 1984). It remains to be seen whether this polymorphism can be related to specific cell populations or disease entities.

The distribution of GFAP in the normal brain has been extensively reviewed (ENG 1980; OSBORN and WEBER 1983; TRAUB 1985) and is summarized in Table 1. It should be pointed out that the

Table 1. Expression of GFAP in normal and neoplastic tissues

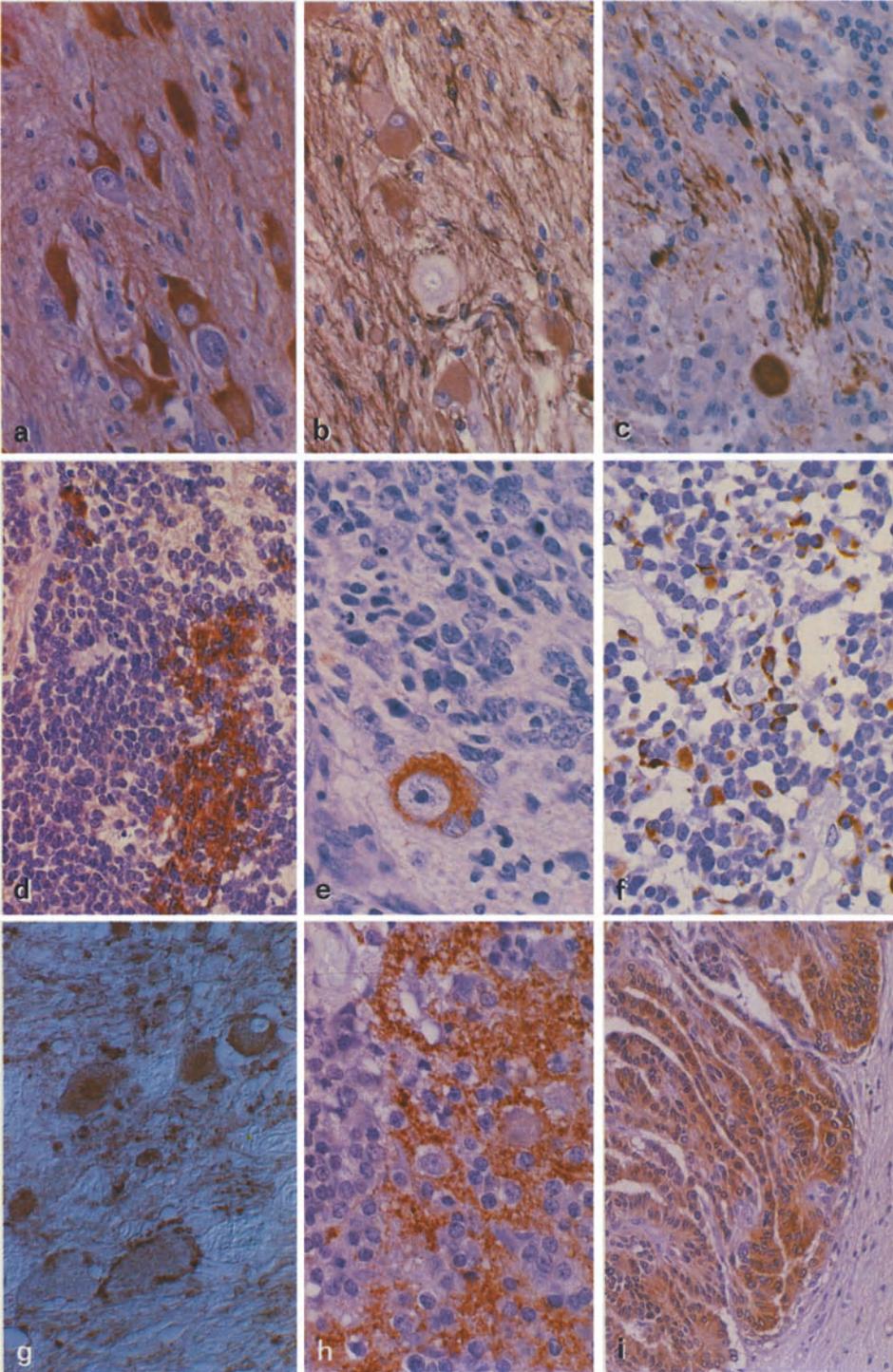
Cell type/Tissue	Comment
<i>Central nervous system</i>	
Astrocytes	More accentuated in fibrous (reactive) than in protoplasmic astrocytes
Bergmann glia	In normal cerebellum and, more markedly, following the loss of Purkinje neurons (SCHACHNER et al. 1977)
Radial glia	In the developing fetal brain (CHOI and LAPHAM 1978; CHOI 1986)
Tanycytes	Subependymal tapering cells (ROESSMANN et al. 1980; SHAW et al. 1981)
Oligodendrocytes	Transient expression during ontogeny (CHOI and KIM 1984)
Ependymal cells	Only during brain development (ROESSMANN et al. 1980)
Rosenthal fibers	Variable staining; amorphous core often negative (JANZER and FRIEDE 1981; SMITH and LANTOS 1984)
<i>Peripheral and autonomic nervous system</i>	
Schwann and satellite cells	Limited expression in human Schwann cells. Antigen may differ from that present in CNS astrocytes (JESSEN et al. 1984; JESSEN and MIRSKY 1985)
Enteric glia	Positively identified in rats (JESSEN and MIRSKY 1980)
<i>Neural tumors</i>	
Astrocytomas	Reliable marker for this tumor type (see KUMAR and MARSDEN 1986)
Oligoastrocytomas	Mainly in neoplastic astrocytes (HERPERS and BUDKA 1984; NAKAGAWA et al. 1986)
Oligodendrogliomas	In neoplastic 'gliofibrillary oligodendrocytes' (HERPERS and BUDKA 1984; NAKAGAWA et al. 1986)
Choroid plexus papillomas	Only focal expression (RUBINSTEIN and BRUCHER 1981)
Ependymomas	Variable expression; often accentuated in perivascular pseudorosettes (DUFFY et al. 1979)
Glioblastoma multiforme	Patchy distribution of GFAP, accentuated in the giant cell subtype
Medulloblastoma	Focal expression in approx. 10% of cases (see Table 2)
Neurofibromas	Only occasionally expressed (MEMOLY et al. 1984)
<i>Extraneural tissues</i>	
Adenohypophysis	Follicle-stellate cells (VELASCO et al. 1982; HÖFLER et al. 1984)
Epiglottis	Epiglottic cartilage (KEPES et al. 1984; BUDKA 1986)
Salivary gland	Myoepithelial cells (ACHTSTÄTTER et al. 1987)
Liver	Perisinusoidal stellate cells of rat liver (GARD et al. 1985)
<i>Extraneural tumors</i>	
Pituitary adenoma	Only one case reported (HÖFLER et al. 1984)
Pleomorphic salivary adenoma	Case report; only few cells were GFAP positive (NAKAZUTO et al. 1982)
Metastatic renal carcinoma	Case report (BUDKA 1986)
Papillary meningioma	Case report; triple expression of GFAP, vimentin and desmin (BUDKA 1986)



extent of GFAP expression is closely linked to the functional state of astroglia and greatly influenced by surrounding tissue elements. Generally, protoplasmic astrocytes contain little GFAP whereas reactive fibrous astrocytes show abundant GFAP expression (Fig. 1a). Exposure to cyclic AMP (SENSENBRENNER et al. 1980), wounding of the cerebral cortex with formation of a glial scar (BIGNAMI and DAHL 1976) and contact with mesodermal tissue (HERPERS et al. 1984) is accompanied by a marked increase in immunoreactive GFAP (Fig. 1d). Glial filament proteins are evenly distributed in the perikaryon and cytoplasmic processes, the cell nucleus usually being spared. Rosenthal fibers, i.e. conglomerates of glial filaments, present in a variety of chronic pathological conditions, show variable GFAP antigenicity but the surrounding rim of cytoplasm is usually strongly GFAP positive (JANZER and FRIEDE 1981; SMITH and LANTOS 1985). Co-expression of GFAP with vimentin is not uncommon and this has led to the suggestion that in response to functional differences, these intermediate filaments may act together in the formation of the cytoskeleton (WANG et al. 1984). There is, however, evidence that certain GFAP-positive cells do not express vimentin and these have tentatively been classified as protoplasmic astrocytes (SHAW et al. 1981). In addition, GFAP is frequently co-expressed with S-100 protein, particularly in reactive astrocytes and differentiated astrocytomas. In several neoplasms, however, immunoreactivities to these marker proteins dissociate and this may be a valuable diagnostic criterion (KIMURA et al. 1986).

Intermediate filament typing in diagnostic oncology is based on the concept that neoplastic cells retain the pattern of intermediate filament proteins typical for the tissue or cell type of origin (OSBORN and WEBER 1983; RAMAEKERS et al. 1983). For GFAP this has been largely confirmed, although the practicing pathologist should be aware of some notable exceptions (Table 1). The role of GFAP in the histopathological diagnosis of gliomas has been extensively reviewed (DECK et al. 1978; VELASCO et al. 1980; TASCOS et al. 1982; BONNIN and RUBINSTEIN 1984; COLLINS 1984; GULLOTTA et al. 1985; KUMAR and MARDEN 1986; SCHWECHHEIMER 1986). Two major limitations to the application of GFAP should be emphasized. First, it does not allow a clear-cut distinction of neoplastic cells from reactive astrocytes in the adjacent brain or from entrapped astrocytes within an invading neoplasm. Although in tumor cells GFAP expression is often restricted to the perikaryon and a few, plump cell processes (Figs. 1f, 2b), it can be very difficult to unequivocally identify astrocytic differentiation in non-astroglial tumors, e.g. oligodendrogliomas (HERPERS and BUDKA 1984; NAKAGAWA et al. 1986) and medulloblastomas (MANNOJI et al. 1981; COFFIN et al. 1983). In hamartomatous lesions, astrocytes may show long, but irregularly formed cell processes (Fig. 1h). Secondly, the histopathological assessment of GFAP in astrocytic tumors is of little assistance in tumor grading.

◁ **Fig. 1.** **a** Infiltration zone of a glioblastoma multiforme. Expression of GFAP by reactive astrocytes. In contrast to neoplastic astrocytes (shown in **b**), reactive astrocytes characteristically have numerous delicate cell processes. **b** Gemistocytic astrocytoma. GFAP-immunoreactivity is accentuated in the perinuclear cell body of neoplastic cells. Note the virtual absence of cell processes. **c** Glioblastoma multiforme. Marked expression of GFAP by large, multinucleated giant cells. **d** Anaplastic ependymoma. Expression of GFAP is accentuated at the border towards surrounding connective tissue. **e** Anaplastic ependymoma. GFAP-immunoreactivity is largely restricted to perivascular pseudorosettes. **f** Malignant glioma with an anaplastic focus lacking GFAP and a sharp transition into a more differentiated tumor area with marked GFAP expression. **g** Glial hamartoma of the temporal lobe. Clusters of undifferentiated small cells show marked immunostaining with S-100 protein. **h** Glial hamartoma (same case as in **g**). GFAP immunostaining shows malformed astrocytes with long, but irregular cell processes. **i** Cerebral metastasis of a malignant melanoma. Most tumor cells are strongly positive for S-100 protein



Although neoplastic progression with increasing anaplasia is usually paralleled by a decrease in GFAP expression (JACQUE et al. 1979; DUFFY et al. 1980), this inverse correlation is too variable to be of prognostic value.

Comparison of results obtained in different laboratories is often difficult since a steadily increasing range of commercially available polyclonal and monoclonal antibodies is in use, with variable specificity and sensitivity. In addition, the immunoreactivity of GFAP in paraffin-embedded sections depends on the dilution of the primary antibody (BIGBEE et al. 1977), the interval between tissue removal and fixation (DEARMOND et al. 1983), on the type of fixative and the length of fixation (DIXON and ENG 1981; JIE et al. 1986). With respect to the latter, it is generally agreed that rapid fixation in neutral buffered 4% formaldehyde (NBF) overnight or short fixation in NBF (1 hr) followed by 70% ethanol, give satisfactory results with most available antibodies (ZHENG et al. 1986). The quenching effect of formalin fixation is probably due to extensive protein cross-linking and can be partially reversed by trypsin digestion (MEPHAM et al. 1979; ENG 1985). Monoclonal antibodies tend to bind less consistently to GFAP in formalin-fixed tissues since each of them recognizes antigenic determinants which may be spatially closely related, but not identical. These epitopes may be unpredictably sensitive or resistant to fixation. It has, therefore, been proposed to use a 'cocktail' of monoclonal antibodies to GFAP. This makes a loss of immunoreactivity due to fixation-induced alteration of antigen structure less likely (MCLENDON et al. 1986). In contrast to polyclonal antisera, such MAB mixtures offer the advantage of continuous, unlimited supply.

2.2 S-100 Protein

A thermolabile, acidic protein of MW 20–25,000 found in bovine brain but not liver was first isolated in 1965 by MOORE. It was named S-100 because of its solubility in 100% ammonium sulphate solution at neutral pH. Later it was shown to consist of two polypeptide chains of 93 and 91 amino acids (alpha- and beta-subunit), sharing 54 identical amino acids. S-100-alpha and S-100-beta form homo- and heterodimers (alpha/alpha = S-100a_o, alpha/beta = S-100a, beta/beta = S-100b) but the physiological role of these dimers has not been determined (ISOBE et al. 1983).

Based on biochemical structural similarity, it was suggested that S-100 was a member of the group of calcium binding proteins. There is a soluble and a membrane-bound form of S-100 (HAGLID et al. 1976). The latter is bound to the cytoplasmic side of mitochondria, endoplasmic reticulum

< **Fig. 2.** **a** Cerebral ganglioglioma. Marked GFAP expression by the neoplastic glial component. **b** Cerebral ganglioglioma. NSE immunoreactivity in numerous neoplastic neuronal cell bodies and processes. **c** Spinal ganglioneuroblastoma. NFP expression by ganglionic cells and their processes. **d** Focal expression of NSE in a medulloblastoma, indicating neuronal differentiation. **e** Medulloblastoma with neuronal differentiation. NFP expression is restricted to mature ganglionic cells. **f** Medulloblastoma with astrocytic differentiation. GFAP immunoreactivity is restricted to a small perinuclear cytoplasmic rim. **g** Ganglioglioma. Synaptophysin stains the neuronal tumor compartment. Note the punctuate accentuation along the plasma membrane, reflecting the distribution of synaptic junctions. **h** Supratentorial primitive neuroectodermal tumor (PNET) with focal neuronal differentiation. Synaptophysin immunoreactivity in these areas shows a granular, network-like pattern. **i** Diffuse expression of carbonic anhydrase C in the cerebral metastasis of an anaplastic choroid plexus papilloma

and Golgi apparatus. It is found in free and membrane-bound ribosomes and can be secreted into the extracellular space (COSGRAVE et al. 1983). In addition to its cytoplasmic distribution, S-100 is expressed in the cell nucleus where a stimulatory effect on RNA polymerase I has been reported (MICHETTI et al. 1976). Its synthesis is cell cycle dependent and highest in quiescent cells (FAN 1982), but nothing is known about a possible gene-regulatory function. In the development of the CNS it promotes neurite extension (KLIGMAN and MARSHAK 1985), and has been implicated in microtubule disassembly (BAUDIER et al. 1982), in cation permeability regulation of lipid membranes (CALISSANO and BANGHAM 1971) and in the regulation of protein phosphorylation and dephosphorylation (KUO et al. 1986). Its detailed biological function, however, is still unknown (for review see DONATO 1986).

Originally thought to be nervous system-specific, S-100 has now been identified in over 20 different cell types of normal tissues and their neoplastic counterparts (DONATO 1986; NAKAJIMA et al. 1984; WEISS et al. 1983). Within the normal nervous system, it is present in astrocytes and oligodendrocytes (LUDWIN et al. 1976), ependymal cells (YAMAGUCHI 1980), in some neurons (HAAN et al. 1982; LOEFFEL et al. 1985; MOLNAR et al. 1985; VANSTAPEL et al. 1985), and in Schwann cells (VANSTAPEL et al. 1985). In contrast to GFAP, it is preferentially expressed in cell nuclei (Figs. 2g, h). The relative amount and the distribution of the S-100 subunits in different human tumors is very variable (TAKAHASHI et al. 1984). More than 90% of the cerebral S-100 belongs to the S-100b fraction (JENSEN et al. 1985). Accordingly, the immunohistochemical staining obtained by heterologous polyclonal antibodies recognizing both subunits is very similar, if not identical, to that seen with monospecific anti-S-100-beta antisera as far as the normal (MOLNAR et al. 1985; LOEFFEL et al. 1985; VANSTAPEL et al. 1985) and neoplastic (VAN ELDICK et al. 1986) nervous system is concerned. S-100 is expressed in astrocytomas, ependymomas, glioblastomas, schwannomas, glial hamartomas (Fig. 1g), and the adamantinomatous areas of craniopharyngiomas. In addition, it is strongly positive in melanocytic lesions and can be expressed in chordomas, chondrosarcomas, lipomas, liposarcomas, fibromatosis, histiocytosis X, eosinophilic granulomas, mixed sweat gland tumors, pleomorphic adenomas of the salivary gland, medullary carcinomas of the breast, bronchioalveolar carcinomas of the lung and teratomas of the ovary (KAHN et al. 1982; NAKAMURA et al. 1983; TAKAHASHI et al. 1984; WEISS et al. 1983). Its principal diagnostic application in neuro-oncology is the differential diagnosis of benign and malignant spindle cell tumors since it is consistently expressed in schwannomas and neurofibromas (WEISS et al. 1983; WEIDENHEIM and CAMPBELL 1986). The strong intracytoplasmic expression of S-100 in all melanocytic lesions, including amelanotic melanomas (Fig. 1i) has also proved very useful. It has recently been reported that the alpha-subunit of S-100 is present in human malignant melanomas, but not in Schwann cell-derived tumors of the peripheral nervous system (ISOBE et al. 1984).

2.3 Neuron-Specific Enolase

Neuron-specific enolase (NSE) was first isolated from brain tissue by MOORE and co-workers (1965) and initially referred to as 14.3.2 protein, based on chromatographic and electrophoretic criteria (MOORE 1975). Later, it was designated neuron-specific protein (MARANGOS et al. 1975) until BOCK and DISSING (1975) discovered that this protein was in fact an isoenzyme of the glycolytic enzyme

enolase (2-phospho-D-glycerate hydrolase, E.C. 4.2.1.11). Like several other glycolytic enzymes, enolase exists as a set of isoenzymes. Each of the 5 dimeric isoenzymes known is composed of two out of three immunologically distinct subunits (α , β and γ) which are coded for by separate genes (PEARCE et al. 1976; CHEN and GIBLET 1976). Unlike liver and skeletal muscle which express mainly a single isoenzyme ($\alpha\alpha$ or $\beta\beta$, respectively) it has been established by chromatographic and immunoassay techniques (MARANGOS et al. 1979; SUZUKI et al. 1980) that nervous tissue contains a mixture of isoenzymes, two homodimers ($\alpha\alpha$ and $\gamma\gamma$) and one heterodimer ($\alpha\gamma$). The γ subunit, present either in the hybrid or $\gamma\gamma$ form, accounts for approximately 2% of total soluble brain proteins (MARANGOS et al. 1979; HULLIN et al. 1980; KATO et al. 1982). Most other tissues (e.g. liver and skeletal muscle) have γ subunit levels three orders of magnitude lower than the brain. Compared to the $\alpha\alpha$ homodimer (MW 87,000), $\gamma\gamma$ enolase is characterized by a lower molecular weight (MW 78,000), a more acidic isoelectric point and greater stability towards chloride inactivation (MARANGOS et al. 1978; SCHMECHEL 1985). Immunochemical studies of the subunit distribution in normal tissues have been performed employing purified antibodies specific for either the α or γ subunits (ROYDS et al. 1982; HAIMOTO et al. 1985). At present, immunohistochemical techniques do not allow discrimination between the neuron-specific ($\gamma\gamma$) and the hybrid ($\alpha\gamma$) enolase.

Within the CNS, γ enolase is almost exclusively localized in neurons whereas α enolase expression is found in glial, meningotheial, endothelial and Schwann cells and has thus been referred to as non-neuronal enolase (NNE) (SCHMECHEL et al. 1978). Neuroblasts also contain NNE, but show a switch of expression from α to γ enolase during the process of differentiation (SCHMECHEL et al. 1980). Immunohistochemical and the more sensitive enzyme-linked immunoassay techniques have demonstrated that γ -enolase (mainly in the hybrid form) is also widely distributed in extraneural tissues. High levels of enolase are present in the adrenal and pituitary gland, in cells of the diffuse neuroendocrine system (SCHMECHEL et al. 1978), and in a variety of non-neuronal and non-neuroendocrine cell populations (HAIMOTO et al. 1985).

In spite of the recognition that this protein is not neuron-specific, γ -enolase is currently employed as a broad-range marker for neoplastic neuronal and neuroendocrine cells (TAPIA et al. 1981; GHOBRIAL and ROSS 1986; TAYLOR et al. 1986). Within the CNS, it has been used as an indicator of neuronal differentiation in neuroblastomas (ODELSTAD et al. 1981), retinoblastomas (NAKAJIMA et al. 1984) and a variety of primitive neuroectodermal tumors, including the medulloblastoma (Fig. 2d). Reservation about its usefulness for the classification of CNS tumors, however, has arisen from the co-expression of GFAP and NSE in reactive astrocytes (VINOES et al. 1984; VINOES and RUBINSTEIN 1985) and the positive immunoreactivity of tumor cells occasionally found in various types of gliomas (HAGLID et al. 1973; NAKAJIMA et al. 1984; VINOES et al. 1984), in meningiomas (NAKAJIMA et al. 1984) and neurinomas (NAKAJIMA et al. 1984). This clearly demonstrates that the γ subunit of NSE can no longer be regarded as a neuron-specific tumor marker. NSE is further expressed in neuroblastomas, ganglioneuroblastomas, gangliogliomas (Fig. 2b), paragangliomas (IRONSIDE et al. 1985) and in the majority of neuroendocrine tumors, including intermediate and small cell carcinomas of the lung. In addition, there are numerous studies reporting NSE-like immunoreactivity in non-neuronal tumors including malignant melanomas, Schwann cell neoplasms, chordomas, fibroadenomas of the mammary gland and a variety of carcinomas of non-neuroendocrine origin (DHILLON et al. 1982; NAKAJIMA et al. 1983, 1984; VINOES et al. 1984; NESLAND et al. 1986; PÅHLMAN et al. 1986).

2.4 Neurofilament Proteins (NFP)

Neurofilaments constitute one of five subclasses of intermediate filaments. All intermediate filaments have a strongly conserved, rod-shaped, alpha-helical 40 kD domain, probably derived from an ancestral intermediate filament gene (SHAW et al. 1984; PRUSS et al. 1981). NFP have a hypervariable amino-terminal head and carboxy-terminal tail on both sides of the 40 kD alpha-helix. The tail ends of the neurofilament proteins, which are unusually long for an intermediate filament, contain a domain with a high content of acidic residues (LIEM et al. 1985a). This "b" domain represents the binding site for the Bodian silver stain, which specifically stains neurofilament proteins (WEBER et al. 1983; GAMBETTI et al. 1981). Expression of neurofilament proteins has, so far, only been recorded in neuronal cells, predominantly in axons (DRAKE and LASEK 1984). Its function is not clear, but may not be essential since it is entirely lacking in the well functioning nervous system of arthropods. Neurofilaments are composed of 3 subclasses of polypeptide chains with molecular weights of 68–75, 140–175 and 180–212 kD (SHAW et al. 1984). Since there is a relatively great interspecies variation in molecular weight the terms H-, M- and L-subunit (for "high", "middle" and "low") are commonly used. These subunits are differentially expressed during brain development (SHAW and WEBER 1982) and have a differential distribution within the neuronal cell (HIROKAWA et al. 1984). Although all three subunits are found in the axon as well as in the cell body and dendrites, phosphorylated epitopes on the H- and M-subunits recognized by monoclonal antibodies have so far only been detected in the axon, but not in cell bodies or dendrites (STERNBERGER and STERNBERGER 1983; GOLDSTEIN et al. 1983). The H-subunit forms cross-bridges between neurofilaments (HIROKAWA 1982) while the L-subunit represents the binding site for cross-links with microtubule associated proteins, including tau-protein (LIEM et al. 1985b). In diagnostic neuro-oncology, commercially available monoclonal antibodies recognizing the H- and M-subunit have preferentially been used. In most reports, it is not specified whether these reacted with phosphorylated or non-phosphorylated epitopes. Despite these limitations, neurofilament protein expression in tumor cell bodies has only been found in neuronal tumors, e.g., pheochromocytoma, ganglioneuroma, ganglioneuroblastoma (Fig. 2c), and neuroblastoma (MUKAI et al. 1986; OSBORN et al. 1986; TROJANOWSKI et al. 1984). In our experience, the presence of neurofilaments is highly specific for neuronal tumors. However, NFP are most consistently expressed in neoplasms with advanced neuronal differentiation and may be difficult to detect in undifferentiated neuroblastomas.

2.5 Synaptophysin

Synaptophysin, an acidic homooligomeric glycoprotein of subunit MW 38,000 was first isolated from rat and bovine brain in 1985 (JAHN et al. 1985; WIEDENMANN and FRANKE 1985). Its expression during brain development correlates with synaptogenesis and nerve terminal differentiation (KNAUS et al. 1986). Recent biochemical characterization has identified this protein as an integral calcium-binding membrane protein of presynaptic vesicles in neurons and similar vesicles in neuroendocrine

cells (WIEDENMANN and FRANKE 1985; REHM et al. 1986). Synaptophysin appears to span the membrane of these vesicles and has an epitope exposed on the cytoplasmic surface to which several polyclonal (JAHN et al. 1985) and monoclonal antibodies have been raised (WIEDENMANN and FRANKE 1985; WIEDENMANN et al. 1986). The epitope recognized by the antibody SY 38 is reliably demonstrable by immunofluorescence in frozen sections (WIEDENMANN et al. 1986; SCHWECHHEIMER et al. 1987) and in conventionally fixed, routinely processed paraffin embedded material (for details see GOULD et al. 1986).

In normal tissues, immunoreactivity is present in all types of neurons, at the neuromuscular junction of cross striated muscle, in the adrenal medulla and certain cells of the dispersed neuroendocrine system (WIEDENMANN and FRANKE 1985; GOULD et al. 1986; WIEDENMANN et al. 1986). Immunostaining characteristically reveals a fine punctuate cytoplasmic pattern which most probably reflects the distribution of synapses and/or neurosecretory vesicles. Accordingly, immunoreactivity in mature CNS neurons is accentuated at the cell periphery (Fig. 2g), whereas in neuroendocrine cells it is spread rather evenly throughout the cytoplasm (WIEDENMANN et al. 1986; GOULD et al. 1987). Within the neuroendocrine system, synaptophysin is present in both "neural" and "epithelial" cell types, indicating its independence from cytoskeletal characteristics or the neuropeptide composition of individual cells (GOULD et al. 1986). Thus, co-expression with the neurofilament triplet, cytokeratins, vimentin or a combination of intermediate filament markers has been observed.

Synaptophysin immunostaining was found to be reliable and specific for a variety of differentiated and undifferentiated neuronal tumors, including neuroblastomas, ganglioneuroblastomas, ganglioneuromas, glio-neuronal hamartomas as well as primitive neuroectodermal tumors (Fig. 2h), including the medulloblastomas (GOULD et al. 1986; SCHWECHHEIMER et al. 1987). Synaptophysin is further expressed in almost the entire spectrum of neural and epithelial neuroendocrine neoplasms, irrespective of their degree of differentiation, cytoskeletal constituents and hormone production. In contrast to NSE, immunohistochemical studies of malignant epithelial and mesenchymal tumors have so far failed to show synaptophysin expression. The consistent negativity of malignant melanomas is particularly remarkable, despite their positive immunoreactivity for NSE and several neuropeptide antigens. The absence of synaptophysin immunostaining in non-neural and non-neuroendocrine cells and in neoplasms derived therefrom indicates that synaptophysin may prove to be a specific and highly reliable marker for the identification of neuroblastic and neuroendocrine cells.

3 Human Brain Tumor-Associated Antigens Defined by Monoclonal Antibodies

Being directed towards a single antigenic determinant, MABs have the potential of recognizing proteins that are unique to certain tumor types and restricted to neoplastic cells. In addition, the continuous production of a homogeneous antibody could result in diagnostic and therapeutic applications *in vivo*. Over the past five years, a great number of murine MABs has been raised, using different approaches for immunization. Their potential use and limitations have recently been reviewed by BULLARD and BIGNER (1985). These authors concluded that the antibodies developed so far have greatly advanced our knowledge of the complexity of antigenic determinants in human brain and brain tumors, but that their practical use is still limited by a variety of inherent disadvantages. These include a marked phenotypic heterogeneity of human neuroectodermal tumors, limited sensitivity, inability to detect certain human histocompatibility complex (HLA) specificities and to evoke in mice the autochthonous human immunological response to tumor-associated antigens.

Following immunization with fetal human brain, KEMSHEAD and co-workers developed the murine antibody UJ13A which selectively binds to tissues of neuroectodermal origin and neoplasms derived

therefrom. This pan-neuroectodermal antibody has been successfully used to distinguish neural tumors from lymphomas and epithelial metastatic lesions but the antigen is inactivated by fixation (ALLAN et al. 1983). This also applies to a panel of MABs to neuroectodermal oncofetal antigens developed by WIKSTRAND et al. (1982) and GARSON et al. (1985) which recognize neuroectodermal tumors as well as human fetal CNS and lymphoid tissues. A similar specificity for neuroectodermal tumors and oncofetal tissues was found in a hybridoma raised against melanoma cells (LIAO et al. 1981). In several studies, antibodies were produced by immunizing mice with cultured glioma cell lines. The MABs BF7 and GE7 demonstrated specific binding to glioma cells in a radioimmunoassay (SCHNEGG et al. 1981) but histologically detectable immunoreactivity was present in both normal astrocytes and a subset of neoplastic glial cells in human gliomas (KRAJEWSKI et al. 1986). Special fixation and embedding techniques were necessary to maintain antigenicity.

A detailed analysis of MABs developed by immunization with human glioma-derived cell lines (HGL) revealed a complex pattern of antigenic heterogeneity (WIKSTRAND et al. 1985). Human glioma lines which reacted most extensively with anti-HGL MABs also had the highest reactivity rate with anti-lymphoid marker antibodies. Antigenicity in frozen sections from human glioblastomas and astrocytomas was found to be even more complex, with immunoreactivity to anti-HGL MABs present in 66–100% of cases. None of the MABs tested was sufficiently specific and reliable for diagnostic purposes. This also holds true for most antibodies raised against neuroblastomas which usually cross-react with haematopoietic cells and neuroectoderm-derived tissues and tumors. The recently developed MAB 5A7 appears to be more selective, showing no reactivity with normal or neoplastic haematopoietic cells (GROSS et al. 1986).

In conclusion, the MABs raised in an attempt to selectively define glioma-associated antigens are at present not very helpful in the histopathological diagnosis of human nervous system tumors, mainly due to extensive cross-reactivity with normal CNS cells and extraneural tissues and their general sensitivity to formalin fixation.

4 Application of Tumor Markers to Diagnostic Neuro-Oncology

4.1 Neuronal Tumors

Highly differentiated neuronal neoplasms do not usually give rise to diagnostic problems. This is particularly true for gangliocytomas, gangliogliomas, ganglioneuromas and ganglioneuroblastomas in which mature ganglionic cells can be identified both by their histological appearance and immunoreactivity for NSE (Fig. 2b), NFP (Fig. 1c) and synaptophysin (Fig. 2g). However, these neuronal marker proteins can be of great assistance in the interpretation of neoplasms with less advanced neuronal differentiation. Thus, retroperitoneal and adrenal neuroblastomas can be difficult to distinguish from other small round-cell tumors (e.g., Ewing's sarcoma, malignant lymphoma, rhabdomyosarcoma, Wilms' tumor). *Neuroblastomas* can be positively identified by their immunoreactivity for NFP, but abundant neurofilaments are only present in the more differentiated neoplasms. In immature neuroblastomas, immunoreactivity for NFP is lower and can easily be abolished by fixation. A recent report by OSBORN et al. (1986) indicates that alcohol fixation and paraffin embedding causes a preferential inactivation of epitopes recognized by antibodies to the low molecular weight NFP. This is at variance with a study by MUKAI et al. (1986) who found that in formalin-fixed, paraffin-embedded neuroblastomas, a polyvalent rabbit antiserum to the 68-kD subcomponent was most reliable and sufficiently discriminating for diagnostic application. NSE is also con-

sistently expressed in neuroblastomas, but is less specific than NFP since it has also been found in a variety of other tumors (VINORES et al. 1984; NAKAJIMA et al. 1984; OSBORN et al. 1986). Synaptophysin (GOULD et al. 1986) and microtubule-associated proteins (ARTLIEB et al. 1985) may soon be established as the most useful marker proteins for neuroblastomas and neoplasms of the diffuse neuro-endocrine system since their distribution appears to be highly specific and their immunoreactivity less affected by fixation. None of these markers has been shown to be of use in the grading of neuroblastomas. In contrast, immunoreactivity to S-100 protein has recently been claimed to be associated with favourable prognosis (SHIMADA et al. 1985).

In the *olfactory neuroblastoma*, both NSE (CHOI and ANDERSON 1985) and synaptophysin (Fig. 2h) are expressed in irregularly shaped cell clusters. Immunoreactive S-100 protein is restricted to cells that form an interconnecting network at the periphery of lobular tumor cell nests (CHOI and ANDERSON 1985). With progressive differentiation, olfactory neuroblastomas also express NFP (TROJANOWSKI et al. 1982). Due to their common origin from photoreceptor cells, retinoblastomas and pineal parenchymal tumors (pineocytomas) express the retinal S-antigen (DONOSO et al. 1985; KORF et al. 1986; PERENTES et al. 1986).

4.2 Astrocytomas

The most valuable marker for normal and neoplastic astrocytes is *GFAP* (RAFF et al. 1979; BONNIN and RUBINSTEIN 1984). In astrocytomas, there is a general tendency for decreasing GFAP expression to accompany increasing dedifferentiation (TASCOS et al. 1982; VELASCO et al. 1980; JACQUE et al. 1979). This is well illustrated (Fig. 1f) by the occasional finding in anaplastic gliomas of sharply delineated foci of small anaplastic subclones with complete lack of GFAP expression within more differentiated areas with correspondingly high levels of GFAP (SCHMITT 1983a, 1983b). However, even in the most anaplastic variants of astrocytoma, there are some tumor cell clusters still expressing GFAP. On the other hand, in highly differentiated astrocytomas there is never GFAP-expression by 100% of the tumor cells. Occasionally, low grade astrocytomas demonstrate a surprisingly meager GFAP-expression whereas frankly malignant lesions may contain a high incidence of GFAP-positive tumor cells (KIMURA et al. 1986). Two immunohistological staining patterns are usually observed. Cytoplasmic staining with perinuclear accentuation is mostly found in protoplasmic and gemistocytic astrocytomas, in the astrocytic component of gangliogliomas (Fig. 2a), and in the bizarre giant cells of anaplastic astrocytomas. A diffuse staining of the fibrillary matrix corresponds to neoplastic cell processes in pilocytic and fibrillary astrocytomas. Small round cells with scanty cytoplasm and processes tend not to express GFAP. Rosenthal fibres are typically found in pilocytic astrocytomas, but are occasionally encountered in other subtypes of astrocytic tumors (HWANG and BORIT 1982). They show a variable immunoreactivity for GFAP but a positively stained cytoplasmic rim is consistently visible (JANZER and FRIEDE 1981; SMITH and LANTOS 1985). Pale eosino-

philic hyaline granules, structures of unknown origin and significance, are often present in pilocytic and fibrillary astrocytomas (SMITH and LANTOS 1985) and stain diffusely positive for GFAP.

The expression of *S-100 protein* in astrocytomas is very similar to that of GFAP. Co-expression has been reported (KIMURA et al. 1986), with a similar staining pattern of perinuclear accentuation in gemistocytic lesions *versus* a preferential staining of cell processes in pilocytic and fibrillary astrocytomas. However, there is often an additional expression of S-100 protein in the nucleus of neoplastic astrocytes, especially in small tumor cells with scanty cytoplasm. A dissociation of immunoreactivity with preferential or selective expression of S-100 protein is found in the subependymal giant cell tumors associated with tuberous sclerosis (KIMURA et al. 1986), in glial hamartomas of the temporal lobe (Fig. 1g) and, occasionally, in malignant astrocytic tumors. As for GFAP, a low S-100 protein content correlates with advanced dedifferentiation (JACQUE et al. 1979).

Another useful marker is *glutamine synthetase*, an enzyme abundant in extraneural tissues like liver, but within the CNS restricted to normal, reactive and neoplastic astrocytes (PILKINGTON and LANTOS 1982). Co-expression with GFAP has been reported. The overall staining pattern is very similar to that found with GFAP and S-100 protein, including the tendency for decreasing expression with increasing dedifferentiation of the astrocytoma. However, there are some differences in the cellular staining pattern. Glutamine synthetase is expressed most heavily in the cytoplasm of protoplasmic astrocytes with a low content of glial intermediate filaments and to a lesser extent in the cell processes of fibrous astrocytes.

The following markers are not specific for astrocytomas. Expression of *NSE* (mostly the alpha-, but to a lesser extent also the gamma-subunit) has been observed in all histopathological variants. In most astrocytic neoplasms, a small proportion of cells co-expresses NSE and GFAP, while in the majority of cells either NSE or GFAP is exclusively present (VINOES and RUBINSTEIN 1985). *Vimentin*, an intermediate filament protein of 57 kD, originally thought to be specifically expressed in mesenchymal cells (ALTMANNBERGER et al. 1981), is consistently expressed in normal (CHIU et al. 1981) and neoplastic (YUNG et al. 1985) astrocytes. Its cellular staining pattern is very similar to that of GFAP. GFAP-positive cells in astrocytomas may also be positive for vimentin, but some vimentin-positive cells in astrocytomas are GFAP-negative (WAI-KWAN et al. 1985). Since vimentin is expressed early in astroglialogenesis (DAHL 1981; PIXLEY and DEVELLIS 1984), one could assume that its presence in astrocytomas indicates a lower degree of differentiation. However, this could not be established in larger series comparing GFAP and vimentin expression in human gliomas (SCHIFFER et al. 1986).

Fibronectin expression in astrocytomas is controversial. *In vivo* studies have failed convincingly to demonstrate fibronectin in locations other than the extracellular matrix produced by leptomeningeal or blood vessel cells. A faint diffuse staining pattern with an intercellular distribution around neoplastic glial cells was interpreted as being due to vessel leakage (PAETAU et al. 1980; BELLON et al. 1985). However, neoplastic cells derived from astrocytomas definitely ex-

press fibronectin *in vitro*, in addition to laminin and collagen (MCKEEVER et al. 1986). Within individual neoplastic glial cells, co-expression of fibronectin with GFAP, S-100 or NSE has never been observed. On the other hand, non-neoplastic astrocytes are able to produce fibronectin and laminin *in vitro*, and co-expression of fibronectin and GFAP within the cytoplasm of embryonic and early postnatal rat brain cells has recently been reported (LIESI et al. 1986). *NFP* and *synaptophysin* are not expressed in neoplastic astrocytes. Staining of entrapped axons is, however, frequently observed.

4.3 Oligodendrogliomas

Several marker proteins have been identified in human and rodent oligodendrocytes, including galactocerebroside (RAFF et al. 1978), cyclic nucleotide phosphodiesterase (KIM et al. 1984), myelin-specific P₂ (TRAPP et al. 1983) and proteolipid proteins (HARTMAN et al. 1982). However, these antigens are not consistently expressed in neoplastic oligodendrocytes. This is similarly true for myelin basic protein (MBP), myelin-associated glycoprotein (MAG) and carbonic anhydrase C (KUMPULAINEN et al. 1982; WELLER et al. 1985; NAKAGAWA et al. 1986). Although some oligodendrogliomas show patchy immunoreactivity for NSE and S-100 protein, these markers are neither reliable nor specific for this tumor type (WELLER et al. 1985; NAKAGAWA et al. 1986).

GFAP immunoreactivity in oligodendrogliomas has been identified in three cell types. As in a variety of other CNS tumors, reactive astrocytes are found to be entrapped within the tumor or around the vascular mesenchymal stroma. These reactive astrocytes are characterized by their stellate morphology and abundant cytoplasm (NAKAGAWA et al. 1986). Neoplastic astrocytes with eccentric nuclei are mainly found in oligo-astrocytomas, often closely intermingled with GFAP-negative neoplastic oligodendroglial cells. In addition, about half of all oligodendrogliomas contain GFAP-positive neoplastic oligodendrocytes which may be scattered singly or in clusters throughout the tumor. These cells are categorized as neoplastic by their typical appearance, with centrally located nuclei surrounded by a small rim of immunoreactive cytoplasm (HERPERS and BUDKA 1984; NAKAGAWA et al. 1986). Although various interpretations have been put forward to explain the presence of GFAP-positive neoplastic cells with oligodendroglial morphology, most authors favour the hypothesis of HERPERS and BUDKA (1984), who related this feature to the transient ontogenetic GFAP expression by oligodendrocytes during myelinogenesis (CHOI and KIM 1984). Alternatively, the possibility of a divergent differentiation based on the existence of a common bipotential glial pregenerator cell for astrocytes and oligodendrocytes (RAFF et al. 1983) has been discussed.

The monoclonal antibody *anti-Leu 7* (ABO and BALCH 1981), a marker for natural killer cells (HNK-1), recognizes a carbohydrate determinant present on a family of neural plasma membrane glycoproteins, including myelin-associated glycoprotein (MAG), the neural cell adhesion molecule (N-CAM), and the L1 and J1 glycoproteins (KRUSE et al. 1985). It is expressed in various components of the nervous system, including oligodendroglia (SCHULLER-PE-

TROVIC et al. 1983). Consequently, anti-Leu 7 has been suggested as a useful marker for oligodendrogliomas (MOTOI et al. 1985). This was confirmed by PERENTES and RUBINSTEIN (1986) and NAKAGAWA et al. (1986) who tested a large series of oligodendrogliomas, as well as a variety of other neuroepithelial tumors. To a variable extent, up to 90% of oligodendrogliomas were stained with anti-Leu 7. Although not specific, it was much more abundant (in terms of the number of immunoreactive tumor cells) in oligodendrogliomas than in other gliomas, primitive neuroectodermal tumors and schwannomas (PERENTES and RUBINSTEIN 1985). Immunoreactivity is restricted to cell membranes and processes, in contrast to the intracytoplasmic immunoreactivity for GFAP. Thus, the demonstration of abundant cell-surface anti-Leu 7 immunoreactivity in combination with interspersed GFAP-positive neoplastic cells may be helpful in the diagnosis of oligodendroglial neoplasms.

4.4 Ependymal and Choroid Plexus Tumors

No marker antigen specific for normal or neoplastic ependymal cells has yet been identified. In analogy to fetal ependymal lining cells (ROESSMANN et al. 1980), ependymomas focally express S-100 protein and GFAP (DUFFY et al. 1979; WELLER et al. 1985; KIMURA et al. 1986; MIETTINEN et al. 1986), preferentially in the perivascular pseudorosettes (Fig. 1e). In our experience, this distribution pattern, rather than the specificity of GFAP expression itself, is a helpful diagnostic feature. If perivascular pseudorosettes are not found, the multiplicity and variability of GFAP expression largely reduces its value as a single immunoprobe for ependymomas. In these cases, the additional expression of vimentin and the lack of immunoreactive cytokeratins may serve to discriminate ependymomas from choroid plexus papillomas and metastatic carcinomas (MIETTINEN et al. 1986).

Choroid plexus papillomas (CPP) are usually distinguishable from other intraventricular tumors (e.g., papillary ependymomas and metastatic adenocarcinomas) by their distinctive histoarchitecture with a layer of isomorphic cuboidal or columnar epithelium resting on a fibrovascular stroma. Rare anaplastic variants and metastatic tumors may, however, lack these typical morphologic features and require immunohistochemical analysis for classification and adequate therapy. In accordance with their histogenetic origin from neuroepithelial (ventricular) precursor cells, choroid plexus neoplasms frequently show immunoreactivity for S-100 protein (NAKAMURA et al. 1983; WELLER et al. 1985; COFFIN et al. 1986; KIMURA et al. 1986). In rare cases, focal NSE expression has also been reported (VINOES et al. 1984; COFFIN et al. 1986). A high proportion of CPP contain either clusters or single GFAP-positive cells which are morphologically indistinguishable from adjacent epithelial cells without GFAP expression (RUBINSTEIN and BRUCHER 1981; TARATUTO et al. 1983; McCOMB and BURGER 1983; WELLER et al. 1985; COFFIN et al. 1986; MIETTINEN et al. 1986; GIANGASPERO et al. 1987). This finding has been interpreted as an indication of focal ependymal differentiation (RUBINSTEIN and BRUCHER 1981; TARATUTO

et al. 1983). The epithelial character of choroid plexus cells is reflected by the expression of epithelial marker antigens. In contrast to ependymomas, most benign and malignant CPP contain cytokeratins (in particular the 'non-epidermal' keratin types 8, 18, and 19) and the desmosome plaque proteins desmoplakin I and II (COFFIN et al. 1986; MIETTINEN et al. 1986). In addition to these markers, anaplastic choroid plexus papillomas may also express the carcinoembryonic antigen (CEA) (COFFIN et al. 1986).

Due to their specialized function in CSF production, normal and neoplastic choroid plexus epithelia contain high concentrations of *carboanhydrase C* (CAC) (ROUSSEL et al. 1979; KUMPULAINEN and KORHONEN 1982; WELLER et al. 1986), an enzyme engaged in active transport processes (MASUZAWA and SATO 1983). Immunohistochemical studies of a large series of primary and metastatic brain tumors (WELLER et al. 1985) have demonstrated that CAC immunoreactivity is conserved in all benign and malignant choroid plexus papillomas (Fig. 2i), but absent in gliomas (including ependymomas) and metastatic carcinomas. CAC thus appears to be the most reliable and specific immunoprobe for choroid plexus neoplasms.

4.5 Glioblastoma Multiforme

All marker antigens detected in astrocytomas can also be expressed in the glioblastoma multiforme (GBM), although usually with a lower percentage of immunopositive cells and a reduced staining intensity. There is no evidence that the extent of GFAP expression in glioblastomas correlates with favorable prognosis. Although major portions of a GBM may not express any of the above mentioned markers, in our experience wider sampling will reveal some tumor cell clusters positive for GFAP or S-100. It is noteworthy that multinucleated giant cells often strongly express GFAP (Fig. 1c) and S-100 protein, whereas the occasional giant cells in anaplastic oligodendrogliomas very rarely do so. The presence of giant cells in glioblastomas has been shown to be correlated with a somewhat better prognosis (BURGER and VOLLMER 1980). Receptors for *epithelial growth factor* (*EGF*) are found in up to 40% of tumor cells of glioblastoma multiforme (LIBERMAN et al. 1984; NOBLE, personal communication), but the prognostic relevance of this finding is uncertain. In the gliosarcoma, the exact nature of the sarcomatous component is still controversial. There is agreement that its most likely cellular derivation is from the vascular wall of tumor vessels. Expression of endothelial cell markers (*factor VIII-related antigen* and *Ulex europaeus I agglutinin*) and of basement membrane components like laminin and fibronectin in the sarcomatous component is consistent with an origin from the endothelial hyperplasia typically found in anaplastic gliomas (SCHIFFER et al. 1984; SLOWIK et al. 1985). Ultrastructural findings and the expression of smooth muscle myosin (KISHIWAKA et al. 1986) suggest the possible participation of myofibroblasts, smooth-muscle cells, pericytes or fibroblasts in the genesis of the sarcomatous component of gliosarcomas.

4.6 Medulloblastomas and Related Primitive Neuroectodermal Tumors (PNET)

Although the cerebellar medulloblastoma has for more than 60 years constituted a well defined clinical and histopathological entity, its histogenesis has long been disputed (for review see RUBINSTEIN 1975). It is now generally agreed that this neoplasm originates from a pluripotent matrix cell population. Some authors maintain that this is most likely to be the external granular layer of the cerebellum. Others have proposed the hypothesis that this tumor type is not restricted to the cerebellum but occurs at all levels of the CNS. Accordingly, the subependymal matrix cells should be regarded as precursor cell population. There is indeed evidence that medulloblastoma-like tumors occur in the cerebral hemispheres and it has been proposed (HART and EARLE 1973) that this group of embryonal neoplasms should be named primitive neuroectodermal tumors (PNET). The conceptual basis for this nomenclature is the assumption that PNETs share a common progenitor population, i.e. the subependymal matrix cells, and that their neoplastic transformation at various levels of the CNS leads to tumors with similar morphology and biology (RORKE 1983). Analogous to non-transformed matrix cells, PNET cells have the potential for neuronal, glial and ependymal differentiation. Although a tendency for various lines of differentiation has been noted in the past, only recent immunohistochemical techniques have allowed us to estimate more reliably the incidence and extent to which this occurs.

Historically, *neuronal differentiation* has been most conspicuous, since neuroblastic (Homer Wright) rosettes can easily be detected in a significant proportion of medulloblastomas. Immunocytochemical studies indicate that NSE constitutes a valuable marker for incipient neuronal differentiation (GIORDANA et al. 1983; VELASCO et al. 1985; BURGER et al. 1987). In a recent evaluation of 133 medulloblastomas (Table 2), we found sharply delineated islands of NSE-positive cells (Fig. 2d) in approximately one third of the cases. Often, the nuclei of these cells had a more vesicular chromatin, occasionally with transition into ganglionic cell types. Medulloblastomas with more advanced neuronal differentiation showed clearly identifiable ganglionic cells and these often expressed neurofilament protein, predominantly in the cell processes but occasionally also in the perikaryon (Fig. 1e). This is in accordance with the findings of VELASCO et al. (1985) but at variance with those of ROESSMANN et al. (1983) and FRIEDMAN

Table 2. Differentiation in medulloblastomas

Neuroblastic rosettes		36%
– With ganglioid cells	13%	
Neuron-specific enolase (NSE) positive clusters		35%
– With neuroblastic rosettes	58%	
– Without neuroblastic rosettes	42%	
GFAP-positive neoplastic astrocytes		10%
Desmoplastic variant		11%

133 Cases of the ongoing SIOP/GPO MED 84 study. For details see BURGER et al. (1987)

et al. (1985) who reported positive NFP staining in small undifferentiated cells whose neuroblastic or neuronal nature was not obvious from their appearance in conventionally stained histological sections. In our experience, there is a continuous line of neuronal differentiation, the earliest characteristic being the focal expression of NSE and formation of neuroblastic rosettes, followed by the appearance of ganglioid cells and, eventually, the expression of NFP (BURGER et al. 1987).

Glial differentiation in the medulloblastoma is much more difficult to define. Since specific and reliable markers have not been identified for neoplastic oligodendrocytes and ependymal cells, GFAP is at present the only marker that allows the estimation of glial differentiation in primitive neuroectodermal tumors. Most medulloblastomas contain GFAP-positive cells but the distinction of neoplastic *versus* reactive cells can be very difficult, particularly when these are sparsely dispersed within the neoplasm. Accordingly, the reported incidence of glial differentiation ranges from absent or very uncommon (COFFIN et al. 1983; SCHINDLER and GULLOTTA 1983) to 50% (ROESSMANN et al. 1983) and more (PASQUIER et al. 1983; PALMER et al. 1981). In our view, the typing of GFAP-positive cells in medulloblastomas as proposed by MANNOJI et al. (1981) is quite useful. These authors define neoplastic GFAP-positive cells as morphologically identical to the ordinary medulloblastoma cell with a hyperchromatic nucleus and a very scanty cytoplasm (Fig. 2f). Reactive, entrapped astrocytes show a sparse chromatin and well-developed cytoplasmic processes. In addition, there is an intermediate type with fairly rich cytoplasm but short, plump cell processes and it is assumed that at least some of these cells are neoplastic. When we applied these stringent criteria to a large series of medulloblastomas (Table 2), we found unequivocal evidence of GFAP expression by neoplastic cells in only 10% of cases, and this figure agrees well with the findings of MANNOJI et al. (1981), SCHINDLER and GULLOTTA (1983) and KUMANISHI et al. (1985). We could not confirm the observation of HERPERS and BUDKA (1985) that the occurrence of GFAP-positive neoplastic cells is restricted to the desmoplastic variant. In contrast to neuronal differentiation, GFAP expression by medulloblastoma cells is usually restricted to a few foci of dispersed cells, with no evidence of a progressive differentiation into mature astrocytes.

4.7 Tumors of the Peripheral Nervous System

The only reliable tumor marker for Schwann cell-derived tumors is S-100 protein. Only the beta-subunit, but not the alpha-subunit is present in these neoplasms (ISOBE et al. 1984). The marker is expressed in all benign schwannomas and neurofibromas and in the majority of malignant Schwann cell tumors (WEISS et al. 1983; DAIMARU et al. 1985) in a diffuse cytoplasmic and nuclear distribution, generally with a stronger expression in Antoni A than in Antoni B areas. The same is true for the occasional Schwann cell tumors, which are NSE-positive (NAKAJIMA et al. 1984). Fibronectin and laminin can be demonstrated in the basement membrane surrounding the tumor cells (GIORDANA et al. 1985). Myelin basic protein and myelin-specific P2 are usually absent in Schwann cell tumors

(CLARK et al. 1985). The presence of axons, as demonstrated by neurofilament staining, may be of help in the differential diagnosis of spindle cell tumors. Rhabdomyoblastic differentiation in malignant schwannomas (Triton tumor) can be verified with anti-myoglobin antibodies.

4.8 Meningiomas

Meningiomas have been histogenetically linked to arachnoidal cap cells (KEPES 1986). Despite the existence of diverse morphological subtypes, all meningiomas have common immunohistochemical characteristics that parallel those of their putative non-neoplastic progenitor cells. Meningeal neoplasms share morphological features of mesenchymal and epithelial tissues and this is reflected by the co-expression of markers for epithelial and mesenchymal cells. All meningiomas so far tested showed cytoplasmic and membrane-bound immunoreactivity for *epithelial membrane antigen (EMA)* (THEAKER et al. 1986; KEPES 1986), a glycoprotein marker of epithelial and mesothelial differentiation (PINKUS and KURTIN 1985). Similarly, meningiomas consistently exhibit a strong positive reaction for the major antigenic constituents of desmosomal plaques, *desmoplakin I and II*, another distinctive diagnostic feature of epithelium-derived tumors (MOLL et al. 1986). Desmoplakin staining yields a fine punctuate pattern confined to cell borders, and ultrastructural immunolocalization has pinpointed reactivity to desmosomal junctions (KARTENBECK et al. 1984; SCHWECHHEIMER et al. 1984).

Although initial reports have emphasized the exclusive expression of *vimentin* in meningiomas (KARTENBECK et al. 1984; SCHWECHHEIMER et al. 1984; HALLIDAY et al. 1985), this concept has subsequently been challenged by an increasing number of observations suggesting a more complex pattern of intermediate filament expression. Although it remains undisputed that fibrillary cytoplasmic vimentin immunoreactivity is present in virtually all meningiomas and thus represents the principal cytoskeletal component (KARTENBECK et al. 1984; SCHWECHHEIMER et al. 1984; HALLIDAY et al. 1985; THEAKER et al. 1986), some authors have also detected focal immunostaining for cytokeratins, in particular adjacent to so-called pseudopsammoma bodies (YUNG et al. 1984; THEAKER et al. 1986). Double-labeling studies have localized cytokeratin and vimentin expression to different cells (THEAKER et al. 1986). Recently, even triple expression of vimentin, GFAP and cytokeratin was reported in a case of malignant papillary meningioma (BUDKA 1986). These studies, however, are outnumbered by those which failed to demonstrate expression of cytoskeletal components other than vimentin, including cytokeratins (SCHLEGEL et al. 1980; KARTENBECK et al. 1984; SCHWECHHEIMER et al. 1984; HALLIDAY et al. 1985), desmin (KARTENBECK et al. 1984; SCHWECHHEIMER et al. 1984), NFP (KARTENBECK et al. 1984; SCHWECHHEIMER et al. 1984) and glial filaments (TASCOS et al. 1982). Since some of these negative results have been substantiated by immunoblot analyses (KARTENBECK et al. 1984; SCHWECHHEIMER et al. 1984; HALLIDAY et al. 1985), the issue remains at present unresolved.

S-100 protein which is rather uniformly expressed in schwannomas, has also been focally detected in meningiomas (NAKAMURA et al. 1983; THEAKER et al.

1986) and thus does not contribute significantly to the differential diagnosis of these two most frequent types of extracerebral intracranial neoplasms. Basement membrane antigens (laminin, fibronectin, type IV and V collagen) which are also abundant in schwannomas were found to be largely restricted to vessel walls, whorl formations and psammoma bodies in meningiomas (KOCHI et al. 1983; BELLON et al. 1985; GIORDANA et al. 1985; MCCOMB and BIGNER 1985).

In view of the consistent expression of mesenchymal (vimentin) and epithelial (epithelial membrane antigen, desmoplakin) markers in normal and neoplastic meningothelial cells, combined probing for these antigens probably represents the most valuable tool presently available to discriminate rare anaplastic or atypical meningiomas from other primary or secondary leptomeningeal neoplasms, such as metastatic carcinomas, gliomas and schwannomas. There are only two other examples of an apparent potential for epithelial differentiation in tumors of presumed mesenchymal origin, i.e., mesotheliomas and synovial sarcomas (CHURG 1985; SALISBURY and ISAACSON 1985).

5 Immunohistochemical Determination of Proliferating Cells

Since the most prominent feature of cells undergoing malignant transformation is a loss of growth control, pathologists have always been eager to estimate the rate of cell proliferation in biopsies from human neoplasms, in particular in slowly growing but ultimately recurring lesions of the CNS. However, the mitotic index cannot be regarded as a reliable parameter since the mitotic phase accounts for less than 10% of the total cell cycle time, and during the interval between tissue removal and fixation, cells may complete mitosis without new cells entering the mitotic phase. Alternative methods have, therefore, been used which give a more reliable estimation of tumor cell proliferation, e.g. tritiated thymidine incorporation in vivo (HOSHINO and WILSON 1979), flow cytophotometry (for review see SPAAR et al. 1986) and immunohistochemical localization of bromodeoxyuridine (YOSHII et al. 1986).

GERDES and co-workers (1983, 1984, 1985) have developed the monoclonal antibody Ki-67 which recognizes a nuclear antigen expressed in the G_1 , G_2 , S and M phases of the cell cycle. Only resting cells (G_0 phase) are consistently negative for Ki-67. The use of this antibody is at present restricted to frozen sections since immunoreactivity is lost during fixation. Estimation of the growth fraction of CNS tumors, as determined by Ki-67 immunoreactivity, can be easily performed and yields reliable data even in tumors with a very low cell turnover, e.g. pilocytic astrocytomas, schwannomas and pituitary adenomas. In a recent report on 40 neoplasms of the nervous system (BURGER et al. 1986), the highest incidence of stained nuclei was found in a metastatic carcinoma (57%). The percentage of stained cells in gliomas correlated with their histologic grading and known biological behavior, the lowest values being found for pilocytic astrocytomas and the highest for the glioblastoma multiforme. In fibrillary astrocytomas, there was a good correlation between the Ki-67 index and the

Table 3. Ki-67 Expression in intracranial tumors

Diagnosis	n	Range	Mean
Metastatic carcinoma	6	2.2–56.7	23.4
Medulloblastoma	1	10.2	10.2
Glioblastoma	32	1.3–27.6	10.9
Astrocytoma, anaplastic	3	1.6– 4.7	3.3
Astrocytoma	7	0.6– 3.2	1.7
Oligodendroglioma	11	0.7– 4.9	1.9
Oligo-astrocytoma	3	1.3– 2.9	2.1
Meningioma	11	0.2– 4.0	1.8
Ependymoma	4	0.2– 1.5	1.1
Astrocytoma, pilocytic	6	0.4– 1.4	0.9
Pituitary Adenoma	32	0.2– 3.7	1.1
Schwannoma	3	0.2– 2.7	1.0

Data are given as percentage of Ki-67 positive nuclei. Modified from BURGER et al. (1986)

degree of nuclear polymorphism. Since this report was published, additional cases have been examined (Table 3). The wide range of Ki-67 immunoreactivity in the glioblastoma multiforme was particularly striking. At the lower end, these lesions did not differ from benign astrocytomas. There was no apparent correlation with the histology of these tumors and it remains to be shown, whether these differences in the growth fraction of glioblastomas correlate with patient survival. Recent investigations by ZUBER et al. (1987) and GIANGASPERO et al. (1987) corroborate these findings, with Ki-67 indices for the glioblastoma ranging from less than 2% to 32%. As expected, pituitary adenomas were shown to have a low percentage of Ki-67-positive cells but a recent survey of 31 cases (LANDOLT et al. 1987) showed a considerable variation. Low values were present in 11 endocrine inactive adenomas (0.1–1%), higher values in 6 patients with Cushing syndrome (1.1–1.5%), whereas 12 prolactinomas covered the entire range from 0.2 to 3.7%. Invasive adenomas demonstrated significantly higher values than non-invasive neoplasms.

6 Concluding Remarks

The immunohistochemical assessment of neural marker antigens has become an integral part of the histopathological diagnosis of nervous system neoplasms. Antibodies presently available for routine application are directed towards cell constituents present in both normal and neoplastic neural cell lineages. They contribute to the determination of the histogenetic origin of neoplasms and their distinction from extraneural epithelial and mesenchymal tumors. However, there are no markers that assist in tumor grading and in the distinction of normal and reactive glial cells from highly differentiated, invading gliomas. Future efforts should be directed towards the development of antibodies that make these distinctions possible.

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Viral Tumor Markers

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1 Introduction

In diagnostic virology, isolation of viruses by culturing infectious material and serological immunoassays have usually met the clinical needs for:

1. accurate identification of the infectious agent,
2. definition of the state of the disease (acute, convalescence, recovery),
3. information on the infectiousness of patients,
4. monitoring the immune status of patients,
5. estimation of the prognosis (KINGSBURY and FALKOW 1985).

Traditional morphological methods (light microscopical recognition of cytopathic effects, ultrastructural detection of virus particles, SEIFERT et al. 1984) usually fail to fulfill these clinical demands. Very recently, DNA hybridization probes have been applied in order to detect viral genomes in cells, tissues and biological fluids (McDOUGALL et al. 1985, 1986; HAASE 1986; SCHUSTER et al. 1986; LÖNING et al. 1986a, b). This methodology – initially carried out on filters after extraction and dissection of nucleic acids (HAMES and HIGGINS 1985) – has gained particular importance for in situ recognition of infectious cells. These techniques are even more relevant in tumor virology, since for some oncogenic viruses (e.g. hepatitis B, papillomaviruses) cell culture studies are

Table 1. Virus infection and human oncogenesis

Virus	Tumor	Cofactor	Putative oncogenic events
HTLV 1	Adult T-cell leukemia	?	tat gene activation
EBV	Burkitt lymphoma Nasopharyngeal carcinoma	Malaria plant extracts	chromosomal rearrangement ?
HBV	Hepatocellular carcinoma	aflatoxins, alcohol, malnutrition	?
HPV	Squamous cell carcinomas	UV, tobacco, alcohol, HSV?	E 6-gene activation

not feasible, and for the particular tumor patient under study, antibody titres may not be detectable because of suppressed Ig production or virus infections hidden in the tissues in a silent form without release of appropriate amounts of antigens. From the practical diagnostic viewpoint, two categories of viruses and virus-associated tumors may be distinguished:

1. Tumors with a high degree of virus replication (e.g. human T lymphotropic viruses (HTLV) – Epstein-Barr virus (EBV) – associated tumors) and formation of viral antigens, which can be maintained *in vitro* and which elicit measurable antibodies *in vivo*.
2. Low(non-)productive tumors (e.g. papillomavirus-/hepatitisB-associated carcinomas), which can not be grown *in vitro* and which do not provoke high titres of antibodies against viral antigens *in vivo* (Table 1).

Of course, for a given disease, demonstration of infectious organisms does not necessarily give the answer as to the pathogenicity of these findings. Interpretation needs to be even more cautious in the complicated case of the initiation, maintenance and progress of neoplasia. However, in this chapter, the primary concern is not directed at the current evidence of oncogenicity of viruses, which is rather circumstantial in most instances, but to the diagnostic and prognostic value of the assessment of virus infection.

2 Methods

In the field of viral pathology, conventional light and electron microscopy and in particular immunocytochemistry represent interesting tools for the recognition of productive virus infections (ALMEIDA 1980; CHERNESKY and MAHONY 1984). For tumors, however, major technical advances rely upon the introduction of the molecular virological detection of foreign DNA/RNA by means of hybridization with cloned nucleic acid probes containing the whole genetic information or subgenomic segments (SHKLAR 1985; PFISTER 1986).

At the very beginning of this process, viral probes have to be labeled. Radioactive probes labeled with tritium, sulphur, phosphorus, remain the first choice

because of their high sensitivity (EDBERG 1985; KULSKI and NORVALL 1985). Biotin, fluorochromes, haptens are further labels, which are now used with great success, and which certainly better meet the demands of most pathological laboratories, since these probes can be stored and handled without the hazard of radioactivity (MYERSON et al. 1984; EDBERG 1985; MCDUGALL et al. 1985). Viral probes are now even commercialized in the biotinylated form (Enzo, N.Y., see SEIFERT et al. 1984). Thus, my technical comments are strictly confined to the handling of tissues, the hybridization itself and the detection systems, which – at least in our experience – can be rapidly and reliably conducted in the routine situation of surgical pathology. Under routine conditions, pathologists can probably not afford the more sophisticated methods of DNA (Southern blot) and RNA (Northern blot) detection, but they are able to establish *in situ*, and *filter* techniques in the simplified form of dot (slot) blot hybridization (KULSKI and NORVALL 1985; HAMES and HIGGINS 1985).

Since these techniques attract the attention of more and more cytologists, our working formulae are described here.

2.1 DNA Extraction

In our experience, dot blot hybridization works better with DNA extracted and purified before hand from surgical samples or cytological scrapings/washings, since the very small channels of the manifold in our vacuum device (Schleicher and Schuell, Dassel, FRG) block up very easily with tissue fragments or cells. For DNA extractions, tissue samples of 0.2–1 g are homogenized with 3 ml TE buffer (10 mM Tris-HCl, pH 8.1, 1 mM EDTA) by an ultraturrax (IKA, Janke + Kunkel, Staufen, FRG). After addition of 1 ml lysis buffer (2% sodium dodecyl sulfate, 70 mM Tris-HCl, pH 8.1, 25 mM EDTA), the mixture is incubated overnight at 37° C with 100 µg/ml proteinase K (Merck, Darmstadt, FRG). Then the nucleic acids are purified by extraction with phenol (2 ×) and chloroform/isoamylalcohol (24:1, 3 ×), and RNA is degraded with 5 µg/ml ribonuclease B (Sigma, St. Louis, Mo, USA). DNA is precipitated with 0.1 vol 3 M Na-acetate and 2.5 vols ethanol for 1 h at –70° C, harvested by centrifugation for 60 min at 15000 rpm (–15° C), lyophilized and redissolved in 1 ml 0.1 mM EDTA (Fig. 1).

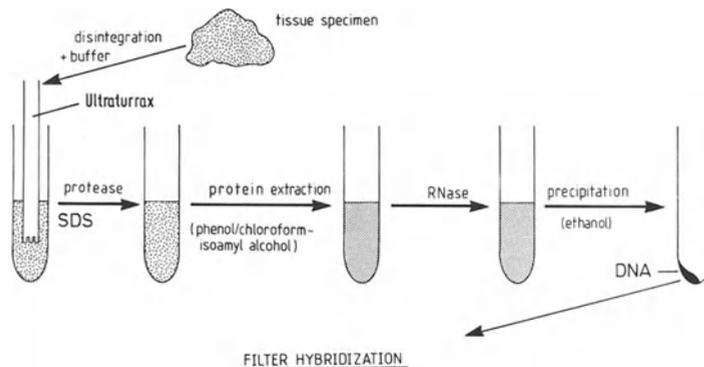


Fig. 1. Tissue processing for DNA extraction and filter hybridization

DOT BLOT HYBRIDIZATION

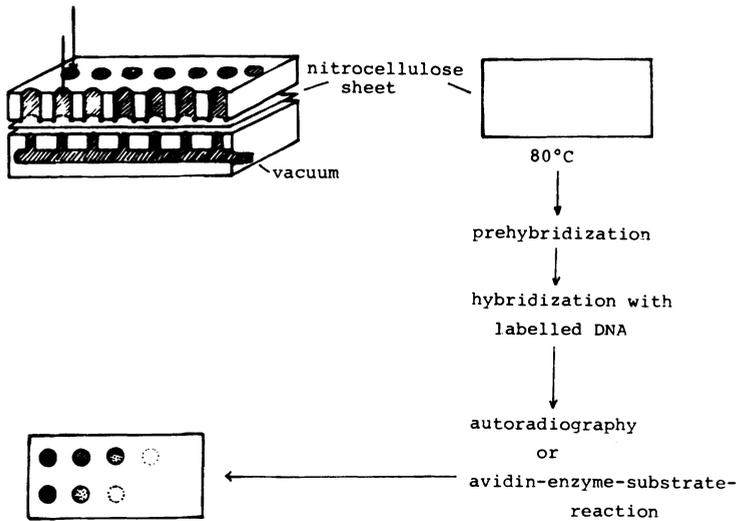


Fig. 2. Spotting and hybridization of cellular DNA

2.2 Dot Blot Hybridization

Aliquots containing 1–20 µg DNA are placed into the wells of a minifold apparatus (Schleicher and Schuell) (Fig. 2) and absorbed to the nitrocellulose membrane (BA 85, Schleicher and Schuell) by suction. The DNA is denatured by placing the membrane for 5 min on a filter paper saturated with 1.5 M NaCl, 0.5 M NaOH, and neutralizing it for 5 min on another filter saturated with 3 M Na-acetate, pH 5.5. Then the air-dried nitrocellulose membrane is baked for 1 h at 80° C.

Prehybridization is carried out for 1–3 h at 42° C in the following solution: 5 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na-citrate, pH 7.0), 50 mM Na-phosphate, 50% formamide, 0.1% sodium dodecyl sulfate (SDS), 0.1% bovine serum albumin (BSA), 0.1% ficoll, 0.1% polyvinyl-pyrrolidone, 250 µg/ml sheared and denatured herring sperm DNA. The hybridization is conducted overnight in the same solution containing 50–100 ng/ml of the respective cDNA probe labeled with biotin (e.g. deoxy Bio-probe, Enzo, New York, USA), which was denatured beforehand for 10 min at 100° C and chilled on ice. After hybridization, the filter is washed in several changes of 2 × SSC, 0.1% SDS at room temperature, then for 30 min at 55° C in two changes of 0.1 × SSC, 0.1% SDS.

For visualization of the hybridized biotinylated DNA, the Blue Gene-kit (BRL, Eggenstein, FRG) containing a streptavidin-alkaline phosphatase conjugate and the substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) is used.

2.3 In Situ Hybridization

Of course histopathologists usually prefer to look at cells and tissues rather than at spots. In recent years, hybridization protocols were created which allow detection of viral nucleic acids even in paraffin-embedded material (BRIGATI et al. 1983; MYERSON et al. 1984; LÖNING et al. 1986b). Our present protocol is described here briefly for frozen as well as paraffin sections:

Frozen sections are air-dried, fixed in methanol/acetic acid (3:1) for 3 min, immersed in boiling phosphate-buffered saline (PBS, pH 7.4) for 15 s, and cooled in ice water. RNAs are degraded

by treatment with 100 µg/ml ribonuclease B from pancreas (Sigma) and 100 units/ml ribonuclease T 1 from *Aspergillus oryzae* (Sigma) for 1 h at room temperature. After a second fixation in methanol for 3 min, the sections are air-dried.

Paraffin sections are adhered on poly-D-lysine-coated glass slides. Sections are dewaxed in xylene, rehydrated and treated sequentially with 0.02 N HCl, 0.01% Triton X-100 in PBS, 1 mg/ml Pronase E (Calbiochem, Frankfurt, FRG) in 0.05 M Tris-HCl, pH 7.6, 5 mM EDTA, 100 µg/ml ribonuclease B from pancreas (Sigma), and 100 units/ml ribonuclease T 1 (Sigma) in PBS, 100 µg/ml avidin (Sigma) in PBS, and 4% paraformaldehyde in PBS. After washing in PBS, the sections are dehydrated and air-dried.

For *hybridization*, each section is covered with 20 µl of the following hybridization solutions: 2 × SSC, 45% (v/v) formamide, 10% (w/v) dextran sulfate, 0.1 mg/ml herring sperm DNA, and 1.5–2.5 µg/ml biotinylated cDNA (HPV probes were kindly provided by Professor Dr. H. ZUR HAUSEN, Heidelberg, FRG, HIV probes were a generous gift of Dr. H.J. BUHK, Robert Koch Institute, Berlin, Bio-Probes TM for HSV, CMV, EBV and HBV were purchased from Enzo, New York, USA). Section and DNA probes are denatured together in a 90° C water bath for 10 min, then hybridized overnight at 37° C. After hybridization, the sections are washed 2 × 10 min in 1 × SSC, 20% formamide at 36° C (non-stringent conditions) or in 1 × SSC, 45% formamide, 42° C (stringent conditions). Then, a further washing is conducted for 3 × 5 min in 2 × SSC at room temperature.

For the *detection of the biotinylated DNA*, the sections are incubated for 1 h at 37° C with rabbit anti-biotin-antibodies (Enzo) diluted 1:100 in 2 × SSC + 1% BSA. After extensive washing in 2 × SSC, they are incubated with a 1:200 dilution of biotinylated anti-rabbit-IgG (Vector, Burlingame, CA, USA) for 1 h at 37° C, followed by washing and a 1:500 dilution of avidin-alkaline phosphatase (Bresa, Adelaide, Australia) in 100 mM Tris-HCl, pH 7.5, 1 M NaCl, 2 mM MgCl₂, 0.05% Triton X-100 for 1 h at 37° C. After further thorough washings in 100 mM Tris-HCl, pH 9.5, 1 M NaCl, 5 mM MgCl₂, the sections are incubated with 0.3 mg/ml nitro blue tetrazolium and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂ for 15 min in the dark. In order to inhibit endogenous alkaline phosphatase activity, 1 µl/ml levamisole (Sigma) is added to the substrate solution. The colour reaction is blocked with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and the sections are photographed without and with counter-staining.

3 Non-Hodgkin Lymphoma (NHL) and Its Association with DNA and RNA Viruses: EBV (Epstein-Barr Virus) and HTLV (Human T Cell Lymphotropic Viruses)

3.1 Oncogenic DNA Viruses (EBV)

Although a common etiological denominator of NHL is not known as yet, and arguments are found for all the major concepts of human oncogenesis (spontaneous somatic mutations, physical (radiation), chemical or viral agents), this tumor group has especially attracted the interest of virologists since members of the herpes virus group were discovered to be associated not only with lympho-proliferative diseases in primates, but also in humans (KLEIN 1982). The historical hallmark was the epidemiological observation of high grade lymphomas as endemic in children of Central Africa (BURKITT 1958), and the later recognition that EBV infection is also usually found in those children and their tumors in 80–90% of cases (HENLE and HENLE 1967; KLEIN 1982; ZUR HAUSEN 1985). The early enthusiasm later declined with the finding that EBV is only involved in 20–30% of similar lymphomas of American or European origin (ZUR HAUSEN 1985). It is the present belief that even in African Burkitt lympho-

mas, EBV is only one actor on the whole stage of B lymphocyte transformation and clonal outgrowth. Recent findings point to three steps of tumor development (KLEIN 1982; KLEIN and KLEIN 1986):

1. Primary EBV infection.
2. Enhanced cell proliferations via exogenous cofactors (e.g. malaria), which – putatively – suppress or switch off immunological growth control mechanisms.
3. Specific chromosomal events: translocation of the distal arm of chromosome 8 to the chromosomes 14, 2 or 22. 8:14 translocation leads to a close spatial relationship of the *c-myc* oncogene (located on the chromosome 8 fragment) and the genes for the heavy chain Igs, which are situated at the breakpoint region of chromosome 14. It is tempting to speculate that *c-myc* activation emerges from this special type of translocation (FUKUHARA and UCHINO 1983; HAMLYN and RABBITS 1983; MCINTOSH et al. 1983). Again, the impact of EBV infection on chromosomal instability and malignant transformation is far from being understood. In patients with sporadic Burkitt lymphomas, 8:14 translocation was seen without any sign of EBV infection, and even in NHL other than lymphoblastic lymphomas of the Burkitt type (e.g. follicular centrocytic-centroblastic lymphomas) chromosome 14 was seen to be a major acceptor site for genomic material from chromosome 8 or other chromosomes (YUNIS et al. 1982; BLOOMFIELD et al. 1983; GAHRTON et al. 1984).

The biological scenario of diseases of the lymphoid system associated with EBV is outlined in the following issues:

1. In young adults, an overt, self-healing EBV infection of B-lymphocytes is well known and called infectious mononucleosis. This benign lymphoproliferative disease takes the usual course of a virus infection including a prodromal phase of virus entrance (via C3d receptors, WEIS et al. 1986), the acute phase with massive virus production and release from B-lymphocytes eliciting high IgM titres against viral antigens, and the convalescent phase with increasing and, later on, persistent IgG titres for EA (early antigen), VCA (viral capsid antigen), MA (membrane antigen), and EBNA (Epstein Barr nuclear antigen). Extrachromosomal persistence and random integration of viral DNA takes place and leads to a life-long latency state in B lymphocytes of all affected individuals (Fig. 3). In West-Germany, 80% of all adults carry antibodies as a sign of previous EBV infections (ZUR HAUSEN 1981, 1985). After long observation periods of up to 30 years, EBV-infected individuals were reported to carry a slightly increased risk of developing malignant lymphomas (CONNELLY and CHRISTINE 1974; ROSDAHL et al. 1974; MUNOZ et al. 1978).

2. Patients with Burkitt lymphomas follow a rapid and fatal course of tumor progress without treatment. High IgG titres are found for all major EBV antigens. The most reliable signs of virus persistence are the demonstration of nuclear antigens (EBNA) and/or viral DNA in tumor cells. Integrated and extrachromosomal viral DNA can be observed within the tumor (ZUR HAUSEN 1981).

3. Further associations of EBV infections were reported on for primary lymphomas of the central nervous system (ZIEGLER 1982; HOCHBERG et al. 1983) and for lymphomas of North American homosexuals (MAGRATH 1984). Again, persistence of the viral genome, appearance of EBNA in tumor cells, and the

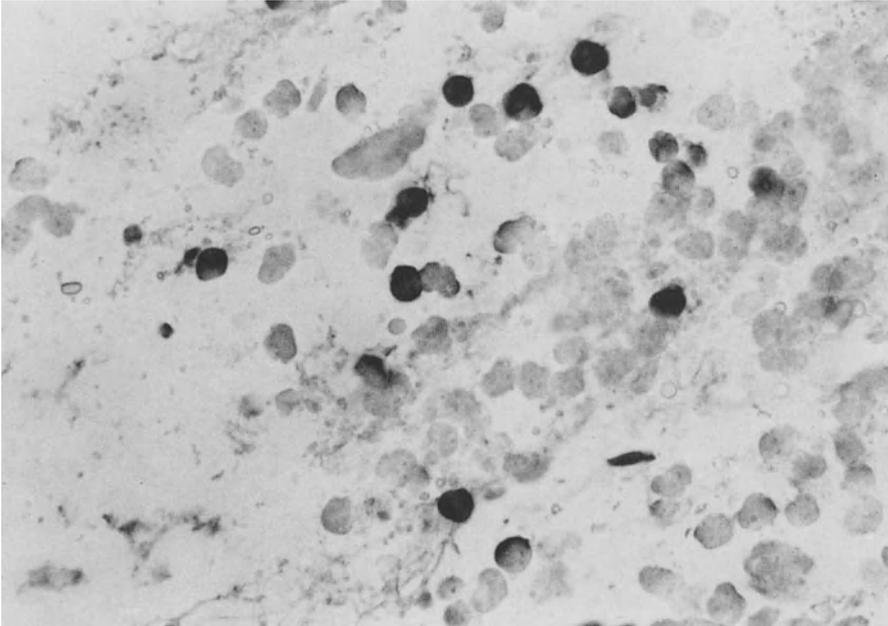


Fig. 3. EBV-DNA harbouring peripheral blood cells (renal transplant recipient). In situ hybridization with a biotinylated EBV probe. $\times 500$

detection of high serum antibody titres for EBV antigens gave rise to the assumption of EBV being one etiological candidate.

3.2 Oncogenic RNA Viruses (HTLV)

Since the discoveries of ROUS (1911) RNA viruses have turned out to be the “classical” source of oncogenic infectious agents, which cause a wide spectrum of sarcomas and numerous neoplasias of the hematopoietic system (including lymphomas), an etiological linkage which even follows the rigorous rules of Koch’s postulates – at least for the animal system – (BISHOP 1982; LACEY 1986; GALLO 1986). Those *animal* studies led to major insights into at least some of the principles of virus-associated tumor formation (GALLO 1982; GALLO and WONG-STAAAL 1982; WONG-STAAAL and GALLO 1985):

1. In acute leucemias, viruses carry their own oncogenes into the host genome. Those oncogenes are the transforming counterparts of normal cellular genes, which – in part – are responsible for the control of normal growth and differentiation (SLAMON et al. 1984; WEINBERG 1985; LACEY 1986). Tumor induction will follow independent of the integration site of the oncogene-harboring viral DNA.

2. In chronic leucemias, viral promotor sequences (probably located within the long terminal repeats) activate cellular oncogenes. Tumor induction needs

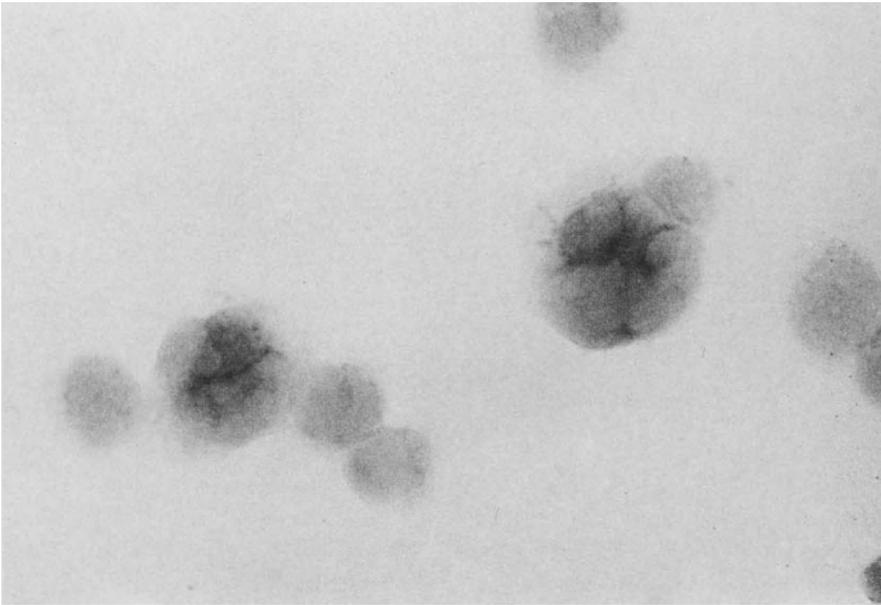


Fig. 4. HIV infected T cell line (H9). In situ hybridization with a biotinylated HTLV III probe reveals positive syncytia. $\times 500$

conserved integration sites in order to bring the viral promotor region next to the cellular oncogene and to influence its function.

3. In some lymphomas, the inserted viral genome codes for regulatory proteins, which are responsible for persistent activation of at least parts of the viral genetic information, and which – in addition – influence the expression of growth conditioning proteins of the host.

In the last five years, the studies of GALLO and associates (see for review: WONG-STAAAL and GALLO 1985) have attracted particular attention, because – for the first time – distinct types of retroviruses (HTLV-human T cell lymphotropic viruses) were found to be closely linked to human T cell leukemias/lymphomas. HTLV isolates were obtained from tumor cells of patients with Mycosis fungoides/Sézary syndrome (BUNN et al. 1983), of individuals with adult T cell leukemia (ATL) in endemic regions of South-Western Japan, the Caribbean Islands, and Southern Italy (isolates commonly called HTLV 1, GALLO 1986), of patients with hairy cell leukemia (called HTLV 2, KALYANARAMAN et al. 1982) and from peripheral blood cells of patients with the acquired immune deficiency syndrome (AIDS, isolates commonly termed (HIV/HTLV 3, BARRÉ-SINOSSI et al. 1983; POPOVIC et al. 1984) (Fig. 4). These HTLV's (HTLV 1,2,3,4) are now defined according to size and structure, nucleic acid homology, the supposed pathogenic principles (e.g. *transacting transcriptional activity controlled by the viral tat-gene: transactivation of cellular genes for interleukin 2 and its receptor*, WONG-STAAAL and GALLO 1985), the cellular tropism (helper

T lymphocytes), and action (HTLV 1,2 infections leading to unlimited cell growth, HIV infections causing cell death) (GALLO 1986; GELDERBLOM and PAULI 1986). The final outcome of HIV infected patients with AIDS is not only governed by recurrent opportunistic infections, but also by the development of peculiar sarcomas (Kaposi sarcomas: MOSKOWITZ et al. 1985), and high grade malignant lymphomas (Lancet Editorial 1986). Cytomegalovirus DNA (in Kaposi sarcomas: FENOGLIO et al. 1982; MCDUGALL et al. 1985; LÖNING and MILDE 1986) and Epstein Barr virus DNA (in malignant lymphomas: Lancet Editorial 1986) have been seen to occur sporadically in those tumors, whereas the presence of HIV has not been unequivocally proved as yet (WONG-STAAAL and GALLO 1985) (Fig. 5).

Further studies must also be awaited before the etiological relevance of serological findings of antibodies against HTLV antigens in other NHL including even chronic lymphocytic leukemia can be ascertained (GALLO 1982; BLATTNER et al. 1983).

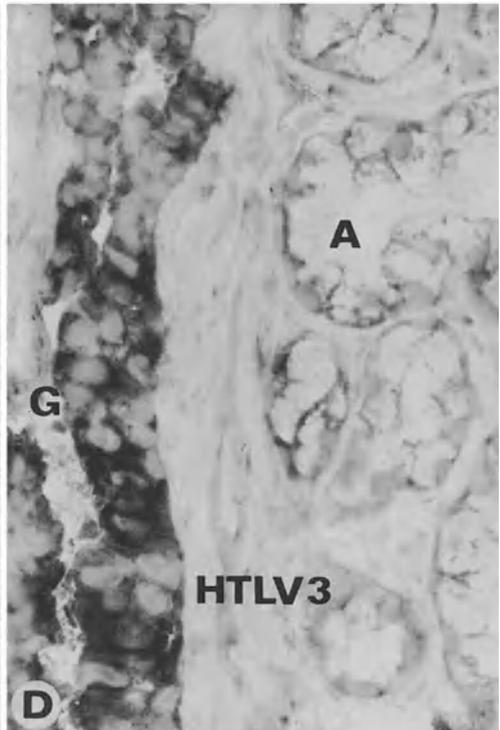
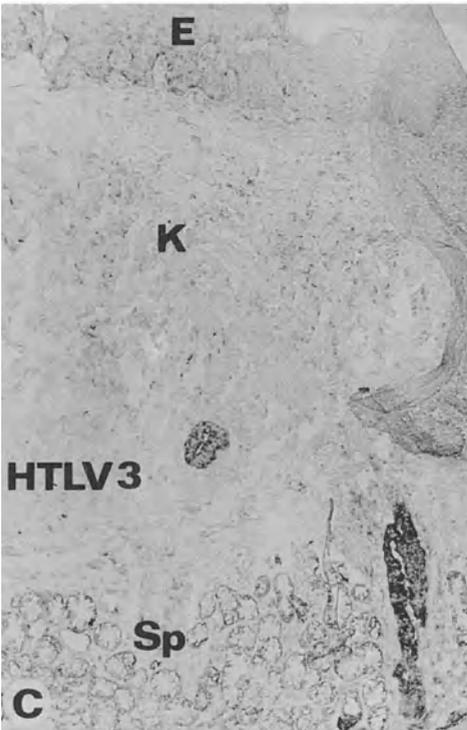
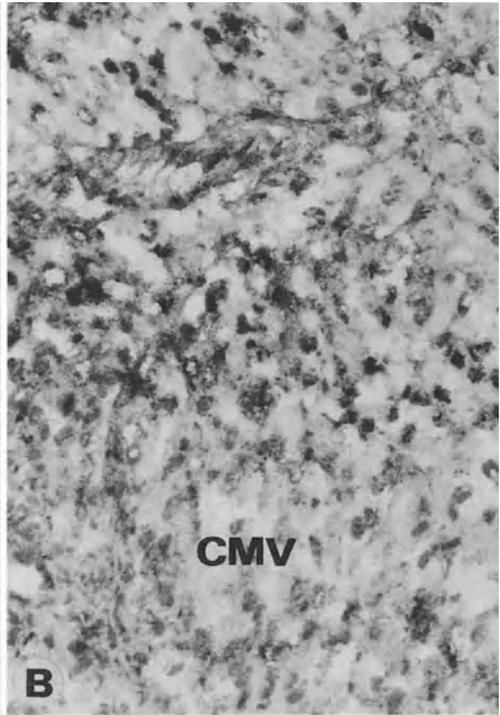
The same reservations must be attached to the observation of retrovirus-related proteins in a cell line derived from diffuse histiocytic (large cell) lymphoma (GOODENOW et al. 1982). Of course, the discovery of the HTLV's throw some light on older epidemiological studies, which stimulated speculations as to an infectious etiology of NHL. Family clustering has been noted in particular for cases of chronic lymphocytic leukemias, and also for other lymphomas/leukemias (GUNZ et al. 1975; BLATTNER et al. 1976; WINTROBE et al. 1981; BOWEN et al. 1982). KIM and associates (1982) reported increased frequencies of lymphomas in couples. However, it is again EBV which remains a potential etiological candidate, too. Thus, in some cases of lymphomas linked to the X-chromosomal recessive lymphoproliferative syndrome, EBV-related DNA was described to be present (PURTILO et al. 1977; PURTILO 1980).

4 Hepatocellular Carcinoma and Its Association with Hepadnaviruses

The hepadna virus family comprises the human hepatitis B virus (HBV), similar viruses of woodchucks and Beechey ground squirrels in North America, and the duck hepatitis virus of Pekin ducks in China, Japan, and the United States (SUMMERS et al. 1978; MARION et al. 1980; MASON et al. 1980; ROBINSON 1980).

Persistent infections with these viruses are common and may result in chronic hepatitis, cirrhosis, and primary hepatocellular carcinomas (PHC, SZMUNESS 1978). In woodchucks and humans, epidemiologic evidence of the association of HBV infection and PHC is very strong (BEASLEY et al. 1981; POPPER et al. 1981), and for humans, this association is more striking than for any other virus-related cancer.

Immunocytochemical studies directed to tumor-burden liver tissues of seropositive patients (HBs-Ag⁺) revealed non-tumorous liver cells to be positive for HBsAg and for HBcAg, but most often failed to detect viral antigens in the tumor itself (KEW 1978). In some studies, scattered HBsAg-positive tumor



cells were observed, while HBcAg was found only exceptionally (SUZUKI et al. 1985).

These reports should be read with caution, since single positive cells are hard to define as true tumor cells (POPPER 1986). In our own studies, we never unequivocally observed tumor cells or complexes to be positive for HBs/HBc, and even in the non-tumorous liver tissue of seropositive patients, we found viral antigens only to the low extent of 20–30% of over 20 PHC (Figs. 6, 7). In contrast to these disappointing immunological approaches, hybridization methods offered the answer as to the frequency of HBV infections in tumor tissues and tumor cell lines (LUTWICK and ROBINSON 1977; SUMMERS et al. 1978; BRÉCHOT et al. 1980; CHAKRABORTY et al. 1980; EDMAN et al. 1980; TWIST et al. 1981; BRÉCHOT et al. 1982; CHEN et al. 1982; SHAFRITZ 1982; MILLER and ROBINSON 1983). From these experiments, the following findings emerged:

1. The majority of PHC of seropositive individuals contain integrated viral DNA.
2. Tumor cells are clonal with respect to the site of DNA integration in a given tumor.
3. For different PHC's, however, integration sites vary to a large extent. Inserts are generally highly disordered.
4. Multiple insertion sites occur and further obscure the etiological relevance of any of these integrations.
5. As yet, integration has not been regularly linked to oncogenes. Very recently, however, integration was seen next to liver cell DNA homologous to *v-erb-A* and steroid receptor genes (DEJEAN et al. 1986).
6. The state of integration in PHC must not be different from that in infected non-tumorous tissue.

From the practical diagnostic viewpoint, HBV-DNA appears to be currently the most reliable viral tumor marker in PHC. In our series of PHC examined by dot blot hybridization, we found HBV-DNA in nearly all cases (Fig. 8).

In contrast – at least to our experience – in situ hybridization is very difficult to conduct, which may be due to the insufficient sensitivity of the technique (LÖNING and MILDE 1986). It was of further interest that we found HBV-DNA in some carcinomas other than PHC's, too (e.g. a low differentiated penile carcinoma). Although HBV-DNA has been reported in cells other than hepatocytes (e.g. pancreas, kidney, skin, lymphocytes: BRÉCHOT et al. 1984) our own very preliminary results have to be further confirmed prior to announce a broader spectrum of HBV infections in human tumors.

◁ **Fig. 5.** **A** H & E section of an oral Kaposi sarcoma shows densely packed mesenchymal cells and slit-like spaces in between. $\times 67$. **B** Same case. Several hybridizing nuclei of mesenchymal cells. In situ hybridization with a biotinylated CMV probe. **C** Oral Kaposi sarcoma (*K*). In situ hybridization with a biotinylated HIV probe. Note the negative epithelium (*E*) and the negative tumor, while some ductal epithelia of underlying minor salivary gland (*Sp*) are positive. $\times 67$. **D** Higher magnification of the salivary gland already shown in **C**. Positive duct (*G*). Negative acini (*A*). In situ hybridization with a biotinylated HIV probe. $\times 420$

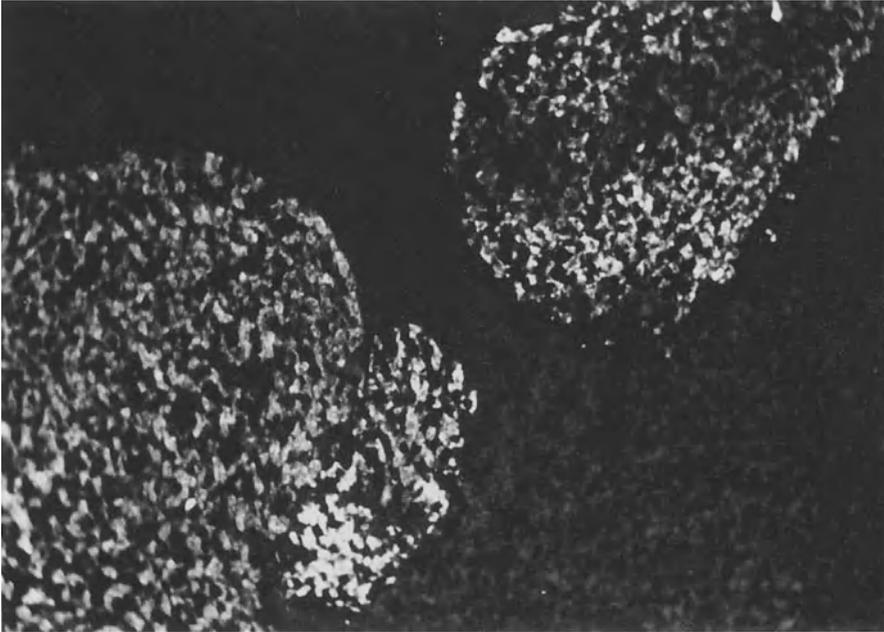


Fig. 6. Non-tumorous liver in the presence of a hepatocellular carcinoma. HBs antigen detected with heterologous antibodies (DAKO). Almost all hepatocytes of cirrhotic nodules are positive. Indirect immunofluorescence. $\times 160$



Fig. 7. Hepatocellular carcinoma. Few HBs-positive cells within tumor nodules. Indirect immunofluorescence. $\times 500$

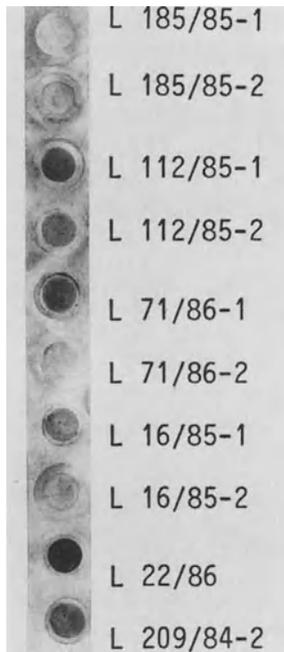


Fig. 8. Dot blot hybridization of six liver carcinomas. Two of the cases (L-209/84-2, L 185/85) represent liver transplants (surgery done because of a hepatocellular carcinoma) anew infiltrated by a cancer, which was discussed to be again hepatocellular carcinoma. This assumption is supported by positive DNA hybridization in tumor tissues in case L 209/84-2, while highly questionable in the negative case L 185/85-1 + 2. The remaining four hepatocellular carcinomas (L 112/85, L 71/85, L 16/85, L 22/86) are also positive for HBV DNA with some variations in different tissue fragments (see the difference of staining intensity in 1/2)

5 Genital Cancer and Its Association with Herpes Simplex Type 2 and with Papillomaviruses

The insights and illusions of science in dealing with the etiology of cancer are best highlighted by the history of infectious organisms accused of being associated with genital cancer. This spectrum ranges from protozoa to viruses (Koss 1979).

5.1 Herpes Simplex Type 2

Herpes simplex type 2 (HSV 2) represents the traditional viral agent assumed to play an oncogenic role, since seroepidemiological studies (NAHMIAS et al. 1974) revealed elevated titres of serum antibodies against viral antigens in females with cervical cancer. Later on, however, it became clear that this observation was largely overemphasized, taking into account the high prevalence of positive HSV serology in non-selected populations (COREY and SPEAR 1986). Moreover, although controversially discussed, viral proteins and viral nucleic acids were claimed to occur in cervical cancer tissues (see for reviews: MCDUGALL et al. 1985; ZUR HAUSEN 1985). Up to 30% of cervical intraepithelial neoplasias (CIN) and cervical carcinomas are said to harbour HSV-specific RNA (MCDUGALL et al. 1980; MAITLAND et al. 1981; WILKIE et al. 1981).

These studies were first done with hybridization probes containing the whole genomic DNA, and putative cross-reactions with homologous sequences of mammalian DNA were not entirely excluded (PEDEN et al. 1982). Later on, however, these experiments were confirmed with subgenomic viral probes free of homologous cellular sequences (MCDUGALL et al. 1985). HSV 2-derived RNA species were shown to be read from only three regions of the viral genome, two of them representing DNA regions with transforming activity (MCDUGALL et al. 1985). These observations are in line with earlier reports (FRENKEL et al. 1972), but contradict the studies of other groups (ZUR HAUSEN et al. 1974).

The controversy, of course, could well be a matter of different sensitivity of the applied methods. MCDUGALL and associates (1985) further substantiated their results by restriction enzyme analysis and found parts of the HSV 2 genome in cervical as well as in anal carcinomas. They also made the interesting observations that, in some anogenital tumors, genomes of HSV 2 and human papillomaviruses (HPV 6b) may coexist (MCDUGALL et al. 1985).

5.2 Human Papillomaviruses

The, as yet, sporadic observations of HSV 2 infections, however, contrast with the overall high incidence of *human papillomavirus (HPV)* infections in genital cancer. It is the merit of the molecular biological studies of ZUR HAUSEN and GISSMANN (ZUR HAUSEN 1986; GISSMANN and SCHNEIDER 1986) that these viruses are now becoming more and more attractive as causative agents of anogenital tumors. The increasing knowledge on HPV infections of those tumors is partly described in the following list (see for review: GISSMANN and SCHWARZ 1986):

1. HPV-DNA is regularly found in cervical precancer and cancer, and in a high proportion of carcinomas of the lower uro-ano-genital tracts (vaginal, vulvar, penile, urethral, anal).

2. Two types of the heterogeneous HPV group are frequently seen in carcinomas. HPV 16 is present in approximately 50%, and HPV 18 in about 20% of samples. In contrast, about 90% of benign condylomatous lesions harbour HPV 6 DNA.

3. Viral DNA persists in an extrachromosomal state in benign and preinvasive lesions, while becoming integrated in cancer and tumor cell lines. Integration takes place in different regions and different chromosomes of the cellular genome of tumors, although, for a given tumor, tumor cells contain only a single integration site in about 2/3 of cases.

4. In HPV 18 harbouring cell lines, viral DNA is active and RNA's of similar origin are produced (early genes E6, E7, and parts of E1). However, in cancer biopsies, continued expression of the viral genome is found in only some of the specimens. It is assumed, that transcription of viral DNA is mostly required at an early stage of malignant transformation, but not for the maintenance of the neoplastic state. Of course, whether the virus is responsible for tumor induction and/or maintenance, or whether the virus is only one of several

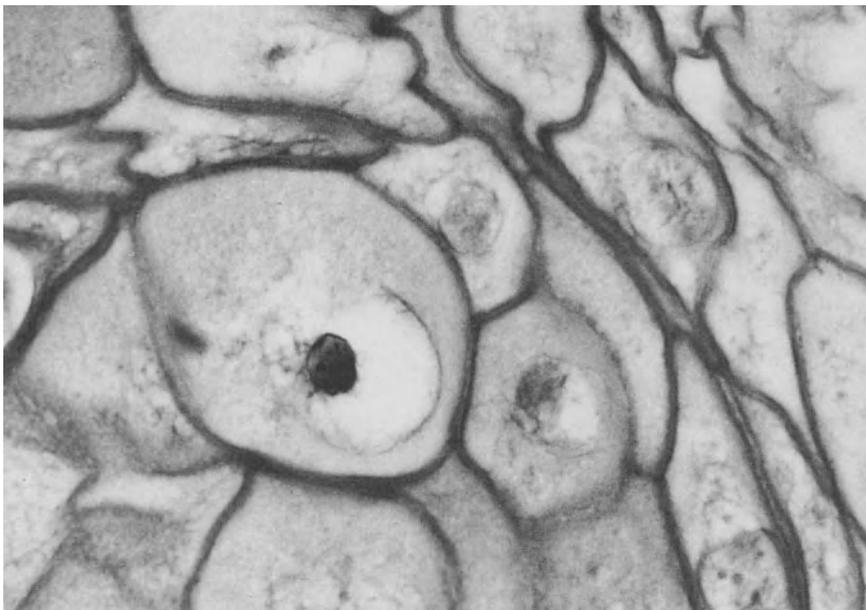


Fig. 9. H & E section showing a typical koilocytic cell with hyperchromatic nucleus and perinuclear halo. $\times 1250$

factors, which simply raise the rate of cell proliferation, makes little difference in a practical diagnostic sense.

5. Among cellular oncogenes, *c-myc* and *c-Ha-ras* genes are amplified in a proportion of HPV 16-harboring cervical cancer. *C-myc* amplification and activation of transcription is mainly seen in advanced cervical cancer of stage 2–4 (RIOU et al. 1985; ORTH 1986).

In recent years, increasing attention has been paid to the more practical questions of the prevalence of HPV infections in *non-selected populations*, and of the diagnostic and prognostic implications. Percentages of the subclinical persistence of HPV now pass 20%, when calculated irrespective of HPV types (LORINCZ et al. 1985; PFISTER 1986). When focussed on HPV 16, the numbers come to 10% (MACNAB et al. 1986). For West Germany it has been calculated from this percentage that only about one in 300 women with cervical HPV 16 infection will develop a carcinoma (PFISTER 1986).

Although there is a general consensus that HPV DNA occurs in perfectly normal-looking epithelia (MCDUGALL et al. 1986), and, thus, precedes the appearance of the characteristic cytopathic reactions (koilocytosis, dyskeratosis, binucleation, LÖNING et al. 1986a), cytology and histology remain of prime importance in the diagnosis of the early stages of cervical premalignancy. The histological continuum between CIN and HPV infection is now agreed upon by most pathologists and, particularly from the studies of REID and associates (1984), it has become clear that no fundamental qualitative differences exist morphologically between subclinical HPV infection and dysplasia (Figs. 9, 10).

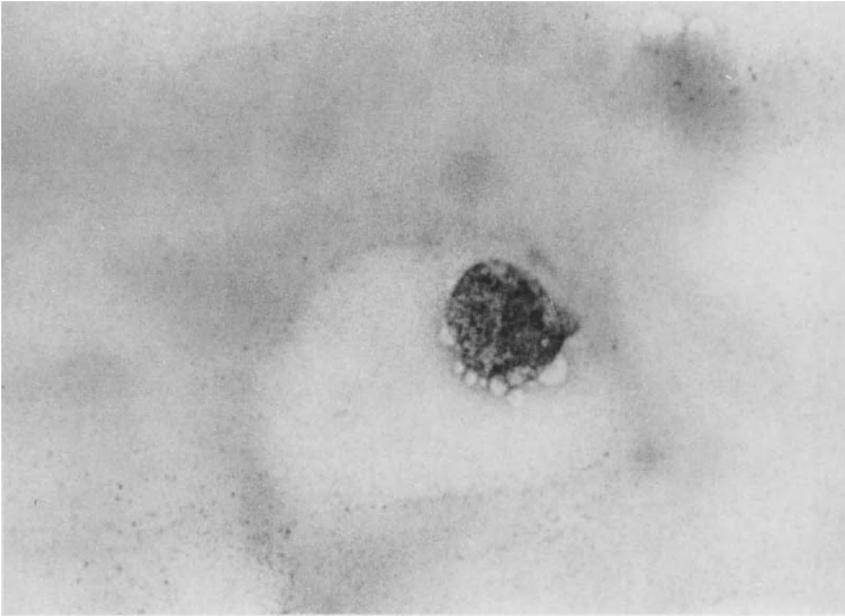


Fig. 10. Koilocytic cell hybridized with HPV 16/18 probe under conditions of high stringency. $\times 1250$

Our own studies included electron microscopy, immunohistochemistry, DNA hybridization (Fig. 11), and DNA cytophotometry, and the following conclusions are drawn from these investigations:

1. Mild cervical dysplasias (CIN 1) show prominent fields of koilocytic cells, contain viral particles and viral antigens in up to 60% of cases, are euploid or sometimes polyploid, and harbour HPV 6/11 or HPV 16/18 DNA.

2. In moderate dysplasias (CIN 2), koilocytic cells, viral particles and viral proteins are found to a minor extent. Lesions show polyploid or even aneuploid patterns, and HPV 16 infections prevail in the latter cases.

3. High grade dysplasias and invasive carcinomas show close morphological relationships, in that the epithelium is highly disordered, and atypia is found throughout the epithelium. This relationship also holds true for the pattern of HPV infection: Koilocytosis is rarely – if ever – seen. Viral particles and viral proteins are usually missed. HPV 16/18 are the predominant virus types. The DNA pattern is almost always aneuploid.

Few prospective studies have seriously scrutinized the biological outcome of HPV 16 infections. The first follow-up study of HPV-associated CIN were recently published by SYRJÄNEN and associates (1986a, b), and showed a striking tendency of HPV 16 lesions to progress (80% of cases), while HPV 6/11 harbouring lesions were seen to regress in up to 45%. Although the reported series of patients (a total of 32 HPV associated cases) is still small and the follow-up period (five years) is probably rather short, investigations of this type will certainly answer the question of whether or not particular HPV infections prime the cervix for neoplastic change.

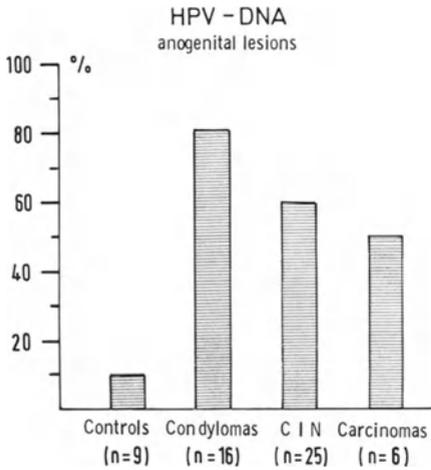


Fig. 11. HPV DNA in anogenital lesions as detected by in situ hybridization (all cases positive for any HPV type, conditions of high and low stringency)

6 Other Carcinomas of the Skin and Mucous Surfaces and Their Association with Viruses (HPV, HSV, EBV)

6.1 Human Papillomaviruses

Among the more than 40 HPV types currently known (Table 2) 11 groups and subgroups are now distinguished on the basis of less than 50% cross-hybridization (COGGIN and ZUR HAUSEN 1979; PFISTER et al. 1986). One of these groups comprise 14 HPV types, which are more or less closely related, and which have all been isolated from patients with *Epidermodysplasia verruciformis* (PFISTER et al. 1986; ORTH 1986). This hereditary generalized verrucosis of the skin represents the first, and still one of the most exciting, papillomavirus-associated human cancer models. It is a particular HPV type (HPV 5) which is most frequently found in primary cancer and metastases of the more than 60 individuals studied as yet (ORTH 1986). Malignant transformation of formerly unsuspecting warts takes place at sunexposed sites in about 1/3 of individuals (ZUR HAUSEN 1985). As yet only casual reports exist to support the association of certain HPV types with other cutaneous preneoplasias and neoplasias (solar keratosis, basal cell carcinoma, keratoacanthoma, even malignant melanoma: PFISTER 1986; ORTH 1986). Moreover, genital types of HPV (HPV 16) were observed in Morbus Bowen in addition to the genital bowenoid papulosis (IKENBERG et al. 1983). Of course, these reports need to be substantiated in molecular biological, pathological and clinical settings. Another source of HPV is being discovered in mucosal tissues of the *upper digestive and respiratory tract* and their benign and malignant tumors (LÖNING et al. 1985; DE VILLIERS et al. 1986; MILDE and LÖNING 1986; COX et al. 1986). Among the benign lesions, oral focal epithelial hyperplasias seem to be peculiar with respect to the supposed genetic background (LÖNING 1984), and the particular HPV type most frequently found (HPV 13: PFISTER et al. 1983; SYRJÄNEN et al. 1984; HENKE et al. 1986).

Table 2. Human papillomavirus types in benign and malignant tumors

Tumor types	Papillomavirus types
Palmoplantar wart	1
Verruca vulgaris	1,2,4,7,26,29
Verruca plana	3,10,27,28
Epidermodysplasia verruciformis (warts)	5,8,9,12,14,15,17,19–25,36,38
Solar keratosis	36
Keratoacanthoma	9,25,37
Morbus Bowen	16
Bowenoid papulosis	16,34,39
Condyloma acuminatum	1,2,6,10,11,16,30
Cervical condylomatous lesion	6,11,16,18,31
Conjunctival papilloma	11
Oral leukoplakia	16
Focal epithelial hyperplasia (Morbus Heck)	13,32
Laryngeal papilloma	6,11
Epidermodysplasia verruciformis (carcinomas)	5,8
Basal cell carcinoma	20
Malignant melanoma	38
Cervical carcinoma	10,11,16,18,31,33,35
Vulvar carcinoma	6,11,16,18
Penile carcinoma	6,11,16,18
Oral carcinoma	2,11,16
Laryngeal carcinoma	30,40
Bronchial carcinoma	16

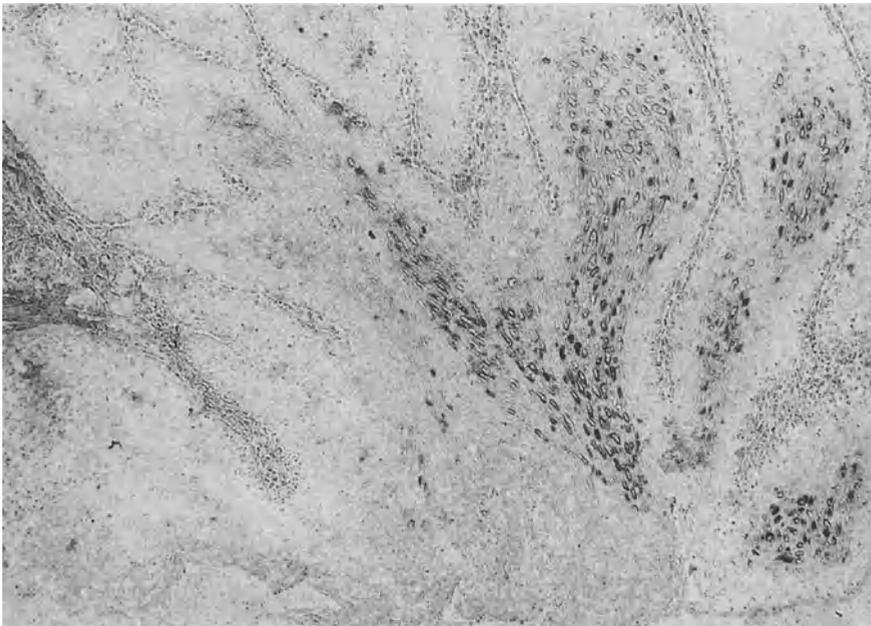


Fig. 12. Oral papilloma. Numerous hybridizing keratinocytes in keratinized parts of the tumor. In situ hybridization with HPV 1,2,6,11,16,18 cocktail under conditions of reduced stringency. $\times 80$

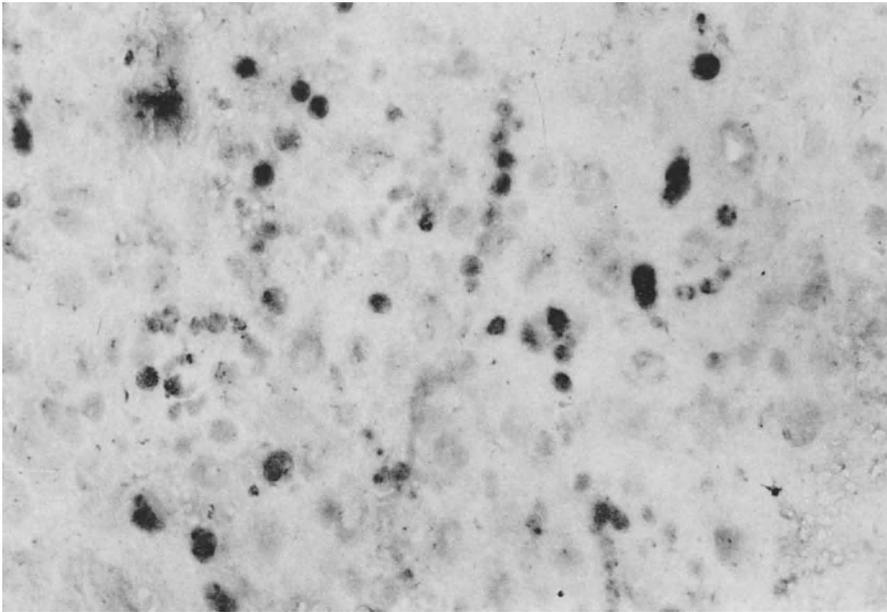


Fig. 13. Oral squamous cell carcinoma. In situ hybridization with HPV 16/18 probe under conditions of high stringency. Scattered hybridizing cells. $\times 500$

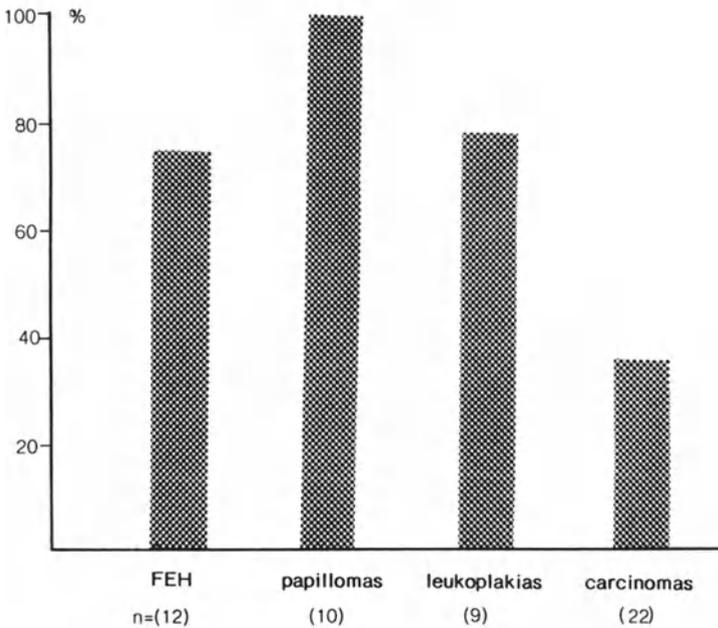


Fig. 14. HPV DNA in oropharyngeal lesions as detected by in situ hybridization (*FEH* = focal epithelial hyperplasia/Morbus Heck). All cases, positive for any HPV type, conditions of high and low stringency

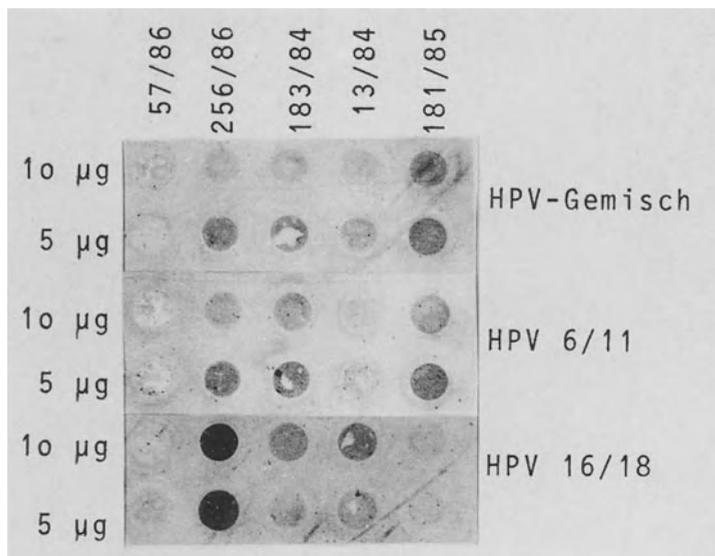


Fig. 15. Dot blot hybridization under conditions of low stringency (HPV-Gemisch = cocktail of HPV 1,2,6,11,16,18-DNA) and high stringency (for HPV 6/11 and HPV 16/18). Cases include two oral squamous cell carcinomas (L 57/86 = negative for any HPV, L 183/84 = positive for both HPV 6/11 and HPV 16/18), one oral leukoplakia (L 13/84 = positive for HPV 16/18), one penile condyloma (L 181/85 = positive for HPV 6/11), and uterine cervix, which was clinically and morphologically unsuspecting (L 256/86 = positive for HPV 6/11 and especially for HPV 16/18). At the left border amount of spotted cellular DNA

Warts and condylomas of the oropharyngeal mucosa are very rare and contain the HPV types of their cutaneous (HPV 1, 2, 3, 4) or genital (HPV 6) counterparts (PFISTER 1986). True papillomas of the mouth, pharynx and larynx were most often seen to harbour HPV 11-DNA (GISSMANN et al. 1982, 1983) (Fig. 12).

In these benign lesions, virus particles, or at least viral structural proteins, were usually observed (JENSEN et al. 1982; LÖNING 1984; LÖNING et al. 1984; LÖNING et al. 1985). Even in some oral leukoplakias without dysplasia, HPV antigens were reported to occur (LÖNING et al. 1984; LÖNING et al. 1985). In our series of cancer of the oral cavity, pharynx, and larynx, we found HPV DNA in 40% of over 20 cases (LÖNING and MILDE 1986) (Figs. 13–15).

We found HPV 6/11 and HPV 16/18 infections to occur and even to coexist in some lesions. In contrast to genital cancer, however, HPV 16 infections were not seen to predominate in malignant lesions. Few mainly casual reports are concerned with the association of HPV infections with esophageal and bronchial carcinomas (HPV 16: STREMLAU et al. 1985; OSTROW et al. 1985). In an extensive analysis of over 200 samples of normal tissues and tumors of different origin and histology, OSTROW and associates (1985) found HPV DNA in a very small percentage of cases (about 2%). Our own data on normal tissues and tumors outside the anogenital and oropharyngeal and laryngeal mucosa are consistent with these findings.

Table 3. EBV-DNA in lymphoepithelial carcinomas

	WOLF and SEIBL (1984)	LÖNING and MILDE (1986) ^a
Epipharynx	100%	60%(10/17 cases)
Tonsil	25%	20%(1/5 cases)

^a This study was done on formalin-fixed, paraffin-embedded material

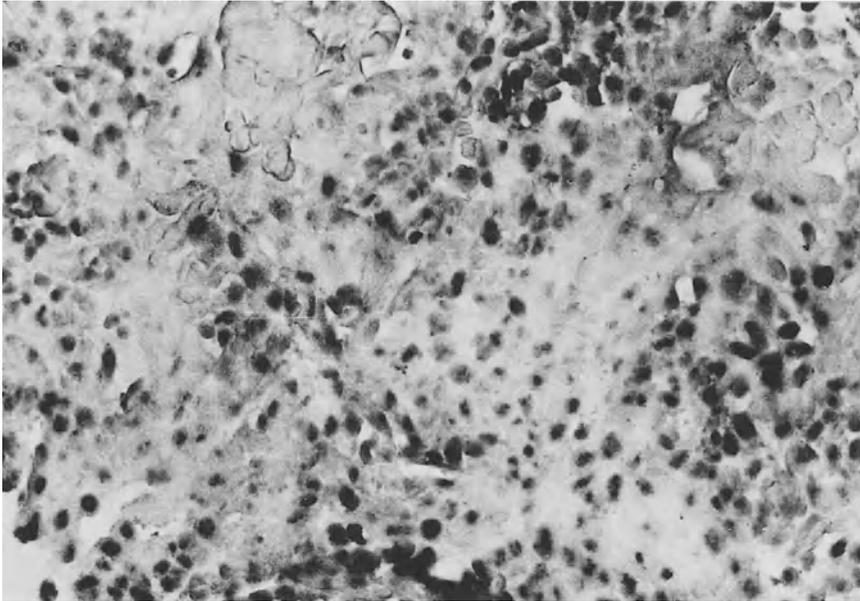


Fig. 16. Epipharynx-carcinoma. In situ hybridization with biotinylated EBV DNA. Hybridizing epithelial and lymphoid cells. $\times 500$

6.2 Herpes Viruses

In addition to HPV, members of the *herpes virus family* must always be kept in mind as etiological (co-)factors for oropharyngeal tumourogenesis. Coexistence has recently been demonstrated for HPV and EBV in AIDS-associated hairy leukoplakia (GREENSPAN et al. 1984, 1985). Coexistence may also occur for HPV and *HSV 1* in oral cancer, since HSV-related RNA was recently claimed to be present (EGLIN et al. 1983). As already discussed for herpes simplex viruses in association with genital cancer, HSV and its relevance for human cancerogenesis (genital and oral cancerogenesis) is very controversially treated in the literature (see for review: SCULLY 1983).

There exists, however, general consensus about another of the herpes viruses (*EBV*) and its very strong association with cancer of the epipharynx (see for

review: WOLF and SEIBL 1984). These carcinomas, formerly called lymphoepithelial tumors (KRUEGER and BERTRAM 1983), almost always contain EBV DNA and the EB nuclear antigen (ZUR HAUSEN 1985; LÖNING and MILDE 1986). In countries with high incidence of nasopharyngeal carcinomas (e.g. Southern China, ZENG et al. 1985), hybridization is hoped to become a diagnostic supplement to usual serological surveys, which include monitoring of IgA titres for VCA and EA. Nasopharyngeal carcinomas together with Burkitt lymphomas are the only human tumors which consistently contain EBV DNA. Recently, we were even able to detect EBV DNA in paraffin-embedded specimens (Table 3, Fig. 16). In contrast to carcinomas of the epipharynx, results on undifferentiated carcinomas of tonsils are not as convincing so far (WOLF and SEIBL 1984; LÖNING and MILDE 1986).

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Cell, Tissue and Organ Specific Tumor Markers: An Overview

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1 Introduction

Immunohistochemistry, it can be said without any hesitation, represents the most significant methodological and conceptual breakthrough and contribution to diagnostic tumor pathology that this discipline has seen in the entire second half of the twentieth century. Although there are many other new techniques and biomedical innovations that are being applied to pathology (SEIFERT 1986; GOULD 1986) only immunohistochemistry has become readily available to pathologists outside of the elite research laboratories, and has firmly become part of the daily practice of histopathology of the eighties (GATTER et al. 1985).

Like all other innovations, diagnostic immunohistopathology has passed through several phases which roughly correspond to the phase of discovery, formulation of general principles and solution of critical technical problems; gradual acceptance by the research community; transition from research into the routine laboratory; and finally acceptance by the community of pathologists and clinicians at large. Each of these phases was marked, as with any other development in science, by a predictable response: incredulity, cautious exploration by those who thought that the technique had some merit and gradual acceptance by those who became personally convinced that the new approach was indeed useful. Thereafter followed a phase of more or less uncritical overen-

thusiasm, which is now replaced by the phase of critical evaluation with weighing of positive and negative aspects and *real* contributions. We are, I believe, now entering that final phase and the stage is being set for the ultimate assessment of the contributions of this method to pathology.

Many aspects of immunohistochemistry have been reviewed and highlighted in this volume. I will thus only add a personal overview of the contributions of immunohistochemistry in an attempt to outline how it has influenced our daily practice of pathology, list briefly some of the major problems that have or have not been solved, and also mention at least some of the disappointments that were inevitable due to the high expectations and overenthusiasm of the original proselytizers.

2 General Approaches and Strategies for Identification of Tumor Markers

Search for tumor markers has concentrated on several basic questions which in essence reflect the major concerns of diagnostic pathologists. Most prominent among these questions are:

1. Are there markers that would allow distinction between normal and neoplastic cells?
2. How to recognize and distinguish benign from borderline and clinically malignant tumors?
3. How to distinguish low grade malignant tumors from their more malignant counterparts?
4. Since tumor diagnosis is primarily based on subjective evaluation of histologic sections, would it be possible to improve the diagnostic precision and make it more reliable, reproducible and objective i.e. quantifiable?

2.1 Markers of Early Neoplasia

Malignant transformation is a complex, unpredictable and poorly understood process that occurs in several successive stages (FARBER 1984a, b; RUBIN 1985; BALMAIN 1985). The phenotype of transformed cells is highly variable. Studies of early experimental neoplasia have reported numerous morphological and biochemical changes associated with malignant transformation (FARBER 1984a). However, these markers of early neoplasia vary depending on the organ involved, the carcinogen applied, the stage of neoplasia and many other poorly defined determinants. Thus, it has been shown that the cells in early neoplastic foci of the liver accumulate glycogen, express high levels of glucose-6-phosphate dehydrogenase and differ from adjacent livers with regards to their ability to accumulate exogenous iron (FARBER 1984a, b). On the other hand, papillomas of the skin express an entirely different spectrum of histochemical markers (FOULDS 1969). In most cases experimental data are not directly applicable to human pathology, and there are few cases in which the markers of early

experimentally induced animal neoplasia have found application in clinical pathology. Alpha fetoprotein (AFP), discovered originally in rats, is probably the most prominent example of such transfer of knowledge from experimental to diagnostic laboratory (ABELEV 1971).

The basic premise underlying the search for markers of early neoplasia is that the tumor cells differ from normal cells. Theoretically, one could thus hypothesize that cancer cells either lack some of the characteristics of fully differentiated mature cells or that they have acquired some new features. Along these lines it has been shown that some tumor cells, in contrast to their differentiated counterparts, may lack antigens (LIMAS and LANGE 1980), express aberrantly or miss antigens of the major histocompatibility complex (MOMBURG et al. 1986; GUY et al. 1986), or express only the backbone of the oligosaccharide complex that carries the blood group determinants (VOWDEN et al. 1986). These markers of incomplete differentiation or maturation belong in general to the group of carbohydrate antigens (HAKOMORI 1984, 1985). Although there is ample evidence that complex cell surface or secreted glycoconjugates are altered in neoplastic cells, and although there are numerous monoclonal antibodies to these antigens, it is questionable whether any "atypical" or altered glycoconjugates could serve as diagnostic markers of early neoplasia. Hence, antibodies to putative markers of incipient neoplasia still remain to be developed.

Acquisition of new characteristics on evolving cells is even more difficult to document. Most of the "new" cell surface tumor markers described in literature have been shown not to be unique to tumors and are thus of limited diagnostic value (FEIZI 1985). With considerable hope, it was thought that the demonstration of potentially oncogenic viruses such as papilloma virus (KIRCHNER 1986) in the cervical canal or Epstein Barr virus in lymphoid cells (KLEIN and KLEIN 1984; PURTILO 1984) could facilitate the morphologic diagnosis of neoplasia. However, soon after antibodies or molecular probes to these viruses were developed it became apparent that the presence of viruses in tissues does not contribute significantly to the diagnosis of preneoplasia or even incipient and overt neoplasia (PILOTTI et al. 1984; FALSER 1984; SINGER et al. 1985; COLEMAN and RICHMAN 1985). It remains to be seen whether the immunohistochemical demonstrability of well documented oncogen products could provide more reliable evidence of neoplasia (HORAN HAND et al. 1984; THOR et al. 1984). However, recent reports indicate that antibodies to oncogen related proteins react not only with malignant cells but also with benign tumors and normal cells, questioning the validity of this diagnostic approach (GHOSH et al. 1986).

2.2 Benign Versus Malignant Tumors

The criteria for the distinction of benign from corresponding malignant tumors are not always precise. Thus, for example, renal cell adenomas are arbitrarily separated from carcinomas on the basis of their size – tumors measuring less than two centimeters in diameter being labeled as benign in contrast to the larger tumors which could have the same histologic appearance but prove to be clinically malignant. Leiomyosarcoma are most reliably distinguished from

their benign counterparts on the basis of mitotic counts rather than on the morphology of tumor cells (KEMPSON and BARI 1970). Indistinct borders between cystadenoma and cystadenocarcinoma of the ovary led to the concept of so-called "borderline-tumors" (for recent studies see e.g. CHAITIN 1985; ERHARDT et al. 1985). In view of these examples, it would be most desirable to have reagents that would distinguish benign from malignant tumors.

Unfortunately, all the efforts in this respect have failed, and most of the studies performed so far have reported only quantitative rather than qualitative differences between malignant and benign tumors. Although such data could still be used in selected cases it is doubtful that quantitative differences detected immunohistochemically could ever be translated into precise diagnostic terms needed for chemotherapeutic or surgical treatment of neoplasia.

Monoclonal antibodies like Ki67 (GERDES et al. 1983), which react with nuclear antigens selectively expressed during mitosis could theoretically be applied to better assess the proliferative tumor cell compartment. Although Ki67 provides more complete and reliable data (BURGER et al. 1986), its application to tumor pathology is only an amplification of the time honored practice of a semiquantitative assessment of the number of mitoses.

Expression of Thomsen-Friedenreich antigen on neoplastic urothelium was found to correlate with the malignancy of the tumors (LIMAS and LANGE 1986). However, the same antigen is expressed on 10% of non-invasive tumors. Accordingly, such histochemical data could contribute to the overall assessment of the tumor but cannot stand on their own without conventional light microscopy.

Reagents that recognize qualitative differences between neoplastic and normal cells have not yet been developed. Judging from the experience of the last fifteen years it appears that such markers will not become readily available in the near future.

2.3 Histochemical Grading of Malignancy

In certain organs, like the urinary bladder, histologic grading of tumors is of paramount clinical importance. Criteria for histologic grading are based on semi-quantitative assessment of several features in tissue sections or cytologic smears (see e.g. HIGHMAN 1986). Thus there is a great need for more objective and less observer-dependent evaluation. Study of the expression of blood group antigens carries considerable promise for objective assessment of malignancy and grading of urinary bladder tumors (LIMAS et al. 1979; LIMAS and LANGE 1980). Cumbersome erythrocyte binding techniques have been replaced by immunohistochemical methods based on polyclonal and monoclonal antibodies (JUHL et al. 1986). Nevertheless despite a considerable body of data accumulated so far this approach has not received wide acceptance and is used only in highly specialized laboratories.

Histologic grading of tumors can be supplemented by several immunohistochemical methods. Thus semi-quantitative assessment of laminin, based on the assumption that malignant tumors produce less basement membrane material than corresponding normal tissues or equivalent benign tumors led to sugges-

tions that basement membrane components be used in overall histochemical evaluation of malignancy (BIREMBAUT et al. 1985; D'ARDENNE et al. 1986; FABER et al. 1986). More malignant, i.e. less differentiated, tumors produce presumably less "luxury proteins" typical of the mature cells in the organ of their origin. However, like all other methods that evaluate the loss of certain characteristics, rather than the appearance of unique new features this approach has obvious disadvantages and is of limited diagnostic value for routine pathology.

Tumor growth rate assessment is of potential clinical significance, and with time this approach could replace the standard semi-quantitative and subjective grading of tumors. Several methods have been tested including autoradiography with tritiated thymidine on freshly dissected tumors (CAMPLEJOHN and AHERNE 1974); labeling of tumor cells with bromodeoxyuridine and immunohistochemical detection of mitotic cells with antibodies to this analogue of uridine (HOSHINO et al. 1986); cytofluorimetry, (HALL and FU 1985; GOH and JASS 1986). Recent reports on the use of antibodies selectively reactive with nuclei of mitotic cells promise to make this approach more reproducible and technically less complex (GERDES 1985). However, it should be remarked that all these techniques are still in experimental phases, often require fresh tumor tissue and are usually not applicable to paraffin embedded material. Thus, there is no indication whether they will ever become routine.

2.4 Monoclonal Antibodies to Tumor Markers

The search for immunohistochemical tumor markers started on a large scale following the discovery by GOLD and FRIEDMAN (1965) of the so-called carcino-embryonic antigen (CEA). The search received a new impetus following the introduction of monoclonal antibody technique to tumor pathology (FOSTER 1982; NEVILLE et al. 1982; DAMJANOV and KNOWLES 1983). However, more than ten years after the seminal technical discovery of KOHLER and MILSTEIN (1975) there are still major problems pertaining to the application of monoclonal antibodies in tumor histochemistry:

a) Some antibodies, specifically reactive with sera of tumor bearing patients, react with many normal tissues. Thus they are of little value to pathologists, although they might be useful in the clinics for serologic detection and follow-up of malignancy (ITZKOWITZ and KIM 1986; SACK and KIM 1986).

b) Some antibodies, when injected into the tumor patients, localize specifically in tumors (SHEN et al. 1984; ALLUM et al. 1986). However, when used histochemically many of these antibodies do not discriminate between normal and neoplastic cells (BARA et al. 1986). Obviously the antigens expressed *in vivo* are different on tumor cells than on normal cells and are either inaccessible to the intravenously injected antibody or do not react with it for unknown reasons.

c) Some antibodies whose biochemical specificity was determined by immunoprecipitation or immunoelectrophoresis on Western blots, when applied to tissues may react not only with the biochemically determined epitope but also show cross reactivity with other substances. For example, some antibodies

against vimentin react also with other intermediate filaments in tissue sections, although biochemically their reactivity may be limited to vimentin.

d) Antibodies produced against a highly purified molecule may be specific for that molecule in various biochemical tests, but could also be reactive with other macromolecules in which the specific epitope is expressed. Thus it was shown that antibodies to carbohydrate components of the epidermal growth factor receptor crossreact with blood group determinants which obviously had the same carbohydrate sequence (PICARD et al. 1985).

Accordingly a few notes of caution seem in order for future work with monoclonal antibodies:

1. Specificity of polyclonal or monoclonal antibodies should be tested both biochemically and histochemically before any antibody is recommended for practical use in immunopathology or serology laboratory.

2. Antibodies useful for one form of immunodiagnosis are not necessarily adequate for others. An antibody highly sensitive in ELISA may not be a good immunohistochemical reagent and vice-versa.

3. There are no short-cuts and foolproof approaches in the search for tumor associated and specific antigens. A targeted effort, supported by multiphasic testing will produce more reliable results than random "fishing expeditions" although there is no good reason for a priori dismissal of the latter approach as well. New avenues of research should be explored. The full potential of human monoclonal antibodies has not yet been explored. For example, antibody "class-switch" leading to change of immunoglobulin isotype (STEPLEWSKI et al. 1985) could make many antibodies more useful. Idiotype-antiidiotype techniques are being used to combine advantages of monoclonal antibody specificity with polyclonal antibody reactivity (HERLYN et al. 1986). Other new modalities remain to be tested and fully evaluated.

3 Contribution of Histochemistry to Histogenetic and Phenotypic Classification of Tumors

Since the earliest days of microscopic pathology, histogenesis of tumors has been one of the most attractive enigmas of oncology. Advent of histochemistry opened some new avenues to this problem (DAMJANOV 1983; GOULD 1986). Classical concepts of histogenesis of many tumors were placed under scrutiny, questioned, or reinterpreted (e.g. MAZUR and CLARK 1983; MIETTINEN and VIRTANEN 1984). However, as soon became obvious, only a few equivocal conclusions were reached and the histogenesis of most tumors encountered in daily practice of diagnostic pathology remains obscure. Pragmatic pattern recognition based on phenotypic identification of specific cell types thus still represents the mainstay of histopathology.

3.1 Cell Lineage Studies

According to the postulates of classical embryology, all human tissues could be developmentally traced back to one of three primordial germ layers—ectoderm,

mesoderm and endoderm – and cell lineages derived from these germ layers (GARDNER 1985). Although these tenets, initially formulated in the study of ascidian and amphibian embryology are not entirely applicable to mammalian embryos (SVAJGER et al. 1986) they have had a profound influence on tumor pathology. The teaching that carcinomas represent tumors of ectodermal/endodermal origin and sarcomas tumors originating from mesodermal tissues is just one of the salient examples of this concept.

Experimental pathologists and embryologists have extensively used immunohistochemistry for the study of cell lineages. To universal disappointment these studies have shown that there are no immutable markers. Most cells in the developing embryo and fetus do not retain the markers of their predecessors and their phenotype, although influenced by the cell lineage, is in most instances finally shaped by the influences operating within developmental fields (LEHTONEN et al. 1985).

Most embryological cell lineage studies performed with immunohistochemical techniques indicate that markers can be used for tracing cell development over short periods. However, even those anterograde prospective studies cannot be performed in all morphogenetic periods, and it is most unwise to use markers retrospectively on differentiated cells to identify their progenitors. Accordingly, it is also risky to make histogenetic assumptions about the progenitors of tumor cells on the basis of their phenotype, even if light or electron microscopic observations are underpinned with immunohistochemical data.

Like the embryologic studies, cell surface markers of tumor cells proved to be developmentally unstable. Markers considered to be unique to endodermal derivatives, were subsequently found on ectodermal or mesodermal cells and vice versa (e.g. LEHTONEN et al. 1985). As in embryogenesis, intermediate filament polypeptides still appear to be the most stable cell lineage markers, although even these show considerable “switching”. Keratin, which is one of the best markers for epithelial cells (OSBORN and WEBER 1983) is expressed in some mesodermal tumors, such as synovial cell sarcoma (MIETTINEN and VIRTANEN 1984; FISHER 1986) or the rhabdoid tumor of the kidney and soft parts (SOTELO-AVILA et al. 1986). Expression of keratin in the normal murine ependymal cells and some human tumors originating in the ventricle (MIETTINEN et al. 1986) cannot be fully explained from present data on the histogenesis of brain and is another example of intermediate filament type switching in development of both normal and neoplastic structures. Likewise, the expression of neurofilament proteins in some endocrine tumors, such as insulinomas of pancreas or parathyroid adenomas (MIETTINEN et al. 1985) originating from neurofilament negative precursors, remains to be fully explained.

3.2 Cell Phenotype Markers

The classification of tumors based on phenotypic recognition of neoplastic cells rather than on uncertain histogenetic principles appears to be the least controversial approach of diagnostic histopathology. Immunohistochemistry has contributed considerably to improvements in the precision with which one can

identify various cell types. Numerous cell type markers have been identified. Overall they can be classified as broader *cell class specific* and more restricted *cell type* or *tissue specific* markers.

3.2.1 Cell Class Specific Markers

Immunohistochemistry is the technique of choice for broad classification of cells. Among various antibodies that belong to this category, those recognizing intermediate filament polypeptides are still top of the list of diagnostically useful reagents. Despite many notable exceptions, keratin is still considered to be one of the best markers for epithelial cells. Neurofilaments are good markers for neural cell tumors (SASAKI et al. 1985), although they appear in some neuroectodermal cell tumors (MIETTINEN et al. 1985a, b) and may be barely detectable or not expressed at all in some primitive neural tumors such as neuroblastomas (OSBORN and WEBER 1983). Desmin has a restricted expression and is found almost exclusively in muscle cell tumors (OSBORN et al. 1986a). The only other cell positive for desmin are endometrial stromal cells.

Glial acidic fibrillar protein is a reliable marker of glial cells (OSBORN and WEBER 1983) and is found outside of the central nervous system only in peripheral nerve tumors (GOULD et al. 1986). Vimentin, a marker of connective tissue cells, is not such a good cell class marker. Thus it is expressed as the sole intermediate filament protein in some neuroectodermal tumors such as melanomas (RAEMAKERS et al. 1983) and is coexpressed with keratin in some normal epithelial cells and carcinomas such as those originating from endometrium, thyroid, kidney, lung and possibly other organs (MIETTINEN et al. 1984; GOWN and VOGEL 1985; MCNUTT et al. 1985; DABBS et al. 1986).

Coexpression of vimentin and GFAP has also recorded in gliomas (SCHIFFER et al. 1986; HERPERS et al. 1986). Vimentin expression has been recorded in epithelial cells adapted to in vitro growth and in metastatic lesions. Thus, vimentin cannot be taken with certainty as a consistent marker or exclusive connective tissue cells, which up until now still do not have a class specific unique marker.

Many epithelial cells and tumors originating from them can be identified with antibodies to the so-called milk fat globule or epithelial membrane antigen (EMA) (ORMEROD et al. 1983; PINKUS et al. 1985). Antibodies to EMA originally raised against human milk fat globule membrane in rabbits were reproduced in monoclonal form in mice (HILKENS et al. 1984) and are readily applicable to paraffin embedded formalin fixed pathology specimens. It should, however, be remembered that they are not tumor specific and may occur in many normal tissues as well (HELLE and KROHN 1986). Unfortunately, poorly differentiated carcinomas often lack this antigen (WILSON et al. 1986).

White blood cells express a high molecular weight glycoprotein known as leukocyte common antigen (CLA) (for recent reviews see e.g. TUBBS and SHEIBANI 1984). Leukocyte common antigen may be partially destroyed or lost during paraffin embedding, although it survives postmortem autolysis of at least 72 hours (PALLESEN and KNUDSEN 1985) and could be of considerable importance in autopsy pathology. In conjunction with antibodies to keratin antibodies

to CLA may help in the analysis of large cell tumors and the distribution of carcinomas from lymphomas (LAUDER et al. 1984).

Neural and neuroectodermal cells and tumors contain synaptophysin, a glycoprotein of restricted anatomic distribution, which thus could be considered a class marker for neural and closely related neuroectodermal and neural crest derived cells (WIEDENMANN et al. 1986). In the same category are chromogranins, a group of proteins found in the neuroendocrine granules (WILSON and LLOYD 1984; ANGELETTI 1986). In contrast to synaptophysin which does not occur outside of neural and neuroendocrine or neuroectodermal cells, chromogranins occur in some other cells. However, since these cells are in quite uncommon locations (ANGELETTI 1986) and rarely give rise to tumors, antibodies to chromogranin have become a useful probe for identification of neuroendocrine tumors and even neuroendocrine components in other tumors (INOUE et al. 1986).

3.2.2 *Specialized Cell and Tissue Specific Markers*

Search for specialized cell specific markers has been constantly marred by major disappointments. Most of the antigens originally considered to be specific for a specialized cell or tissue turned out in final analysis to be less restricted in their distribution than originally thought. Chorionic gonadotropin, originally thought to be trophoblast specific was found not only in germ cell tumors but in many tumor cells (BRAUNSTEIN et al. 1986; FUKAYAMA et al. 1986; MEREDITH et al. 1986). S-100, initially considered neural tissue specific, was detected in numerous normal and neoplastic cells (VANSTAPEL et al. 1986). Antibodies to the so-called neuron-specific enolase react with numerous non-neural tissues and tumors (HAIMOTO et al. 1985). Prostatic acid phosphatase may be found in carcinoid tumors (SOBIN et al. 1986). Anti-Leu 7 monoclonal antibody, a reliable marker of human natural killer cells (TRINCHIERI and PERUSSIA 1984), was found subsequently to react with normal human nervous system, neurogenic tissue, peripheral nerve sheath tumors, and neuroepithelial tumors (PERENTES and RUBINSTEIN 1986). Even alpha fetoprotein, one of the earliest tissue specific markers, originally thought to be an exclusive marker of yolk sac tumors and hepatocellular carcinomas, was later reported in other tumors such as e.g. gastric carcinomas (ISHIKURA et al. 1986) and pancreatoblastoma (ISEKI et al. 1986).

Polypeptide hormones, normally secreted by specific cells in distinct anatomic locations, have for a long time been considered as markers of specific cell types. While this assumption holds in general true for most normal tissues, many hormones or hormone like substances are ectopically synthesized by aberrant tumor cells. More extensive immunohistochemical studies could on the other hand disclose that some of the previously held assumptions on the restricted distribution of cells synthesizing a certain hormone in normal tissues may be incorrect. As the best example one could take calcitonin. This calcium regulating hormone, originally extracted from the thyroid was subsequently described in bronchial and bronchiolar mucosa, in the thymus, parathyroids and even the prostate and the epithelium of the anal canal (FETISSOF et al. 1986a, b). Nevertheless, antibodies to hormones, if properly used, are still excel-

lent markers for endocrine tissues, and some like thyroglobulin (WILSON et al. 1986) are highly specific for certain organs.

New antibodies to specialized cell specific antigens are still being reported and only time will show whether they are as specific as claimed by the scientists who have discovered them. The specificity of such new antibodies as those to melanoma (GOWN et al. 1986), renal tumors (OOSTERWIJK et al. 1986) or the diagnostic value of antibody to retinal S-antigen in the diagnosis of pineocytomas (KORF et al. 1986) and many others remain to be determined.

Despite the fact that most claims about "exclusive" or "unique" tissue specific markers for specialized cells or antigens proved to be over-optimistic, there is no reason for complacency. On the other hand, even if the original claims do not withstand more critical evaluations, antibodies produced to these antigens could still be useful diagnostic reagents if applied critically and under well-defined conditions. Thus, antibody to S-100 and natural killer cells are still useful reagents for the diagnosis of melanoma (HAGEN et al. 1986) and antibodies to calcitonin, if applied to thyroid tumors, are still most valuable for distinction of medullary carcinoma from other thyroid tumors (SOBRINHO-SIMOES et al. 1985). The value of antibody to a leukocyte antigen (Leu M1) in the study of solid tumors has also been demonstrated (SHEIBANI et al. 1986).

3.2.3 *Differentiation Markers*

Differentiation and maturation of cells in certain constantly proliferating tissue compartments, such as skin, intestine or bone marrow occurs in a well regulated predictable manner. Immunohistochemistry has made major contributions to the understanding of normal differentiation of lymphoid and myeloid cells, skin or intestine, and other organs. Since the malignant transformation can occur at any stage of the normal proliferation-differentiation sequence, it was possible histochemically to phenotype tumors and compare them with cell stage in normal histogenesis at which the neoplasia has arisen. Most remarkable progress was made in the lymphoma research (TUBBS and SHEIBANI 1984; KNOWLES et al. 1986; WEISS et al. 1986; ANDREESSEN et al. 1986). However, similar phenotyping of tumors is in order for all other organ systems and it is a matter of time before this becomes a reality.

4 **Diagnostic Immunohistopathology**

The extent of future use of immunohistochemistry in diagnostic pathology will depend on many factors. This volume contains many examples of how this most valuable addition to the armoury of the contemporary pathologist has increased our understanding of tumor biology and increased the precision of morphologic diagnosis. Hence, only selected aspects of practical tumor histopathology will be addressed here.

4.1 Histochemical Differential Diagnosis of Tumors

“Is it a carcinoma, sarcoma, melanoma, neuroepithelial tumor or lymphoma?” This question, usually asked by pathologists confronted with a tumor, is usually answered in 85–90% of routine cases upon microscopic study of hematoxylin and eosin stained slides. The remaining 10–15% of the cases, representing the usual “problem” cases, fall into several categories:

- A. Poorly differentiated tumors, showing limited histiotypic or organotypic differentiation;
- B. Tumors presenting an unexpected or highly unusual pattern;
- C. Rare tumors, newly recognized pathologic entities, or sundry conditions that are not commonly encountered in everyday practice;
- D. Metastatic tumors with no obvious primary.

Morphologically, most of the “problem” cases or tumors that cannot be definitively or unequivocally diagnosed by light microscopy occur in four histologic patterns:

- A. Small blue cell tumors,
- B. Spindle cell tumors,
- C. Large or medium sized polyhedral cell tumors, and
- D. Pleomorphic tumors.

The immunohistochemical approach to these tumors will primarily depend on the initial histopathologic impression, the experience and the routine of the pathologist, the anatomic location of the tumor and the differential diagnostic possibilities considered from the clinical information available at the time of examination. In practical terms the approach will also depend on the technical capabilities of the laboratory, availability of antibodies and also on the nature of material that is still available for examination. Certain antibodies are not applicable to routine paraffin embedded formalin fixed tissues and for their optimal usage frozen tissues are a *sine qua non*. Necrotic tumors, or inadequately fixed and autolyzed tumors are not suitable for all immunohistochemical studies, although many antigens apparently survive various forms of maceration and are demonstrable even in inadequately preserved tissues (PALLESEN and KNUDSEN 1985).

To obtain maximum benefit from immunohistochemical studies it is essential to ask the right questions and select the antibodies only after the differential diagnosis of the lesion has been defined as narrowly as possible by standard light microscopy, and the conventional histochemical techniques used in routine pathology laboratories. Standard criteria of tumor histopathology should not only serve as general guidelines, but should also serve as the principal basis for classification of tumors to which all the other ancillary results should be referred and correlated for the formulation of the final diagnosis. If the immunohistochemical studies are inconclusive, it is still best to base the final decision on the light microscopic diagnosis and the overall evaluation of the case.

The approach to each problem should be individually designed and the empirical criteria best suited for the laboratory and the pathologist in charge. On the basis of empirical data from many pathology laboratories algorithms

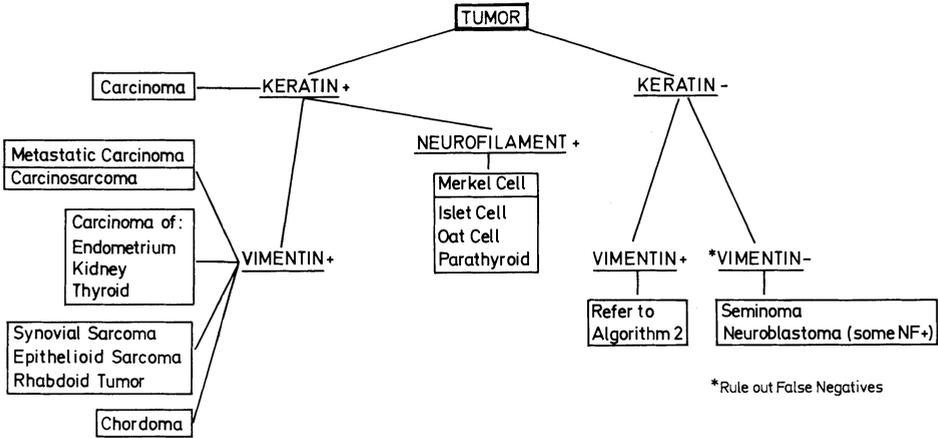


Fig. 1

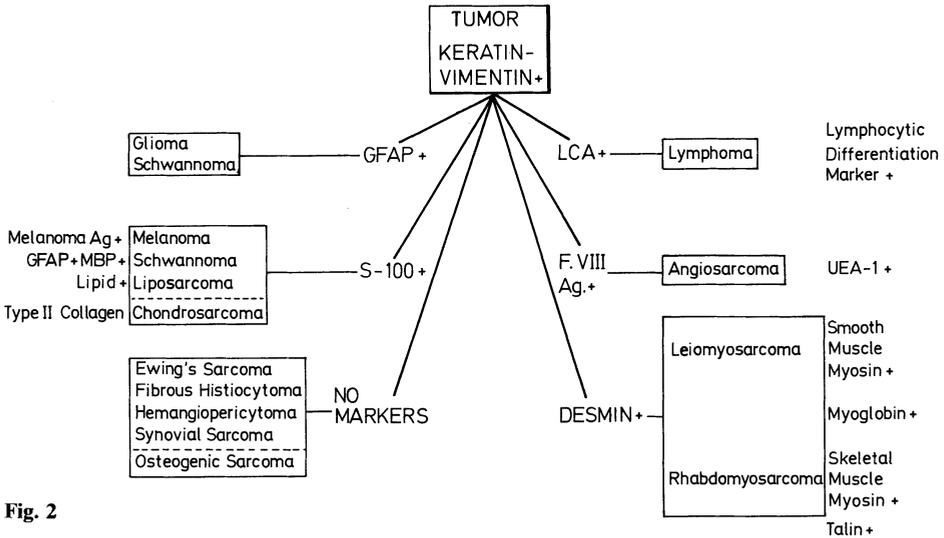


Fig. 2

have been proposed for a step by step approach and these, with some modifications, could be applied to most, if not all, solid tissue tumors. Such a flow chart, modified from ROHOLL et al. (1985) is present in Figure 1 and Figure 2 and is based on the premise that most, if not all, epithelial tumors express keratin polypeptides as major components of their intermediate filament cytoskeleton (OSBORN and WEBER 1983). Epithelial membrane antigen (EMA) could be used instead of keratin as the marker for epithelial cells in the initial screen and the combined application of antibodies to keratin and EMA seems to be even more productive (BATTIFORA 1984).

Keratin positive tumors represent in most instances carcinomas. Appropriately fixed or freshly frozen specimens can be further analyzed for the expression of specific keratin polypeptides (OSBORN et al. 1986b) or vimentin. Vimentin,

a typical marker of mesenchymal cells, if expressed as the only intermediate filament protein is a strong indication that the tumor is not a carcinoma and is thus either sarcoma, melanoma or a hematopoietic neoplasm. In that case a battery of tests with antibodies to specific mesenchymal cell markers should be performed as outlined in Figure 2. Keratin positive, vimentin positive tumors could be carcinomas, and there are several organs, such as endometrium, kidney and thyroid that give rise to such tumors (GOWN et al. 1985; MIETTINEN et al. 1985). Metastatic carcinomas, like the carcinoma cells explanted in vitro may express both keratin and vimentin positive cells. Sarcomatoid carcinomas of the esophagus are another example, although in these tumors one could find both coexpression of vimentin and keratin in the same cells and distinct vimentin and keratin positive cell populations.

It is important to note that certain tumors of soft tissues, most notably biphasic synovial cell sarcomas, (MIETTINEN and VIRTANEN 1984), epithelioid sarcomas (MIETTINEN et al. 1985c) and rhabdoid tumors (SOTELLO-AVILA et al. 1986) coexpress vimentin and keratin in the same cells.

Keratin positive cells may in some tumors coexpress neurofilament proteins. Thus, it has been shown that keratin and neurofilaments are coexpressed in some oat cell carcinomas and carcinoids of the lung (LEHTO et al. 1985) islet cell carcinomas of pancreas, especially insulomas (MIETTINEN et al. 1985b), parathyroid adenomas (MIETTINEN et al. 1985a). Merkel cell carcinomas of the skin coexpress keratin and neurofilaments with great regularity (GOULD et al. 1985). The coexpression of several types of intermediate filament proteins in the same cells is biologically a most intriguing phenomenon which so far has not received full explanation (GOULD 1985). However, such aberrant expression of markers should be kept in mind to avoid overdogmatic interpretation of immunohistochemical findings. Keratin negative, vimentin negative tumors should be evaluated most carefully to exclude spuriously negative results. Vimentin is poorly preserved in routinely processed tissues embedded in paraffin and negative staining with antibodies to vimentin should thus be interpreted with utmost caution. Nevertheless some tumors, such as seminoma or those that have very little cytoplasm like some neuroblastomas appear unreactive with antibodies to all intermediate filaments (OSBORN and WEBER 1983).

Vimentin positive, keratin negative tumors comprise mostly benign and malignant soft tissue neoplasms, melanomas and neuroectodermal neoplasms, gliomas and schwannomas. Other intermediate filament proteins, such as glial fibrillary acidic protein and desmin may be used to further characterize these tumors (ROHOLL et al. 1985). Other markers for these tumors are reviewed elsewhere in this volume. New markers, such as titin for rhabdomyosarcomas (OSBORN et al. 1986) or smooth muscle specific myosin (LONGTINE et al. 1985) could be of additional use.

There are still no universally accepted markers for several sarcomas. This group includes most prominently fibrous histiocytomas, Ewing's sarcoma, hemangiopericytoma, synovial cell sarcoma. The nature of stem cells of these tumors has not yet been defined and it is not known whether the polymorphism in some of these represents abortive differentiation or progression into a more anaplastic form of neoplasia (BROOKS 1986).

5 Perspective

This volume contains numerous examples illustrating the diagnostic value of immunohistochemistry in routine pathology. Antibodies, such as those against intermediate filaments, epithelial membrane antigen, hormones and organ specific polypeptides and many others are already widely used by pathologists worldwide. New antibodies are being developed and tested. Diagnostic pathology has not yet reached its peak, and it is reassuring to note that immunohistochemistry is here to stay and that it is on the best way to realize its full potential.

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