Virchows Arch [Pathol Anat] (1985) 406:237–251

Virchows Archiv A

Epithelial markers for paraffin-embedded human tissues

Immunohistochemistry with monoclonal antibodies against milk fat globule antigens

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Summary. About 200 human tumours and corresponding normal tissue samples were investigated by immunoperoxidase tests for the expression of MAM-3, MAM-5 and MAM-6 antigens, which had previously been defined by monoclonal antibodies to human milk fat globule membranes. All tissue specimens had been treated for routine histopathology, i.e. fixed in formalin and embedded in paraffin. One of the antigens, MAM-6, appeared to be an important epithelial marker, present in all normal and neoplastic breast tissue samples, in about 80% of non-mammary normal tissues and in more than 90% of non-mammary epithelial tumours. It could never be detected in normal and neoplastic mesenchymal and neuroectodermal structures. Direct comparison with the distribution of Carcinoembryonic Antigen (CEA), Tissue Polypeptide Antigen (TPA) and keratin provided clear evidence that MAM-6 is different from these well known epithelial markers. MAM-3 proved to be an additional important marker exhibiting a characteristic distribution pattern in those epithelial tissues investigated. In contrast to MAM-6, it could never be detected in renal cell cancers and carcinomas of the prostate gland, thus allowing differential diagnosis on the basis of immunohistochemistry. MAM-5, known to be associated with lactoferrin, was mainly detectable in secretory organs and their tumours. In the group of breast tumours, its expression was mainly seen in lobular cancers. These findings suggest a use for these new markers for routine histopathology.

Key words: Epithelial markers – Monoclonal antibodies – Immunohistochemistry

Out of a panel of monoclonal antibodies (MoAbs) raised against the human milk fat globule (HMFG) membrane (Hilkens et al. 1982; 1984a, b), three

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proved to be of special value for immunohistochemistry on paraffin-embedded tissues. These antibodies (67D11, 67D9, 115D8) were found to react with different molecules, designated as MAM-3, MAM-5, and MAM-6, respectively. Because of the striking association of these antigens with epithelial structures, we studied their tissue distribution in about 200 human tumours of different organ origin and in the corresponding normal tissues. The data strongly suggest a role for the MoAb-defined MAM antigens, especially of MAM-6, as epithelial markers useful in routine pathology.

Material and methods

Monoclonal antibodies. The MoAbs selected for this study were raised in fusions performed with P3/NS1/1-Ag4-1 (fusion 67) or Sp2/0 myeloma cells (fusion 115) and spleen cells from BALB/cHeA mice as detailed elsewhere (Hilkens et al. 1984a). MoAbs 67D11, 67D9 and 115D8 were found to belong to the IgG subclasses 1, 2a and 2b, respectively (Hilkens et al. 1984a).

Tissue samples and immunoperoxidase procedure. Biopsy and resection preparations were fixed in 4% neutralized formaldehyde immediately after surgery and embedded in paraffin. Deparaffinized 5 µm slices were preincubated with normal rabbit serum for 20 min (room temperature, moist chamber), decanted and incubated thereafter with appropriately diluted MoAbs for 4 h (antibody dilutions in phosphate-buffered saline, PBS, containing 1% of normal rabbit serum: 67D9 (culture supernatant) 1:20; 67D11 (ascites fluid) 1:1,000; 115D8 (ascites fluid) 1:10,000). After blocking the endogenous peroxidase (methanol with 0.1% H_2O_2, 5 min), the sections were covered with POD-labeled rabbit anti-mouse immunoglobulin antiserum from DAKO, Denmark (1:100 in normal rabbit serum) at 37° C for 30 min. For enzyme localization, 3,3′ diaminobenzidinhydrochloride (Boehringer, Mannheim, FRG) was used as substrate. Counterstaining was done with haematoxylin. Washings were performed after all incubation steps (except after the covering with normal rabbit serum) in PBS (pH 7.6) under stirring for 10 min. Optimal antibody concentrations and incubation times had been found by thouroughly pretesting the system. Positive controls (antigen-positive sections) were included in each test series. Parallel slices from all tissue specimens were processed with PBS instead of MoAbs as negative controls and, for comparison, with antibodies to CEA (Zotter et al. 1985).

Results

Breast gland and mammary carcinomas. The Tables 1 and 2 summarize the incidence of positive reactions obtained on non-tumorous mammary tissue and breast cancer specimens. The data demonstrate that MAM-6 is expressed in all the normal samples of the resting mammary gland. Staining was observed with large and terminal ducts as well as with the alveoli (Fig. 1). The same antigen was also detectable in all of the 37 breast cancers studied. In normal breast tissue, MAM-3 and MAM-5 were mainly detectable in ductal epithelia. About half of the mammary cancers expressed MAM-3. Two anaplastic carcinomas were negative for this antigen. Interestingly, invasive lobular carcinomas, but not the ductal ones, revealed a comparatively high frequency of MAM-5 expression.

All three antibodies gave rise to cytoplasmic and membranous staining as well as to the labeling of intraluminal secretion products in normal tissue samples and tumours (Figs. 1–4).

Table 1. Presence of MAM antigens in resting normal breast glands

Type of tissue	No. of cases (positive/tested)		
	MAM-3	MAM-5	MAM-6
Large milk ducts	9/9	6/9	12/12
Terminal ducts (intralobular)	16/19	7/13	23/23
Acini	3/11	3/8	12/12

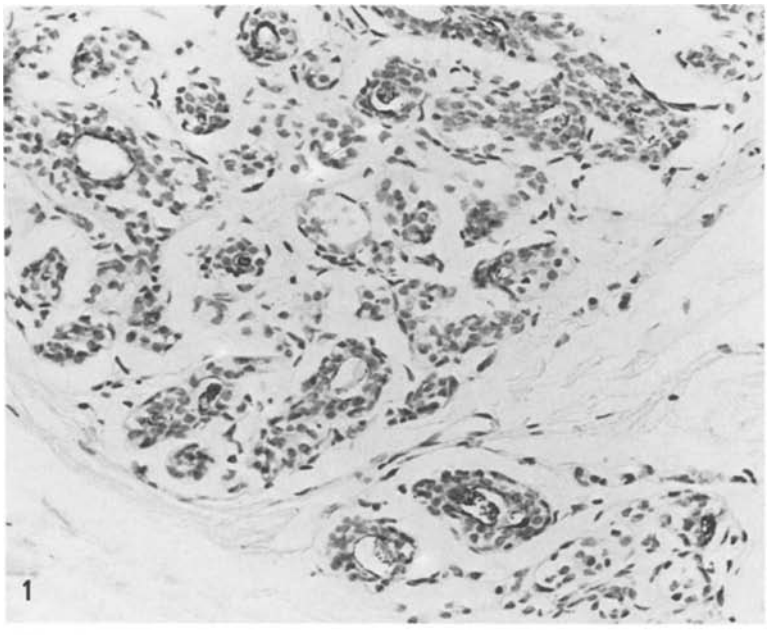
Table 2. Detection of MAM antigens in breast cancers

Histological type of tumour	No. of cases positive			No. of cases tested
	MAM-3	MAM-5	MAM-6	
Invasive ductal carcinoma	10	1	22	22
Invasive lobular carcinoma	6	5	9	9
Medullary carcinoma	2	1	2	2
Tubular carcinoma	1	1	2	2
Anaplastic carcinoma	0	0	2	2
Total: No. (percent)	19 (51.4)	8 (21.6)	37 (100)	37

It is noteworthy that, in general, the reaction obtained by the MoAbs was irregularly focal, sometimes leading to the detection of antigen-positive tissue regions directly surrounded by completely negative ones (and *vice versa*). In ductal or glandular structures, a remarkably prominent reaction with the apical or luminal cell membranes was often observed with MoAb 115D8 (Figs. 1, 4).

Non-mammary tissues and tumours. The data obtained with epithelia in non-mammary normal and neoplastic tissue specimens are summarized in the Tables 3 and 4. As in the mammary gland and its tumours, the reaction pattern observed was again often focal or inhomogenous. Therefore, all samples with clear-cut staining of at least some cell complexes were scored positive in these Tables.

MAM-6 was detected in about 93% of the epithelial tumours and in 82% of normal epithelial tissues. Interestingly, in some cases, especially in the large intestine, normal tissue appeared as MAM-6 negative, whereas the corresponding tumours gave strong positive reactions. In the series of 107 tumours listed in Table 4, the only completely negative samples were 1 adenoid cystic carcinoma of the parotid gland, 1 small cell lung cancer, 3 squamous cell carcinomas (epidermis, mouth, larynx), 1 hypernephroma of special histology (large cell variant with granulated acidophilic cytoplasm), 1 anaplastic prostate gland cancer and 1 granulosa cell tumour of the ovary.



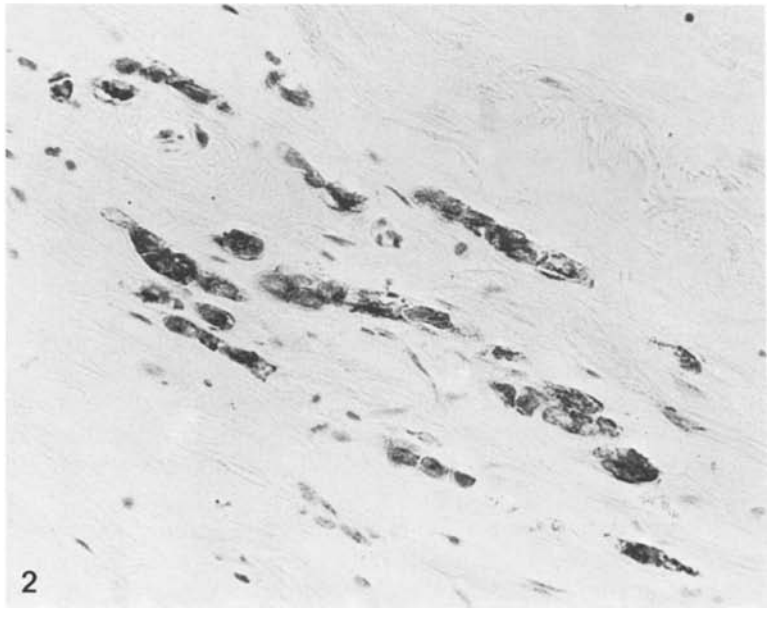
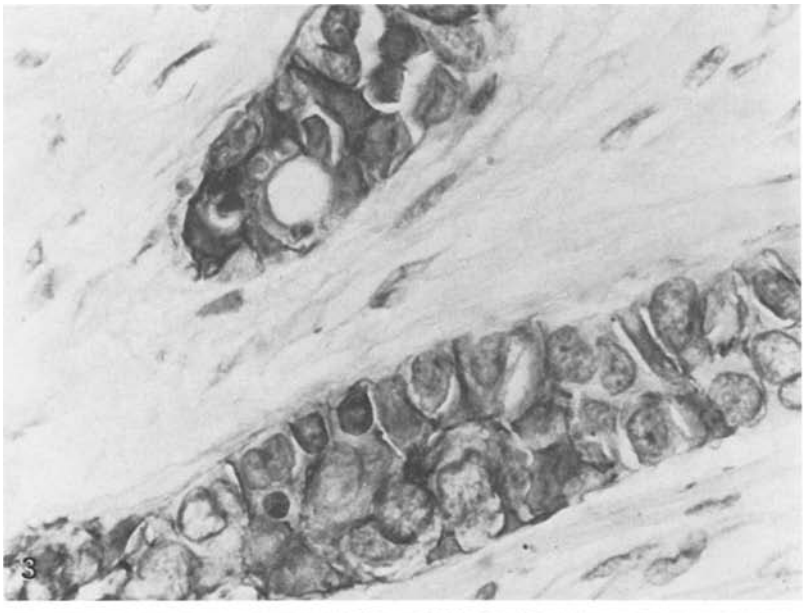


Fig. 1. Resting mammary gland. Moderate reaction for MAM-6 with some acini, mainly at the apical cell surface and with secreted material present in the lumina (MoAb 115D8). × 210

Fig. 2. Invasive lobular breast carcinoma. Strong cytoplasmic staining for MAM-5 in tumour cells arranged in characteristic indian-file pattern (MoAb 67D9). × 320



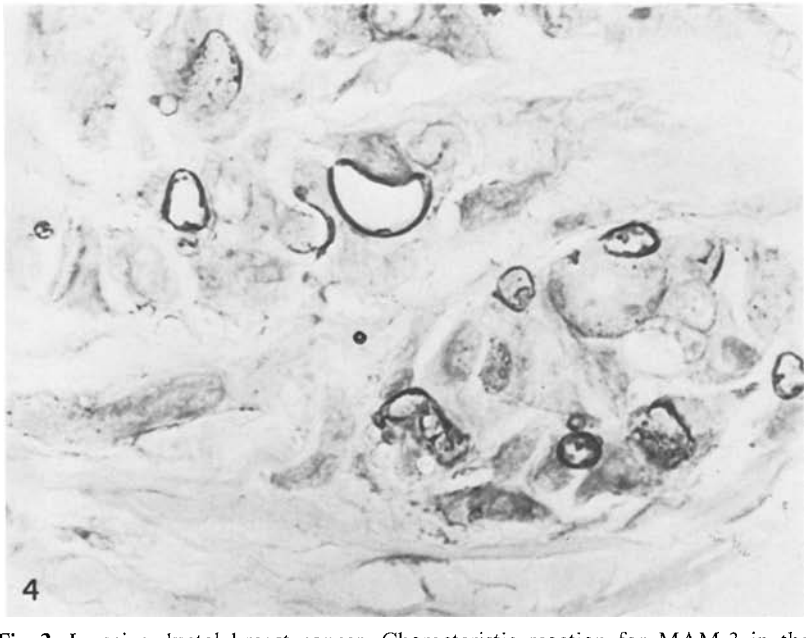


Fig. 3. Invasive ductal breast cancer. Characteristic reaction for MAM-3 in the cytoplasm and at the cell membrane of most of the tumor cells. × 370

Fig. 4. Detection of MAM-6 in an invasive ductal cancer (same tumour as in Fig. 3; MoAb 115D8). Note the prominent reaction with numerous large and small lumina or vacuoles and the comparatively weak reactivity with the cytoplasm. × 370

Table 3. Expression of MAM antigens in normal epithelial tissues of various organs

Type of tissue	No. of cases positive			No. of cases tested
	MAM-3	MAM-5	MAM-6	
Colonic mucosa	4	0	0	8
Stomach mucosa, corpus	4	1	4	4
Stomach mucosa, cardia	3	1	4	5
Parotid gland	4	2	3	5
– Excretory ducts	2	1	1	5
– Intercalated ducts	4	0	4	5
– Acini	2	1	1	5
Salivary glands	7	8	9	9
– Excretory ducts	0	0	2	4
– Serous cells	7	8	9	9
– Mucinous cells	6	0	2	9
Mucosa of the respiratory tract	11	9	14	14
Bronchus glands	4	4	5	5
– Serous cells	1	4	5	5
– Mucinous cells	3	0	1	5
Pulmocytes	2	1	12	12
Squamous epithelium	9	0	11	13
– Entodermal	8	0	11	11
– Epidermis	1	0	0	2
Sebaceous gland	0	0	2	2
Prostate gland	5	2	7	9
Urothel	5	2	7	7
Renal tubuli	3	1	5	5
Endometrium	2	1	4	4
Cervical mucosa	1	1	1	1
Mucosa of the Fallopian tube	1	0	1	1
Testis, tubuli contorti	0	0	0	5
Rete testis	0	0	2	2
Epididymis	1	0	1	1
Total: No. (percent)	66 (58.9)	33 (29.5)	92 (82.0)	112

MAM-3 was less often detected in the same series of normal tissues and tumours. The incidence of positive reactions was about 60% in normal and over 50% in neoplastic samples. Highest frequencies of positive reactions for MAM-3 were observed in the malignant tumours of the gastrointestinal tract and the endometrium. Interestingly, renal cell cancers (hypernephromas) and malignant prostate gland tumours were completely negative for MAM-3.

Reactivity for MAM-5 was about 30% in normal epithelial tissues and 11% in those cancers included in Table 4. It was mainly organs (and their tumours) with secretory functions which were positive for that antigen.

Tests performed with germ cell tumors (Table 5) gave data demonstrat-

Table 4. Detectability of MAM antigens in epithelial tumours of different non-mammary organs

Type of tumour	No. of cases positive			No. of cases tested
	MAM-3	MAM-5	MAM-6	
Colon cancer	9	1	10	10
Stomach cancer	6	2	10	10
Parotid gland tumour	9	5	9	10
– Pleomorphic adenoma	5	2	6	6
– Adenoid cystic carcinoma	2	1	1	2
– Cystadenolymphoma	2	2	2	2
Lung cancer	10	2	14	15
– Small cell cancer	3	1	4	5
– Large cell cancer	1	0	1	1
– Squamous cell cancer	1	1	4	4
– Adenocarcinoma	4	0	5	5
Squamous cell cancer	2	0	9	12
– Cancer of the mouth	0	0	2	3
– Oesophagus cancer	1	0	3	3
– Cancer of the larynx	0	0	2	3
– Anal cancer	1	0	2	2
– Cancer of the epidermis	0	0	0	1
Renal cell cancer	0	0	9	10
Transitional cell cancer of the urinary bladder	7	0	10	10
Prostate cancer	0	1	9	10
Ovarian tumour	3	1	9	10
– Papillary carcinoma	2	1	6	6
– Undifferentiated carcinoma	0	0	1	1
– Endometrioid carcinoma	1	1	1	1
– Mesonephroid tumour	0	0	1	1
– Granulosa cell tumour	0	0	0	1
Endometrioid adenocarcinoma of the corpus uteri	9	0	10	10
Total: No. (percent)	55 (51.4)	12 (11.2)	99 (92.5)	107

Table 5. Expression of MAM antigens in 15 germ cell tumours

Tumour component	No. of cases positive			No. of cases tested
	MAM-3	MAM-5	MAM-6	
Embryonic carcinoma	0	0	0	11
Malignant teratoma	9	4	7	9
Choriocarcinoma	0	1	2	4
Endodermal sinus tumour	0	0	0	5
Seminoma	0	0	0	2

Table 6. Mesenchymal and neuroectodermal tissues and tumours which are immunohistochemically negative for MAM antigens

Normal tissue[a]	Tumor tissue (No. of cases)
Connective tissue	Malignant fibrous histiocytoma (4)
	Alveolar soft-part sarcoma (1)
Blood vessels	Haemangiopericytoma (1)
Lymphatic vessels, lymph nodes	
Smooth muscle	Leiomyosarcoma (1)
Striated muscle	Rhabdomyosarcoma (5)
Chondroid tissue	Chondrosarcoma (2)
Bone, bone marrow	Ewing's sarcoma (1)
Brain	Astrocytoma (4)
	Oligodendroglioma (2)
	Glioblastoma (1)
	Medulloblastoma (1)
	Ependymoma (2)
Peripheral nerves	
Melanophores, naevus cells	Malignant melanoma (10)
	Mesenchymal structures in germ cell tumours (15)[b]

[a] Present in many specimens, number of cases not indicated
[b] For structures being positive in germ cell tumours see Table 5

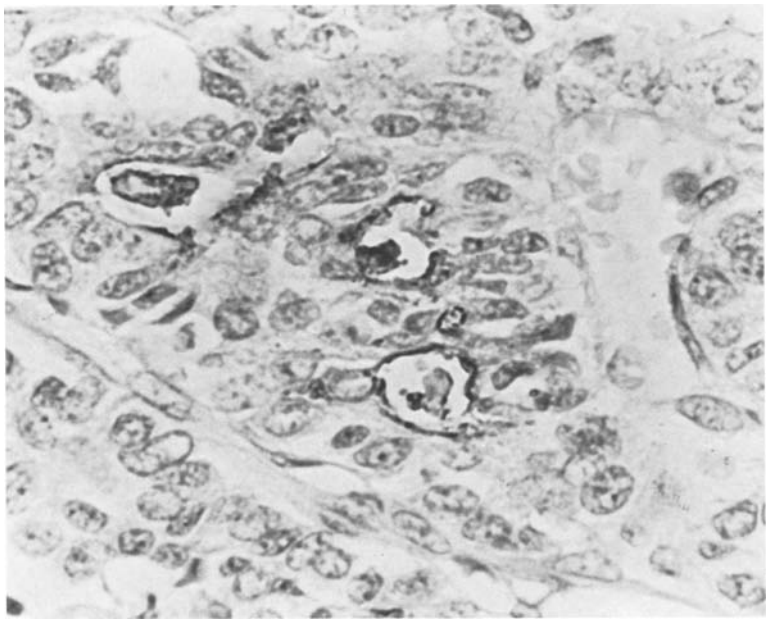


Fig. 5. Adenocarcinoma of the stomach. Prominent reaction for MAM-6 at the apical surfaces of those cancer cells lining the secretory lumina. ×900

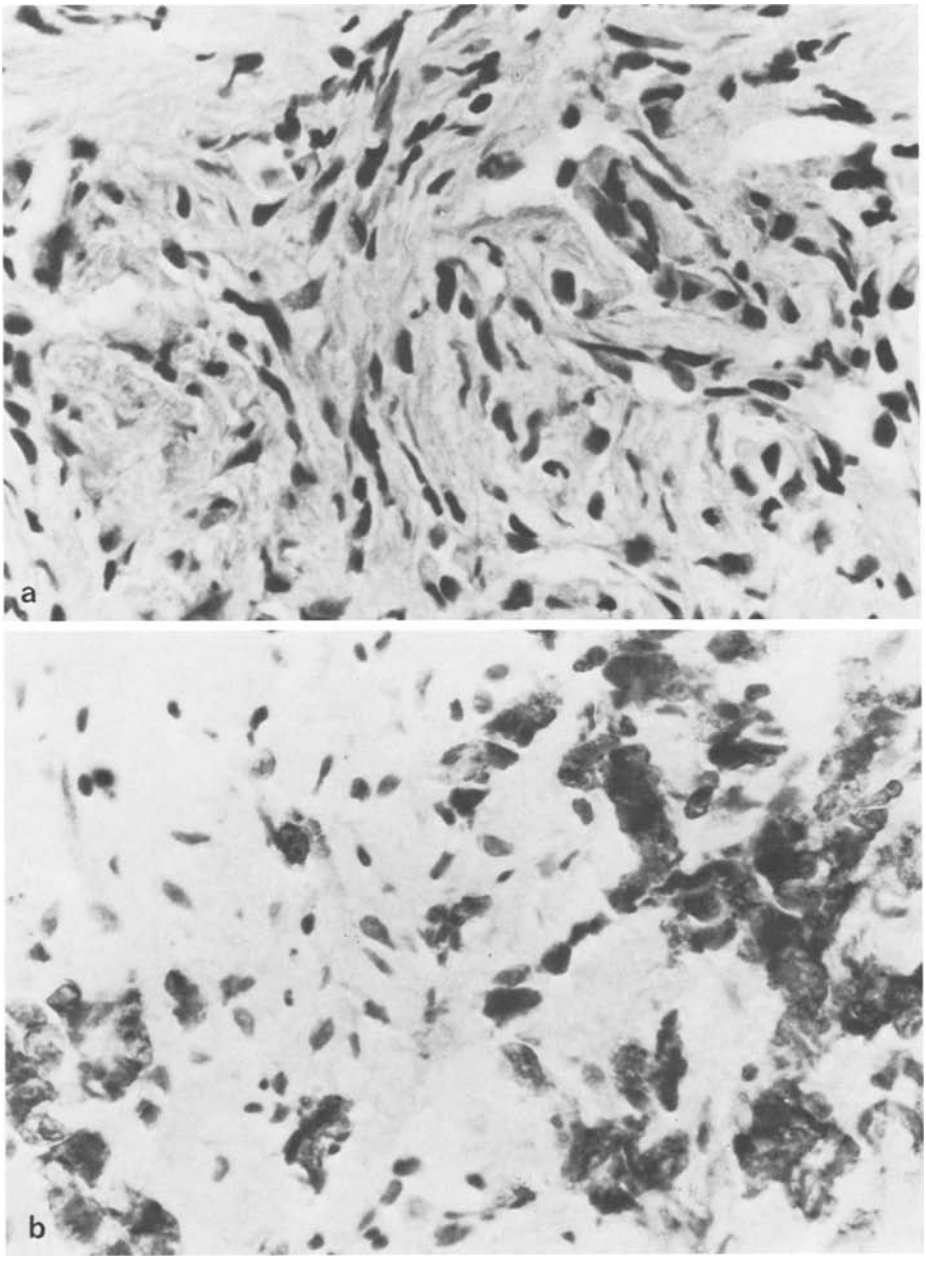


Fig. 6a, b. Biopsy from a bone lesion in a patient suffering from a poorly differentiated invasive ductal breast cancer. Focal fibrosis and unidentifiable cells exhibiting hyperchromatic and moderately polymorphic nuclei detectable by haematoxylin-eosin staining **a**. Identification of those cells as epithelial ones, i.e. metastatic cancer cells, by an immunoperoxidase test performed with MoAb 115D8 **b**. Note the heavy cytoplasmic staining of many cells appearing to have little cytoplasm in **a**. × 600

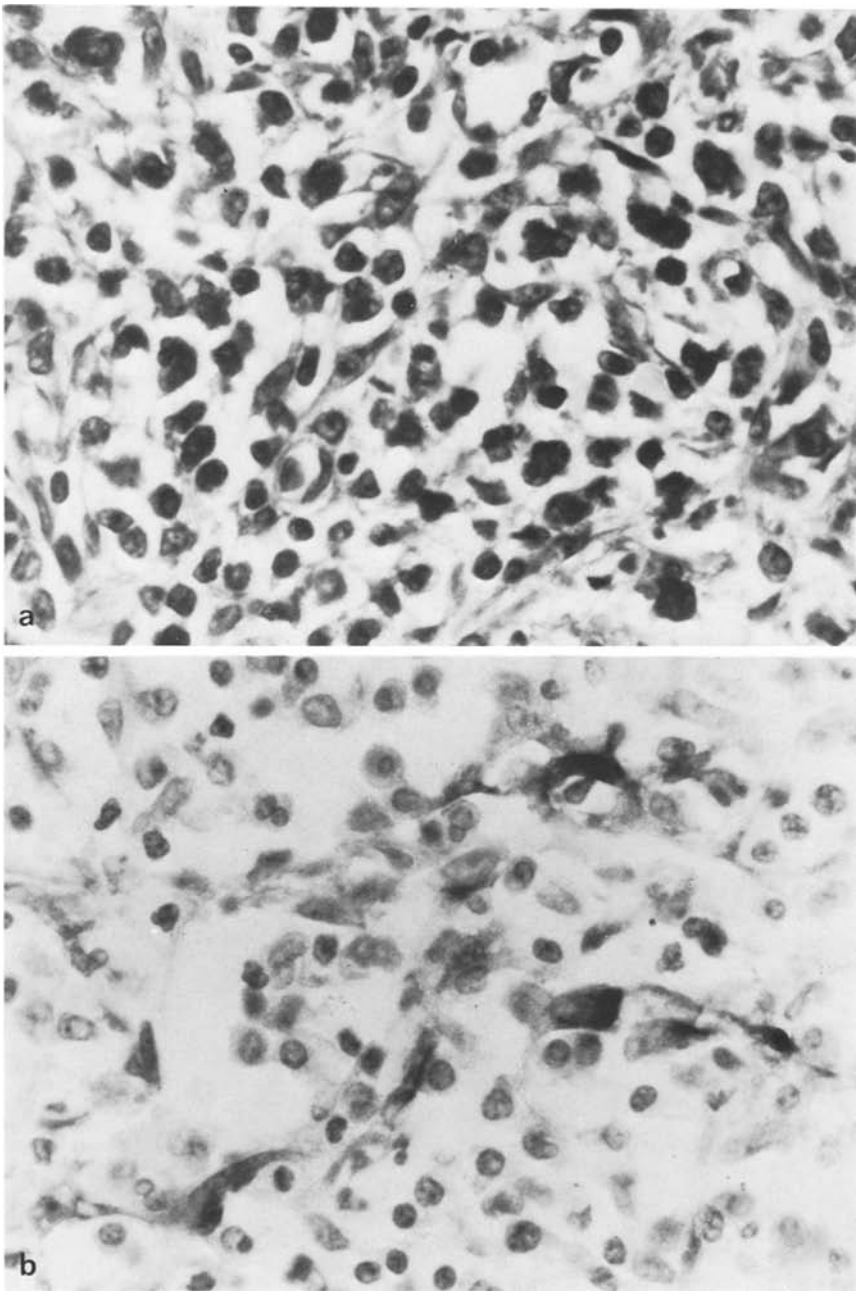


Fig. 7 a, b. Biopsy from an enlarged cervical lymph node. Proliferation of fibroblasts, histiocytes and endothelial cells intermingled with lymphocytes and granulocytes is detectable throughout the lymphnode after HE staining **7 a**. Note the presence of some unidentifiable cells exhibiting large hyperchromatic nuclei. Staining of single irregularly shaped cells for MAM-6 after immunoperoxidase reaction with MoAb 115D8 **b**. 10 months after this biopsy a microcarcinoma of the epipharynx (undifferentiated nasopharyngeal cancer, so-called lymphoepithelial carcinoma) as well as further metastases in cervical nodes had been diagnosed. The primary and the metastases were found to be positive for MAM-6 by the immunohistochemical studies performed afterwards. The dark single cells demonstrated in Fig. 7b are clearly to be considered as distinct early metastasis. × 600

ing the consistent correlation of reactivity of the antibodies with epithelial differentiations. Remarkably, however, embryonic carcinomas and seminomas were completely MAM-6 negative. In this connection, it is noteworthy that tubuli contorti in five testes investigated did not react with MoAb 115D8. Chorionepitheliomatous structures within the germ cell tumours were occasionally stained by the antibodies reactive with MAM-5 and MAM-6.

Table 6 summarizes those mesenchymal and neuroectodermal tissues and tumours studied. No positive reactions were obtained in this group of tissue specimens.

Discussion

Three selected MoAbs against human milk fat globule antigens were examined for their reactivity on a series of paraffin-embedded normal and neoplastic tissues which had already been investigated for their expression of CEA (Zotter et al. 1985), Tissue Polypeptide Antigen (TPA) and keratin (Zotter et al. unpublished; specific antisera to TPA and keratin kindly provided by Dr. L. Fischer, AB Sangtec, Bromma, Sweden, and Dr. P. Brandtzaeg, Oslo, Norway). Properties of these antibodies were previously described by Hilkens et al. (1984a, b). They had also been applied to some special immunohistochemical studies of various organs and tumours (Arends et al. 1984; Hageman et al. 1982; Koldovsky et al. 1984b; Rasmussen et al. 1982; Tsubura et al. 1985; Wagenaar et al. 1984). However, their usefulness in routine pathology deserves to be accentuated, as is done by this independent investigation performed with biopsy material.

The most impressive finding of this study is the almost perfect role of MAM-6 as an epithelial marker in paraffin-embedded tissues. The epitope "a" of MAM-6, recognized by MoAb 115D8, was seen to be expressed in about 80% of non-neoplastic human epithelial tissue samples and in more than 90% of the epithelial tumour specimens. There was no clear-cut positive reaction in mesenchymal tissues nor in tissues of neuroectodermal origin (Table 6). Obviously, a wide range of methods are applicable for its detection, because the immunoperoxidase procedure used in this study and those techniques applied by Hilkens et al. (1984a) and in the reports mentioned above differ remarkably in antibody dilution, incubation time and other details. Variation induced by fixation and embedding on the detectability of MAM-6 by MoAb 115D8 have been ruled out by Rasmussen et al. (1984).

The restrictions to be made in considering the practical usefulness of this marker are: 1) Some groups of epithelial tumours were negative for MAM-6 in our present experience, i.e. embryonic carcinomas and seminomas (tubuli contorti of the testes were also negative). 2) There are particular MAM-6 negative cancers even in the main tumour groups (in this study, e.g., single cases of small cell cancers of the lung and squamous cancers and a hypernephroma of special histology). 3) MAM-6 can occasionally be expressed in activated mesothelia, an important finding which deserves further detailed investigation (Hilkens et al. 1984a). 4) The reaction pattern

is sometimes only focal. Therefore, negative reaction with small tumour parts does not necessarily exclude the epithelial nature of the tissue. It is noteworthy that the antigen expression can be remarkably strong even if only small cell groups are positive. A similar heterogeneity had also been reported for other MoAb-defined breast-associated antigens (Foster et al. 1982a and b; Nuti et al. 1982; Horan Hand et al. 1983; Mariani-Costantini et al. 1984). It might preferably be explained by the presence of different cell clones in one tumour, influences of the cell cycle and/or the cell differentiation.

Interestingly, there is a tendency for the antigen to be expressed at the apical surface of cells oriented to a given lumen, especially in the breast gland and its tumours, but also in normal and neoplastic cells of other organs (compare Figs. 1, 4, 5). However, if this orientation is lost, e.g. in undifferentiated tumours, a more diffuse cytoplasmic staining can often be observed (compare Fig. 6b, 7b). This might be indicative of a correlation between the differentiation of cells, especially their secretory function, and the site of antigen expression.

Out of the monoclonal anti-HMFG membrane antibodies reported in the literature, only HMFG-1 (Taylor-Papadimitriou et al. 1981; Arklie et al. 1981) appeared to detect an epithelial-associated antigen of similar distribution to MAM-6. This antibody seemed to detect the same determinant as MoAb 115D8 (Hilkens et al. 1984a). Interestingly enough, the tissue specificity of one of the antibodies (MBr1) generated against the MCF-7 breast cancer cell line by a group in Milan (Mènard et al. 1983; Canevari et al. 1983) has recently been shown to be rather similar to that described herein for 115D8 (Mariani-Costantini et al. 1984). MoAbs raised by fusion of lymphocytes from breast cancer patients with mouse myeloma cells (Schlom et al. 1980) and murine monoclonals against breast cancer preparations produced by the same group (Colcher et al. 1981; Nuti et al. 1982; Horan Hand et al. 1983) were mainly selected according to their breast cancer specificity and are clearly different from MoAb 115D8.

Another remarkable finding is the lack of detectable MAM-6 in some normal epithelia, the tumours of which express the antigen. This was especially evident in the colon. In this organ, however, the production of MAM-6 could also be observed during fetal stages of development (Gerö, Hilgers et al. unpublished; Zotter et al. unpublished). Thus, the antigen expression in the colon is strongly resembling the situation with CEA. Interestingly, HMFG-1 did also give positive reactions with the intestine of embryos (Koldovsky et al. 1984a).

The biochemical nature of MAM-6 is not yet completely understood. It is evident from the tissue distribution that MAM-6 is different from CEA, TPA and keratin (data not detailed herein; for CEA see Zotter et al. 1985). Obviously, MAM-6 is represented by or belongs to a high-molecular-weight glycoprotein of more than 400 Kd (Hilkens et al. in preparation). Japanese authors have recently described the analysis of such a large molecule isolated from HMFG membranes (Shimizu and Yamauchi 1982). Some of the MoAbs against milk fat globule antigens produced by other groups

were also found to react with a 400 Kd antigen (Burchell et al. 1983; Ceriani et al. 1983).

Remarkably, the Epithelial Membrane Antigen (EMA) described by an English group (Heyderman et al. 1979; Sloane and Ormerod 1981; Ormerod et al. 1982) exhibits a tissue distribution very similar to that of MAM-6. EMA has been defined by a rabbit antiserum to defatted human cream and has a molecular weight range of 200 to 500 Kd (Sloane and Ormerod 1981).

The reactivity of MoAb 67D11 (detecting MAM-3) with epithelial tissues and tumours is more restricted when compared with that of MoAb 115D8 (about 50% of breast cancers and other carcinomas). However, the high percentage of positive outcomes with colon cancers and the complete lack of reactivity with hypernephromas and prostate cancers had already been proven to be of value in differential diagnosis on routine paraffin sections. Moreover, this antibody did not react with activated mesothelia (Hilkens et al. 1984a; Zotter et al. unpublished).

MAM-5, an antigen recognized by MoAb 67D9, was mainly detected in secretory organs and tumours of the breast and of the parotid gland, which is consistent with its association with lactoferrin (compare Hilkens et al. 1984a). The comparatively high incidence of positive tests for MAM-5 in invasive lobular mammary carcinomas, in contrast to the ductal ones, might be explained by the possible "higher"- cell differentiation in the lobular tumours. In this sence, lactoferrin might be regarded as differentiation marker, and MoAb 67D9 appears as a potent reagent against it.

The main conclusion from this paper is the impressive nature of MAM-6 as an epithelial marker easily detectable by immunoperoxidase technique on paraffin sections. It is present in about 80% of non-neoplastic epithelial tissues and more than 90% of epithelial tumours, which could make the MoAb 115D8 an extremely useful reagent for immunohistochemistry. In Fig. 6 and 7, the identification of cancer cells by this antibody in biopsy specimens is shown as examples out of those cases in which MoAb 115D8 proved helpful already for routine histopathology. The restricted, but well known tissue specificity of MoAbs 67D11 and 67D9 will be of additional value in the rapidly developing field of immunohistochemistry with monoclonal antibodies to cell differentiation antigens.

Acknowledgements. Gratitude is expressed to Mrs. Ingrid Peterson (Institute of Pathology, Dresden) for skillful and diligent technical assistance. The courtesy of Dr. Lave Fischer (Bromma, Sweden) and Dr. Per Brandtzaeg (Oslo, Norway) to provide antisera to TPA and keratin, respectively, is gratefuly acknowledged.

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Accepted January 22, 1985