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Expression of cytokeratins and vimentin in salivary gland carcinomas as revealed with monoclonal antibodies

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Summary. The expression and distribution of cytokeratins and vimentin in fifteen malignant salivary neoplasms were examined by immunocytochemical techniques using, five monoclonal antibodies (mAbs) against different epitopes of Cytokeratins (CKs) (mAbs PKK1, PKK2, and PKK3, identifying CKs 8, 18 and 19, CKs 7, 17 and 19, and CK 18, respectively) and Vimentin (mAbs V9 and V24). Antibody PKK1 gave strong reactions in all neoplasms showing the similarity of these tumours to other digestive system adenocarcinomas. Three general staining patterns of the neoplasms were recognized with respect to the reactivity of mAbs PKK2, PKK3, and V9. Mucoepidermoid cancer, salivary duct carcinoma and a clear cell carcinoma had a higher relative content of CKs 7, 17 and 19 than of CK 18. Adenoid cystic carcinoma showed the same CK pattern but in the periphery of the tumour cords vimentin was readily detected. In two acinic cell carcinomas, the relative content of CK 18 was higher than that of CKs 7, 17 and 19. Furthermore vimentin was expressed in the tumour cells. However, one mucoepidermoid carcinoma showed vimentin expression and two acinic cell carcinomas were vimentin negative and more reative for PKK2 than PKK3. Pecularities in CK expression were seen: squamous areas of mucoepidermoid carcinomas were stained by mAb PKK3 although CK 18 is not present in normal squamous epithelia or in squamous cell carcinomas of tongue and skin. In conclusion, the different salivary neoplasms can be distinguished on basis of IFP content. Such a differentiation fits with current theories of histogenesis, i.e. vimentin is seen in tumours presumed to arise from intercalated duct reserve cells, whilst the vimentin negative neoplasms would be expected to arise in excretory duct reserve cells.

Key words: Intermediate filaments – Immunohistochemistry – Salivary gland neoplasms

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Introduction

Intermediate filaments (IF), with a diameter of approximately 10 nm, are made up of a group of cytoskeletal proteins which show tissue-type specificity. In all epithelial cells, the IF are formed of cytokeratins (CK) whereas in most mesenchymal cells, the IF are built up of vimentin. Of special importance is that IF in tumour cells are composed of IF proteins of the tissue of origin (Osborn and Weber 1983; Miettinen et al. 1984; Virtanen et al. 1985). Although this initially was accepted as an axiom, a few exceptions have been observed in which epithelial tumour cells have also contained vimentin (Miettinen et al. 1984a, b; Caselitz et al. 1982, 1984; Krepler et al. 1982; Holthöfer et al. 1983; Herman et al. 1983; McNutt et al. 1985; Gustafsson et al. 1986a, b) or where neuroendocrine or endocrine tumours have acquired neurofilament proteins in addition to CK (McNutt et al. 1985; Miettinen et al. 1985a, b). Epithelia of different kinds express individual CKs in different combinations of polypeptides. A given epithelium or epithelial cell tumour can, therefore, be characterized by the specific pattern of the CK components (Osborn and Weber 1983; Quinlan et al. 1985).

Few organs can give rise to such a wide spectrum of tumours as the salivary glands. Furthermore, the individual tumours may display areas of widely different morphology. This is, in general,

Table 1

Case	Diagnosis	Age	Sex	Location	Primary, recurrence or metastasis	Duration before Operation	Preop. irradiation
1	ACC	16	f	r. parotid	P	1y	–
2	ACC	71	f	l. parotid	M (5 y later)	5y	49 Gy
3	ACC	74	f	l. parotid	P	2y	–
4	ACC	75	f	l. parotid	P	1y	–
5	AdCC	47	f	r. parotid	R (8 y later)	8y	40 Gy*
6	AdCC	26	f	r. parotid	R (24 y later)	24y	65 Gy*
7	MEC	68	f	hard palate	P	1y	50 Gy
8	MEC	13	f	soft palate	P	2m	–
9	MEC	59	m	root of tongue	P+M (neck)	1y	–
10	MEC	70	f	l. sublingual	P	4m	–
11	MEC	60	f	hard palate	P	1y	–
12	SDC	59	m	r. ethmoid sinus or lacrimal sac	R (2 y later)	2y	65 Gy
13	SDC	45	m	l. sublingual	P	6m	–
14	CCC	66	f	lower lip	m (neck)	2y	–
15	MPAC	58	m	l. parotid	P+M (neck)	1.5 y	50 Gy

ACC = acinic cell carcinoma, AdCC = adenoid cystic carcinoma, MEC = mucoepidermoid carcinoma, SDC = salivary duct carcinoma, CCC = clear cell adenocarcinoma, MPAC = mucus-producing adenopapillary carcinoma. Gy = Gray * = irradiated at primary operation

Table 2. Reactivity of mAbs to different tumour types

Case	Diagnosis	Celltype	PKK 1 (8, 18, 19)	PKK 2 (7, 17, 19)	PKK 3 (18)	V 9	V 24
Staining type A							
1	ACC		3	2	3	0–2	0
2	ACC		3	2	3	0–2	0–2
Staining type B							
5	AdCC	central	3	3	1	0	0
		peripheral	3	3	0	2	1
6	AdCC	central	3	3	0–1	0	2
		peripheral	2	2–3	0	2	2
7	MEC	central	3	3	1	0–2	0
		peripheral	3	3	1	3	1
Staining type C							
3	ACC		2–3	3	1–2	0	0
4	ACC		3	2	1	0(–2)	not tested
8	MEC		1–3	1–3	0–2	0	0
9	MEC		2–3	2–3	0–2	0	0
10	MEC		2–3	2–3	1	0	0
11	MEC		2–3	2–3	1–2	0	0
12	SDC		3	3	2	0	0–1
13	SDC		1–3	1–3	1	0	0
14	CCC		3	3	1	0	0–1
15	MPAC	central	3	3	2	0	0
		peripheral	3	3	0	0	1

In cases 5, 6, 7, and 15 central and peripheral cells in tumour sheets or cords are analysed separately. Numbers indicate relative staining intensity. (0 = no staining, 1 = weak staining, 2 = moderate staining, 3 = strong staining). If more than one number is given outside brackets each number represent more than 20% of tumour cell population. Figures in brackets in the heading below PKK1–3 indicate cytokeratin with which the respective antibodies react
ACC = acinic cell carcinoma; AdCC = adenoid cystic carcinoma; MEC = mucoepidermoid carcinoma; SDC = salivary duct carcinoma; CCC = clear cell adenocarcinoma; MPAC = mucos-producing adenopapillary carcinoma

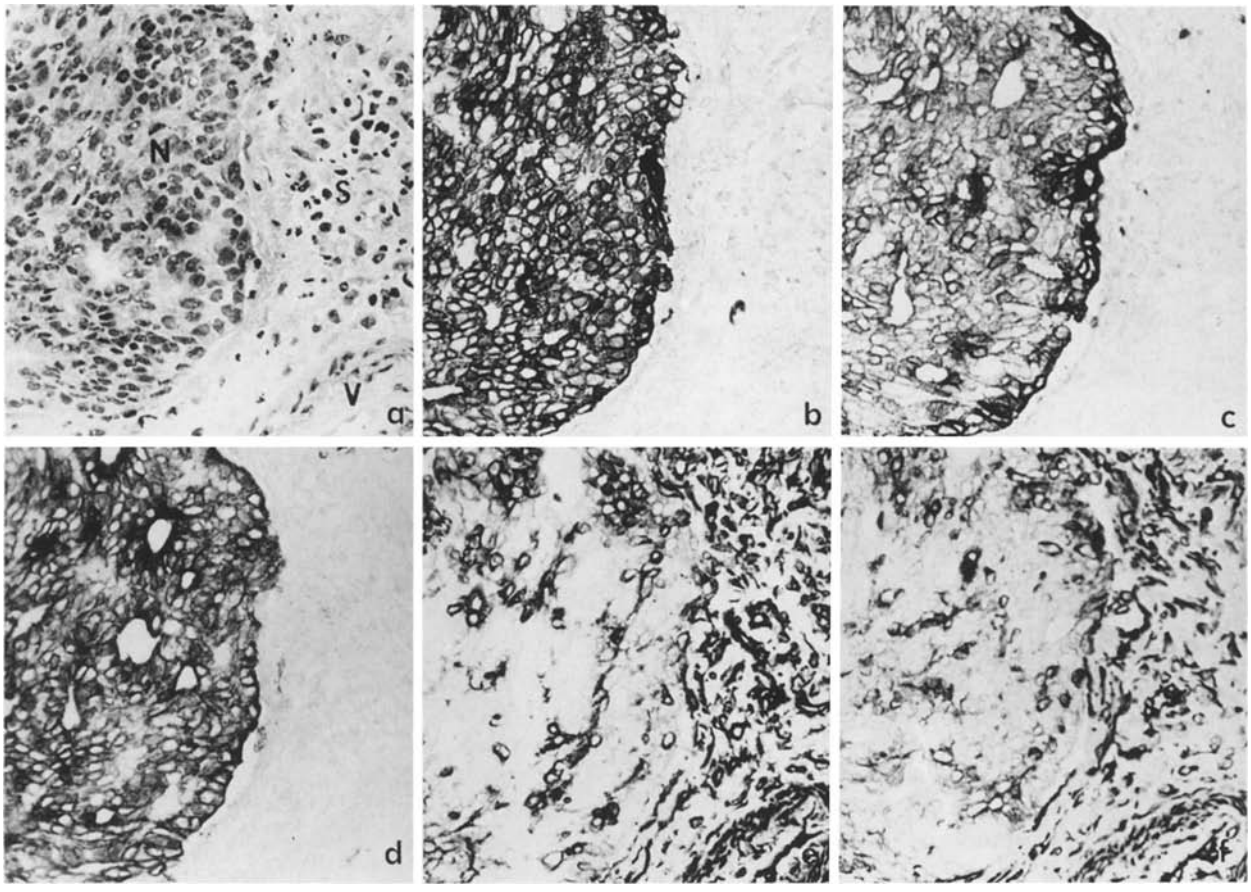

Fig. 1. Acinic cell carcinoma (case 2). Haematoxylin – Eosin (**a**) and immunoperoxidase staining with PKK1 (**b**), PKK2 (**c**), PKK3 (**d**), V9 (**e**) and V24 (**f**). PKK1 and PKK3 stain more intensely than PKK2. The two vimentin antibodies stain some neoplastic cells moderate to strong while others are negative. *N* = neoplasm, *V* = vessel, *S* = stroma, × 250

attributed to the presence of myoepithelial cells which have both mesenchymal and epithelial features (Hamperl 1970; Hübner et al. 1971; Batsakis 1983). The presence of CKs has been demonstrated in all epithelial tumours tested (Caselitz et al. 1981, 1982, 1986; Krepler et al. 1982; Gustafsson et al. 1985, 1986a, b; Palmer et al. 1985; Kahn et al. 1985; Erlandson et al. 1984; Saku et al. 1984; Dardick et al. 1984; Warner et al. 1985). However, no attempts have hitherto been made to distinguish between the different kinds of CKs in malignant salivary neoplasms. Coexpression of CKs and vimentin has been observed in tumour cells of pleomorphic adenomas (Caselitz et al. 1982; Krepler et al. 1982; Gustafsson et al. 1986a), adenoid cystic carcinomas (Caselitz et al. 1984; Gustafsson et al. 1986b) and carcinomas in pleomorphic adenomas (Gustafsson et al. 1986a).

The aim of the present study was to investigate the pattern of expression of intermediate filament proteins in a variety of salivary gland tumours with glandular differentiation, using monoclonal antibodies against vimentin and CKs, and correlate it with the histological picture.

Material and methods

Fifteen malignant salivary gland neoplasms were studied. Specimens were collected at operation and were immediately frozen in isopentane, precooled in liquid nitrogen. Most of the tumours were routinely processed for pathological diagnosis. The sections of the tumours were re-examined by the same experienced pathologist (F.B.) and the tumours were classified according to WHO criteria (Thackray and Sobin 1972) or according to Batsakis (1979). The patients' data and diagnoses are presented in Table 1.

Three mouse monoclonal antibodies (mAbs) directed aginst CKs were used: PKK1, PKK2 and PKK3. These antibodies have been characterized elsewhere (Holthöfer et al. 1983; Virtanen et al. 1985; Miettinen et al. 1985a). In terms of the current numbering system for CKs (Moll et al. 1982; Cooper et al. 1985) PKK1 reacts in normal human parotid gland tissue with a Mr 40 kD polypeptide, corresponding to CK 19, a Mr 45 kD polypeptide corresponding to CK 18 and a Mr 52 kD polypeptide corresponding to CK 8. Antibody PKK2 binds strongly to a polypeptide with Mr 40 kD (CK19), to a polypeptide with Mr 46 kD (CK 17), and to a polypeptide of Mr 54 kD (CK7). Antibody PKK3 reacts solely with a poly-

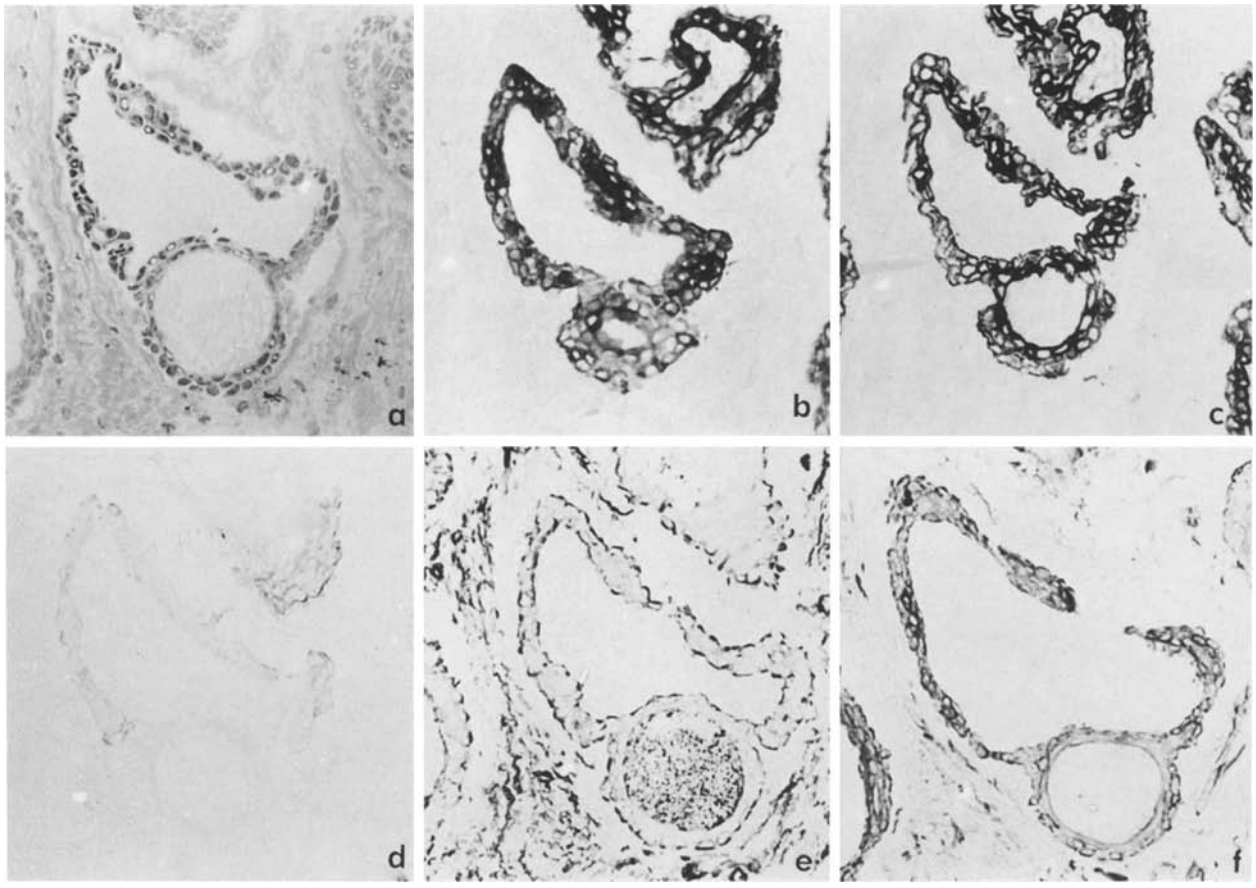

Fig. 2. Adenoidcystic carcinoma (case 6) Haematoxylin-eosin and immunoperoxidase staining with PKK1 (**b**), PKK2 (**c**), PKK3 (**d**), V9 (**e**) and V24 (**f**). Strongly positive reactions are achieved with PKK1 and PKK2, particularly the central ones, while PKK3 only faintly reacts with a few central cells. V9 reacts strongly with the marginal cell both towards the stroma and towards the pseudocyst. In contrast, V24 gives a moderate staining of the whole tumour cells, × 250

peptide of Mr 45 kD corresponding to CK 18. In normal parotid sections, PKK1 reacts with acinar cells, duct cells and myoepithelial cells, PKK2 reactivity is directed at myoepithelial cells and duct cells, whilst PKK3 can be seen in acinar and duct cells (Gustafsson et al. 1988).

Two mouse monoclonal antibodies to vimentin were used: one was raised against bovine lens vimentin (V9) and was purchased from Sanbio (Nistelrhode, The Netherlands) and the other monoclonal antibody was against vimentin from human fibroblasts from the clone, FV24, BA6 (V24). Its preparation and reactivity pattern has been described elsewhere (Virtanen et al. 1985, 1986a). In immunoblots of human parotid gland tissue, V9 and V24 only recognized the polypeptide of Mr 58 kD (vimentin). Antibody V9 does not react with any salivary epithelial cells whilst V24 recognizes myoepithelial cells and basal cells of excretory ducts (Gustafsson et al. 1988).

Serial frozen sections were used for immunostaining after being fixed in acetone at −20° C for five min. The primary antibodies were used at a concentration of 20–50 µg/ml at room temperature. After a thorough washing, the sections were exposed to fluorescein isothiocyanate (FITC)- or tetramethyl-rhodamine isothiocyanate (TRITC)-coupled antibodies or to antibodies coupled with horseradish peroxidase followed by antibodies against peroxidase likewise coupled with peroxidase (PAP method). The sections were mounted in Moviol 4–88 (Hoechst, Frankfurt am Main, FRG), pH 8.5 and viewed in a Leitz Orthoplane microscope equipped with epifluorescent optics or in an Olympus VANOX microscope.

Results

Cytokeratins were present in all tumour cells; these being stained at least with one of mAbs PKK1, PKK2, and PKK3. However, different combinations of cytokeratins were present since the different mAbs gave different staining patterns in the tumour cells (summarized in Table 2).

In two acinic cell carinomas (cases 1 and 2), the tumour cell populations stained homogeneously for the three types of CK mAbs; PKK1 and PKK3, however, giving a stronger positive staining than PKK2 (Fig. 1 b–d). In the adenoid cystic carcinomas (cases 5 and 6), most tumour cells were strongly reactive for PKK1 and PKK2, but for PKK3 a difference in staining was seen between cells adjacent to the connective tissue or pseudocysts (peripheral cells) and those adjacent

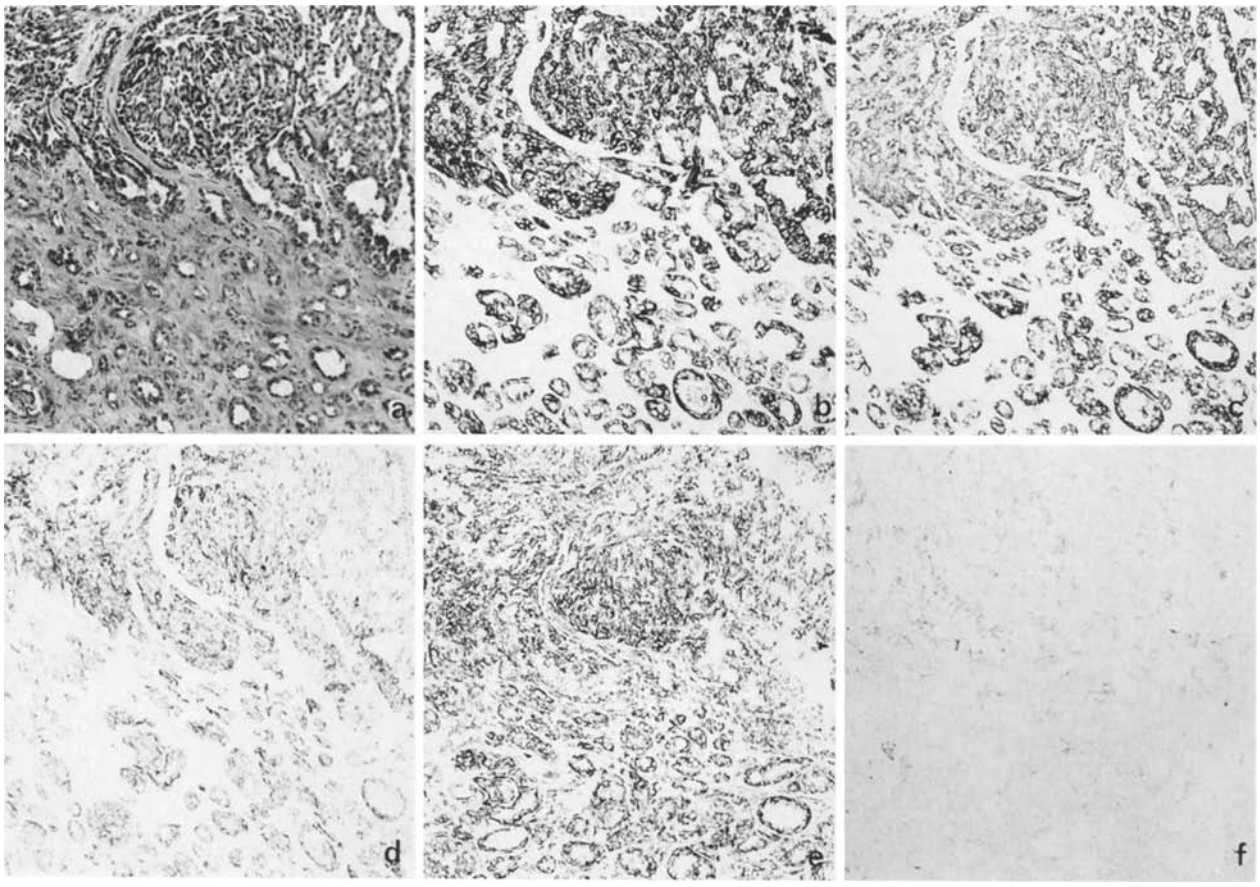

Fig. 3. Mucoepidermoid carcinoma. (case 7) Haematoxylin-eosin (**a**) and immunoperoxidase staining with PKK1 (**b**), PKK2 (**c**), PKK3 (**d**) V9 (**e**) and V24 (**f**). PKK1 and PKK2 stain cells strongly while PKK3 only gives faint reactions. Strongly reactive V9 positive cells are seen peripherally, × 100

to secretory lumina (central cells) (Fig. 2b–d). In only the latter cell type, a faint reaction was obtained with PKK3. A similar reactivity pattern was seen in a mucous producing adenopapillary carcinoma (case 15), where all neoplastic cells stained intensely with PKK1 and PKK2, but only cells lining duct lumina showed a moderate staining with PKK3.

In the remaining neoplasms (two acinic cell carcinomas, mucoepidermoid carcinomas, two salivary duct carcinomas, and one clear cell carcinoma) (cases 3, 4, 7–14) tumour cells reacted intensely to moderately to mAbs PKK1 and PKK2 and moderately to faintly to PKK3.

The two vimentin antibodies showed a different pattern of tissue reactivity. In general the stromal and vascular tissue stained more intensely with mAbs V9 than with mAbs V24 (Figs. 3e, f and 5e, f). In two acinic cell carcinomas (cases 1 and 2), mAbs V9 and V24 stained some tumour cells moderately, whereas others where unreactive (Fig. 1e, f). The tumour cells of the other two acinic cell carcinomas (cases 3 and 4), examined were not stained by the mAbs against vimentin. In the adenoid cystic carcinomas (cases 5 and 6), cells adjacent to basement membranes, as in the periphery of tumour cords or lining the pseudocysts were strongly vimentin reactive, whilst the "central" cells were vimentin negative (Fig. 2e, f). In four mucoepidermoid carcinomas, two salivary duct carcinomas, one clear cell carcinoma and one mucus-producing adenopapillary carcinoma (cases 8–15), V9-reactivity was not seen (Figs. 4e, f, 5e, f and 6e, f), but in one salivary duct carcinoma, one clear cell carcinoma and one mucus-producing adenopapillary carcinoma (cases 12, 14 and 15), a very faint indistinct (probably unspecific) V24 reactivity could sometimes be detected in a few cells. One mucoepidermoid carcinoma (case 7) differed markedly in stainability from the other mucoepidermoid carcinomas. Most tumour cells contained vimentin, especially in the peripheral cells of the tumour cords (Fig. 3e, f) like in the adenoid cystic carcinomas (cases 5 and 6).

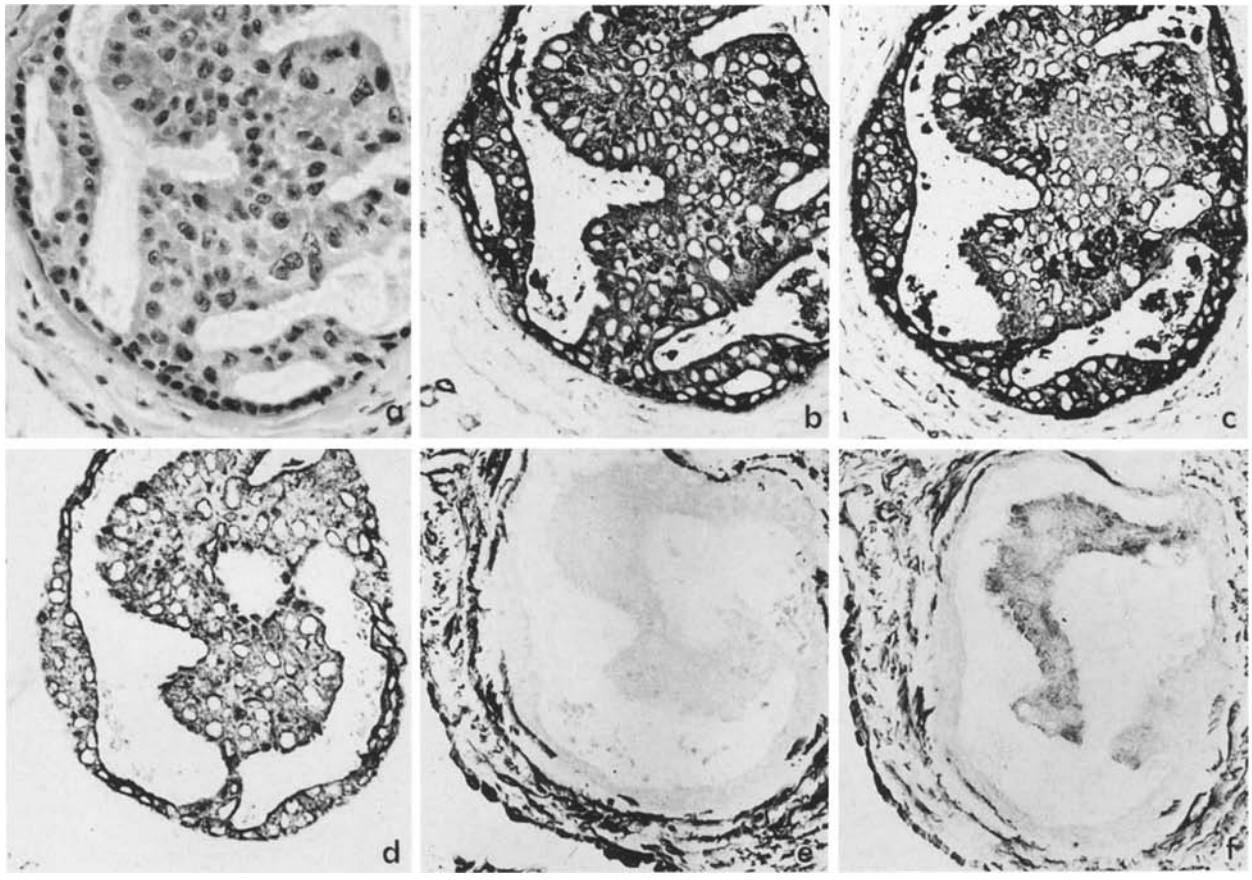

Fig. 4. Salivary duct carcinoma (case 12). Haematoxylin – Eosin and immunoperoxidase staining with PKK1 (6), PKK2 (**c**), PKK3 (**d**), V9 (**e**) and V24 (**f**). PKK1 and PKK2 stain intensely, PKK3 moderately while no definite tumour staining are achieved with vimentin antibodies, ×250

Discussion

In the present study of salivary gland tumours, characteristic patterns of cytokeratin and vimentin staining were distinguished in the different tumours. In general three different patterns of IF expression were recognized in the salivary gland tumours as depicted in Table 2. For 50% of acinic cells carcinomas, the relative content of CK 18 (=45 kD) was higher than that of CKs 7, 17 and 19 (=54, 46 and 40 kD). Furthermore, expression of vimentin as revealed by mAbs V9 was also typical. For adenoid cystic carcinomas, the opposite CK-pattern was found, whereas vimentin could be demonstrated in the periphery of the neoplastic cords. In mucoepidermoid carcinomas and adenocarcinomas, a similar CK pattern as for adenoid cystic carcinomas but without vimentin expression was found. In general, the IFP-staining supported routine pathological typing of these salivary neoplasms. However one mucoepidermoid carcinoma was vimentin positive and two acinic cell carcinomas showed a staining pattern similar to the other adenocarcinomas.

The PKK1 stainability confirms the similarity of salivary neoplasms with other adenocarcinomas as this antibody identifies CKs 8, 18 and 19 which are the cytokeratins most common for most gastrointestinal adenocarcinomas (Moll et al. 1982). However, there is a fundamental difference between squamous areas of mucoepidermoid carcinomas that are PKK3 positive (i.e. contain CK 18) and squamous cell carcinomas of skin and tongue which are CK 18 negative (Moll et al. 1982).

In the normal salivary gland, acinar cells react with PKK1 (CK 8, 18, and 19) and PKK3 (CK 18), myoepithelial cells react with PKK1 and PKK2 (CK 7, 17 and 19), and ductal cells with PKK1, PKK2 and PKK3. No epithelial cell react with V9, but myoepithelial cells and basal cells of larger ducts react with V24.

For the neoplasms in the first two groups, the CK-patterns are in line with the morphological appearances. Acinic cell carcinomas, in which acinar

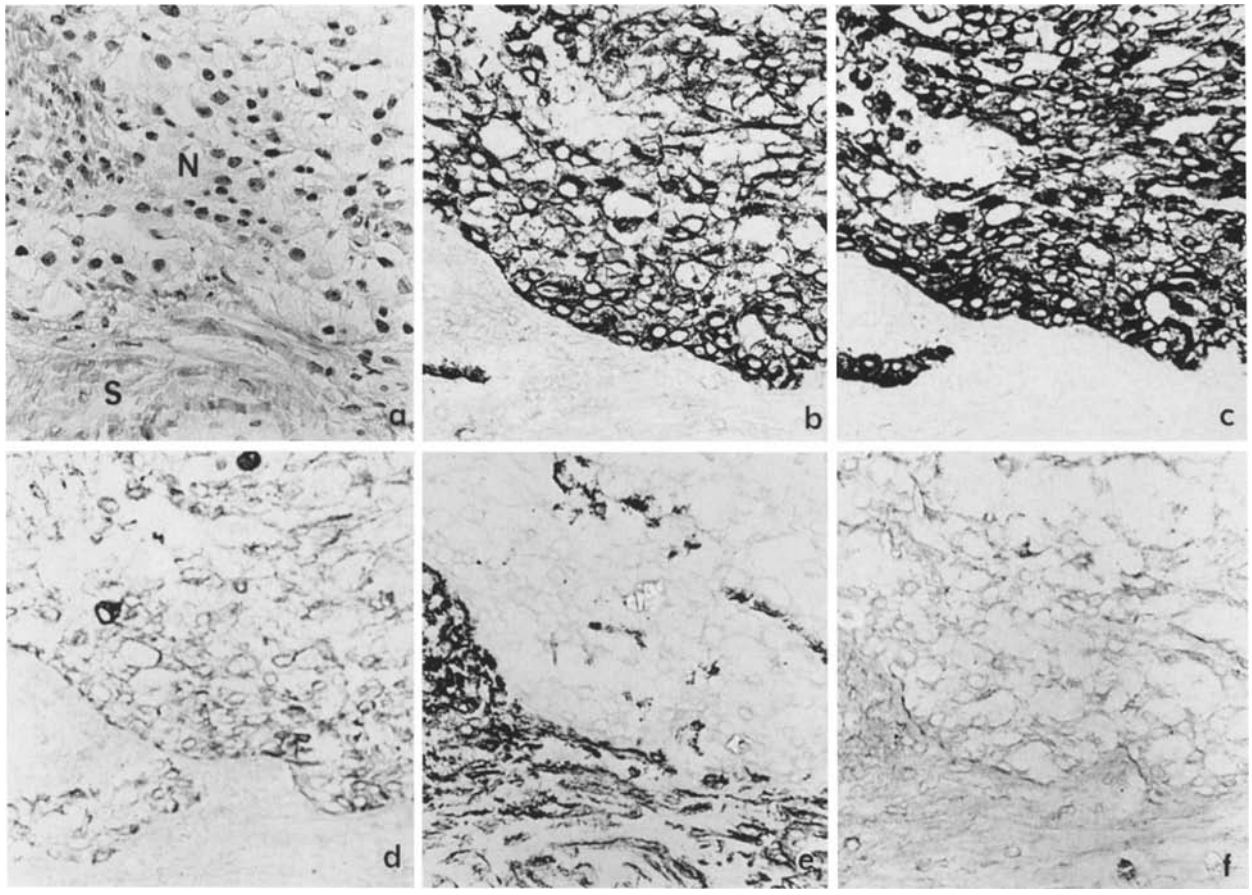

Fig. 5. Clear cell adenocarcinoma (lymph node metastasis) (case 14) Haematoxylin – Eosin (**a**) and immunoperoxidase staining with PKK1 (**b**), PKK2 (**c**), PKK3 (**d**), V9 (**e**), and V24 (**f**). PKK1 and PKK2 stain intensely and PKK3 only faintly. Vimentin is not demonstrated. *N* = neoplasm, *S* = stroma (lymphnode tissue) × 250

cells dominate, have the CK-pattern of normal acinar cell. In adenoid cystic carcinomas, the peripherally positioned cells are normally referred to as myoepithelial, and these cells have the same reactivity pattern for CK-monoclonal antibodies as the non-neoplastic myoepithelial cells. However, in neither of these types of tumour can the presence of V9-reactive cells be explained by the participation of myoepithelial cells, a they are V9-negative in normal glands. In the present study where mAbs PKK3 stained the tumours inhomogeneously, the staining was mostly weaker at the periphery and stronger close to the lumen formations, compared with staining for vimentin, which showed the opposite pattern. This may be regarded as an equivalent to the gradual transition often seen in normal multilayer epithelia, for example normal squamous epithelia (Ramaekers et al. 1985), and is not necessarily a sign that the peripheral cells are "myoepithelial".

Mucoepidermoid carcinomas are very inhomogeneous neoplasms, and a large number of cell types have been described both at the light and electron microscopic level (Bienengräber 1978; Nicolatou et al. 1979). However, both adenomatous and squamous areas seem to have similar CK patterns, CK 18 being present in both types of areas in contrast to the adjacent normal squamous epithelium of the tongue base in case 14.

The present results provide no clear means of discrimination betwen individual salivary adenocarcinoma types or between adenocarcinomas and mucoepidermoid carcinomas. Proliferative squamous epithelium and squamous cell carcinomas are definitely positive for PKK2 (Virtanen et al. 1986). However, in the present study, not only duct structures but also tumours with glandular structures only were positive. Reactivity to PKK3 in normal tissue was found only in simple epithelial or gland structures and not in squamous epithelia. On the contrary, PKK3 reacted in mucoepidermoid carcinomas with squamous cells as well as in those of the glandular or intermediate type. As the difference between the last two types of tumour

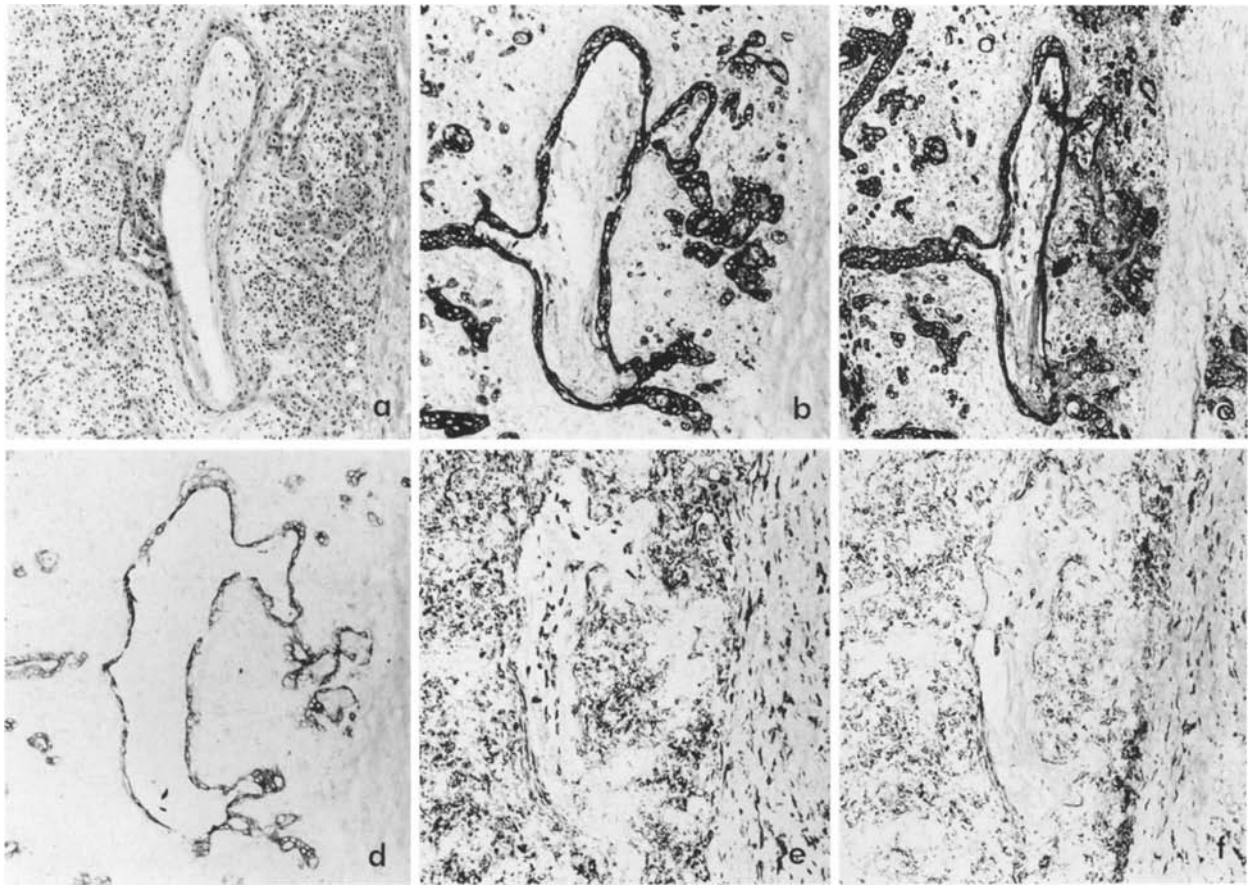

Fig. 6. Mucusproducing adenopapillary carcinoma (case 15) Haematoxylin – Eosin (**a**) and immunoperoxidase staining with PKK1 (**b**), PKK2 (**c**), PKK3 (**d**), V9 (**e**) and V24 (**f**). PKK1 and PKK2 reactions are strong for all cells while only a minority reacts moderately with PKK3. The gland formations are negative for V9 (**e**) but a few peripheral cell are positive for V24 (**f**). The isolated vimentin-positive cells in (**e**) and (**f**) probably represent inflammatory cells, × 100

is the existence of squamous differentiation, a specific Ab should identify squamous epithelium. For that purpose the use of Abs against CK pairs 48/56 kD (=CKs 16 and 6) or 50/58 kD (=CKs 14–15 and 5) (Gould 1986) or other proteins found solely in squamous epithelial cells, such as involucrin (Rice and Green 1979; Osborn 1984) might be preferable. The reactivity in salivary gland tissue of a number of mAbs against CKs have recently been characterized (Palmer et al. 1985; Caselitz et al. 1986; Geiger et al. 1987). They may also give a further contribution in distinguishing between mucoepidermoid carcinomas and various adenocarcinomas.

In the present study the two different mAbs against vimentin were found to recognize vimentin differently. This may be explained by variations in organization; some antigenic epitopes being hidden in one cell type but not in others (see also Kjörell et al. 1987). This finding that monoclonal antibodies against vimentin give rise to different staining patterns might cause considerable confusion in the future if it is not taken into consideration when comparing results on tumour IFP pattern described by different research groups, using different batches of antibodies.

Some of the neoplasms in the present study had been irradiated preoperatively (cases 7 and 15), others had been irradiated in previous treatments (cases 2, 5 and 6) whilst others were not irradiated (cases 1, 3, 4, 8–11, 13 and 14). So far no systematic investigation on the effects of irradiation has been reported, to our knowledge. From the present study we conclude that irradiation does not seem to influence the expression of intermediate filament proteins. No difference could be observed between irradiated and non-irradiated tumours for acinic cell carcinomas, mucoepidermoid carcinomas or salivary duct carcinomas. Furthermore, no difference was found between mucoepidermoid carcinoma specimens from metastases or primary tumours (case 9) or between specimens of

acinic cell carcinoma obtained from primary tumours (case 1) or from metastases (case 2). These observations further demonstrate the stability of IFP-expression in various conditions.

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