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Immunofluorescence study of secretory epithelial markers in pleomorphic adenomas

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Summary. Amylase (Am), lactoferrin (Lf), lysozyme (Ly), secretory component (SC), epithelial IgA, and epithelial IgM were traced by paired immunofluorescence staining in ethanol-fixed specimens from 15 pleomorphic adenomas of the parotid gland. Epithelial elements positive for some of the markers were detected in a variable number of the specimens (Am, 0; Lf, 11, Ly, 2; CEA, 6; SC, 11; IgA, 9; and IgM, 6); their expression seemed to depend on a certain degree of glandular differentiation. Variable co-expression of secretory epithelial markers probably reflected different degrees of differentiation, indicating that clonal diversification may explain the histological complexity of pleomorphic adenomas. The most consistent expression (in almost 75% of the specimens) shown by Lf and SC might further reflect histogenetic relationship to intercalated ducts in which these antigens are normally found in largest amounts.

Key words: Pleomorphic adenomas – Immunohistochemistry – Lactoferrin – Carcinoembryonic antigen – Secretory component

Introduction

Conventional histopathological studies of salivary-gland tumours are generally sufficiently informative to aid diagnosis but have contributed little to the understanding of the biological behaviour and histogenesis of these tumours. Application of tumour markers may turn out to be extremely useful to this end and may, in addition, become a valuable adjunct in the differential diagnosis of unclear neoplasms.

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The first studies along these lines focussing on morphological salivary gland markers have recently been published. Lactoferrin (Lf) and lysozyme (Ly) (Caselitz et al. 1981a and 1982a), carcinoembryonic antigen (CEA) (Korsrud and Brandtzaeg 1979; Caselitz et al. 1981b and 1981c), actin, keratin, vimentin and desmin (Caselitz and Löning 1981; Caselitz et al. 1982b; Krepler et al. 1982; Löning et al. 1982; Nathrath et al. 1982), S-100 protein and glial fibrillary acidic protein (Nakajima et al. 1982; Nakazato et al. 1982; Kahn et al. 1983), and amylase (Am) (Caselitz et al. 1983) were localized immunohistochemically in normal, inflammatory and various neoplastic conditions of human parotid glands. Independently, the distribution of the secretory epithelial cell marker antigens Lf, Ly, Am, secretory component (SC), secretory IgA (sIgA) and secretory IgM (sIgM) was systematically mapped in normal human parotid and submandibular glands by paired immunofluorescence staining (Korsrud and Brandtzaeg 1982). In the latter study, Am was consistently localized exclusively to the acini and Lf and Ly chiefly to the intercalated ducts (but additionally to some acini), whereas SC and IgA (and sometimes also IgM) were found in both acini and ducts of the intercalated and striated varieties. The staining for SC and IgA was characteristically enhanced at the basolateral margins of the cells and particularly apically close to the lumen.

With the latter study as a baseline, we wanted to evaluate the expression of these functional marker antigens in pleomorphic adenomas of the parotid gland. These tumours show an unusual histological complexity which may either reflect derivation from different cell types or different routes of differentiation from the original neoplastic cell. CEA was included as an additional secretory marker since it has been found in normal human saliva (Martin and Devant 1973) and in the epithelium of acini and intercalated ducts of normal parotid glands (Caselitz et al. 1981b).

Materials and methods

From 15 consecutive patients (12 women and 3 men; median age, 47 years; range, 29–75 years) operated for pleomorphic adenomas, small tumour specimens were removed at surgery, immediately placed in ice-chilled PBS (0.01 M phosphate buffer, pH 7.5, containing 0.15 M NaCl), brought directly to the laboratory and subdivided into small pieces (about $3 \times 3 \times 3$ mm) which were fixed for 48 h in 96% ethanol before paraffin embedding (Brandtzaeg 1974).

Serial sections were cut at 6 μm and incubated for 30 min with various pairs of rhodamine- and fluoresceine-labelled immunohistochemical reagents monospecific for human Am, Lf, Ly, CEA, SC, IgA or IgM. These fluorochrome conjugates have been prepared in our laboratory; details about their characteristics and staining properties are reported elsewhere (Rognum et al. 1980; Brandtzaeg 1981a; Korsrud and Brandtzaeg 1982).

Fluorescence microscopy was performed with a Leitz Orthoplan microscope equipped with an HBO 200 W lamp for rhodamine excitation, an XBO 150 W lamp for fluorescein excitation, and Leitz immersion objectives. A Ploem-type vertical illuminator combined with interference filters were used for narrow-band excitation and selective filtration of red and green colours. Findings were recorded on Kodak Ektachrome ASA 400 daylight film; double exposures was used to document results of paired staining.

Table 1. Tumours (percentage) showing expression of secretory epithelial markers

Amylase	0
Lactoferrin	73 (+++)[a]
Lysozyme	13 (+)
CEA	40 (++)
SC	73 (+++)
IgA	60 (++)
IgM	40 (+)

[a] Relative staining intensity of positive elements graded subjectively from + (negligible) to +++ (bright)

Results

Individual tumours showed positive staining for several secretory products with the exception of amylase (Table 1). The expression of these epithelial markers was apparently not influenced by the proportion of epithelial and stromal components, which characteristically varied from one tumour to another and also within different parts of the same tumour. Nevertheless, the number of positive elements increased with the degree of histological differentiation shown by the tumour epithelium.

Lactoferrin. Eleven tumours were positive for Lf, which was found apically in tumour cells forming duct-like structures (Fig. 1a) and in luminal accumulations of secretion. More rarely single cells or solide groups of cells were likewise Lf-positive (Fig. 1a). Lf was, in addition, observed in granulocytes which were concomitantly positive for Ly. Large variations in the numbers of these cells was noted without any apparent relation to the histological tumour differentiation or the proportion of epithelial and stromal components.

Lysozyme. Only in two tumours was Ly weakly expressed by duct-like structures (Fig. 1b).

Carcinoembryonic antigen. Six tumours were positive for CEA which appeared apically in epithelial cells forming duct-like structures, either as a narrow rim (Fig. 2a) or as cytoplasmic staining of cells piled up in papillary luminal projections. Sometimes also luminal accumulations were positive for CEA. Paired staining demonstrated that CEA was usually present together with SC (Fig. 2); conversely, less than 50% of the SC-positive cells were concomitantly stained for CEA.

Secretory component. SC was expressed by epithelial tumour cells forming duct-like structures and appeared in luminal accumulations of eleven tumour specimens (Fig. 2b, 3a). The number of SC-positive elements varied largely from one specimen to another; some sections contained only two to three positive elements and others several hundred.

Secretory immunoglobulins. Epithelial IgA was demonstrated in nine specimens and was always associated with SC-positive tumour elements (Fig. 3b),

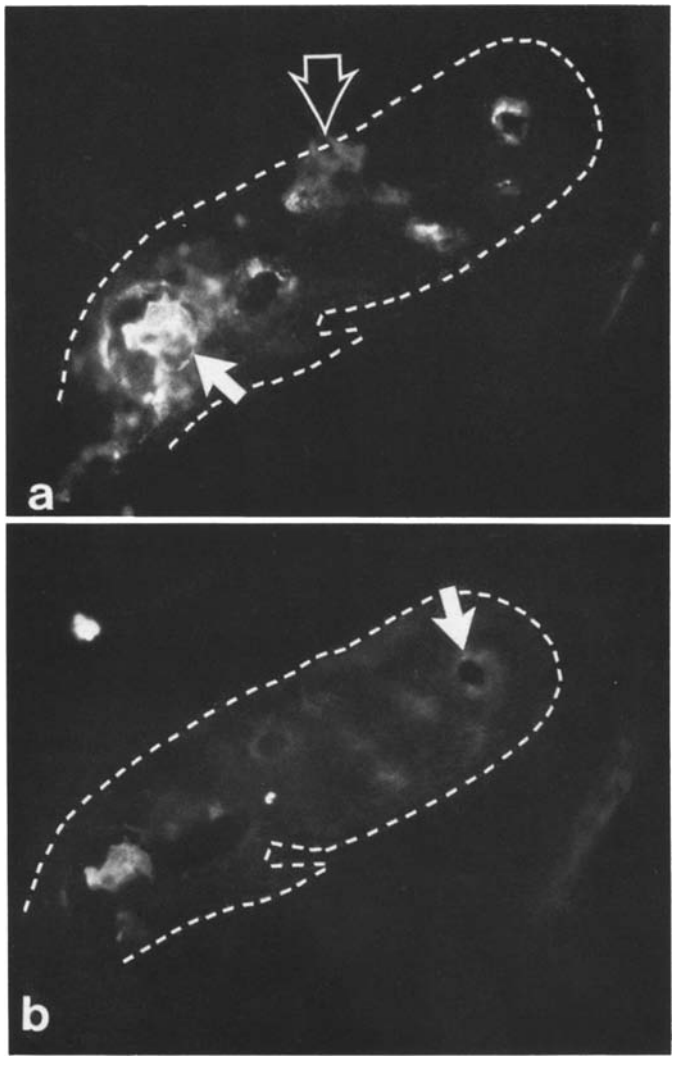

Fig. 1 a, b. Paired immunofluorescence staining for Lf (**a**, fluorescein) and Ly (**b**, rhodamine) in section from pleomorphic adenoma. **a** Note that Lf appears mainly as luminal positive rim. Luminal accumulations (*arrow*) and some epithelial cells (*open arrow*) also show faint staining for Lf. **b** Ly positivity is observed in luminal accumulation and as faint staining of luminal rim (*arrow*)

although generally showing staining of less intensity than the latter marker. IgA was, in addition, distributed variably throughout the stromal ground substance and along basement membrane zones in all of the specimens. In two of them, IgA was absent from the SC-positive elements despite being present interstitially as usual. The opposite situation, absence of SC in IgA-positive epithelial cells, was never observed. IgA alone was found in plasma cells scattered in the stroma of five specimens (Fig. 3b).

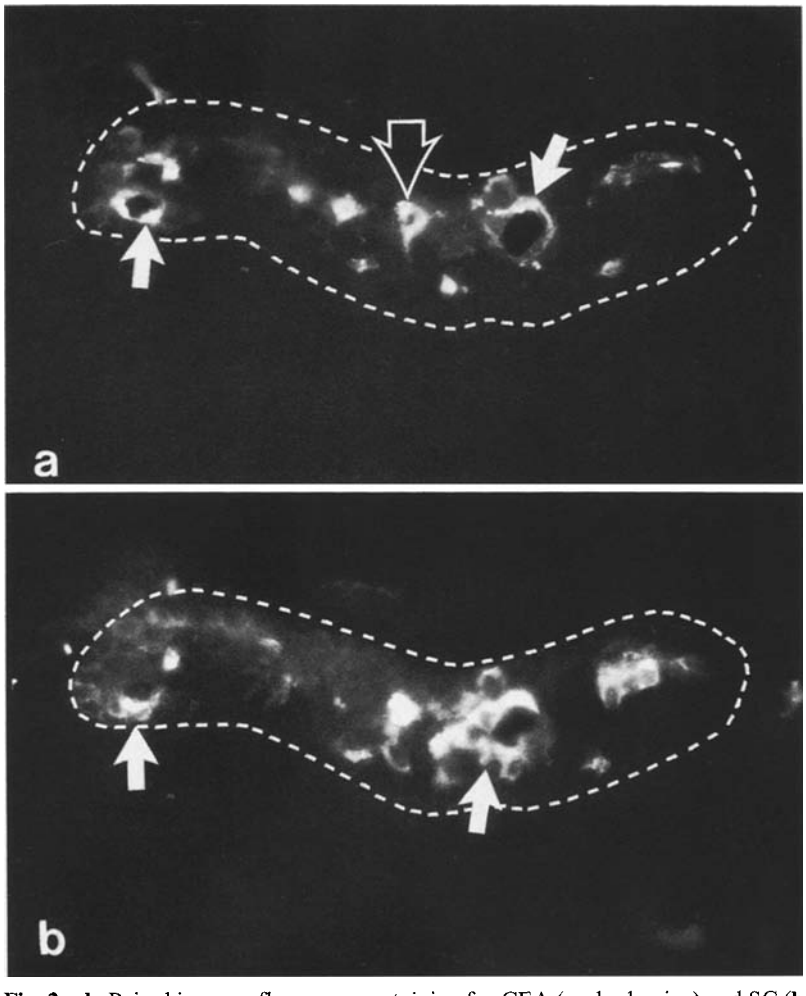

Fig. 2a, b. Paired immunofluorescence staining for CEA (**a**, rhodamine) and SC (**b**, fluorescein) in tissue section from pleomorphic adenoma. **a** CEA is present mainly in luminal rim (*solid arrows*) but also in single cells lining longitudinally sectioned part of duct-like element (*open arrow*). **b** SC positivity is demonstrated as faint diffuse staining in same duct-like element with increasing intensity apically (*solid arrows*)

Epithelial IgM was revealed by very faint glandular staining in those tumours that showed the most intense expression of secretory IgA – altogether six specimens. Faint interstitial staining for IgM was seen in fourteen specimens, but plasma cells positive for this isotype were not observed.

Discussion

In this study the secretory epithelial markers Am, Lf, Ly, SC, IgA, IgM and CEA were for the first time collectively mapped with regard to cellular

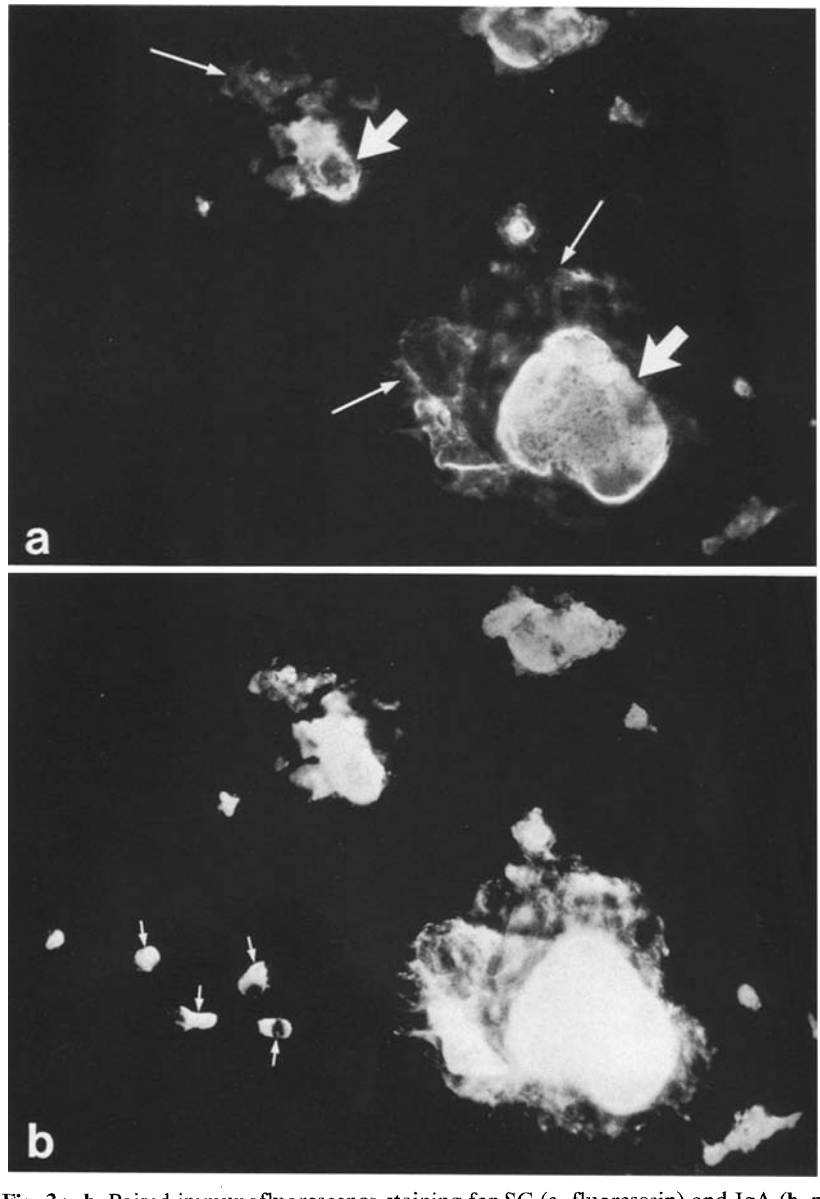

Fig. 3a, b. Paired immunofluorescence staining for SC (**a**, fluorescein) and IgA (**b**, rhodamine) in tissue section of pleomorphic adenoma. **a** SC is seen in luminal accumulations (*heavy arrows*) and in solid strands of epithelial tumour cells (*slender arrows*). **b** IgA is present together with SC in tumour epithelium. In addition, four IgA-producing plasma cells (*small arrows*) are present in the stroma

expression in pleomorphic adenomas. Some of these markers have been individually (Caselitz et al. 1981a, 1981c, 1982a and 1983; McDicken and Scott 1981) or collectively (Korsrud and Brandtzaeg 1979 and 1984) studied in salivary gland tumours before. An interesting finding in the present study was the fact that these marker antigens were variably co-expressed in individual tumours, indicating various degrees of epithelial differentiation. The number of stained elements differed from only a few to several hundreds per tissue section. Their distribution was uneven and no attempt was made to quantitate them because a section would probably be poorly representative of the whole tumour. In agreement with previous reports on various types of salivary-gland tumours (Caselitz et al. 1981a, 1981c and 1982a; McDicken and Scott 1981), we found that the expression of secretory products generally required a tendency towards glandular development as revealed by the formation of duct-like elements.

The latter observation applied even to the expression of CEA; and Caselitz et al. (1981c) reported lack of this antigen in anaplastic parotid carcinomas. Nevertheless, the same authors suggested that CEA might be helpful as a marker of carcinoma in pleomorphic adenoma; but this possibility is clearly limited by the absence of CEA in 57% of such carcinomas (Caselitz et al. 1981c) and by the fact that we found CEA in 40% of the benign tumours.

The relationship between the expression of CEA and neoplastic development seems to be complex. In colonic carcinomas CEA is found most abundantly in moderately differentiated tumours (Rognum et al. 1982a) and particularly in those with a distinctly aneuploid DNA profile (Rognum et al. 1982b). It has, moreover, become apparent that CEA should be regarded as a proliferative rather than a neoplastic marker; its expression is thus enhanced both in hyperplastic inflammatory epithelial lesions in longstanding ulcerative colitis (Rognum et al. 1982c) and in proliferating ducts present in chronically inflamed parotid glands (Caselitz et al. 1981b). Moreover, we have observed strong staining for CEA in papilliferous luminal patches of epithelium in adenolymphomas (Korsrud and Brandtzaeg 1979 and 1984), probably indicating areas with high proliferative activity.

The complete lack of Am along with the expression of Lf and SC in almost 75% of the tumour specimens hints to a histogenetic relationship between pleomorphic adenomas and intercalated ducts in which the latter secretory products are normally found in largest amounts. The variable co-expression of different secretory markers by duct-like elements and single epithelial tumour cells could reflect different routes of differentiation within the same neoplastic clone rather than derivation from different cell types. This would be in agreement with the observation that some duct-associated small myoepithelial and indifferent pleomorphic tumour cells seem to co-express keratin and vimentin (Caselitz et al. 1982b; Krepler et al. 1982); it has been suggested that their normal counterpart may be duct-associated basal stem cells (Caselitz et al. 1982b). Neoplastic development from such a cell type might conceivably, by clonal diversification, result in both duct-like and myoepithelial elements and could thus explain the complexity of

pleomorphic adenomas. This hypothesis is supported by ultrastructural studies which have demonstrated a continuum of neoplastic elements with cytoplasmic features ranging from epithelial to mesenchymal cells, apparently depending on the type and degree of gene expression (Mills and Cooper 1981). Thus, at one end of the spectrum there are pleomorphic adenomas with mainly epithelial type of differentiation (Dardick et al. 1982) whereas in other cases prominent tumorigenicity has been shown to be carried by elements with ultrastructural features of myoepithelial cells (Shirasuna et al. 1980).

In intercalated ducts of normal major salivary glands there is usually co-expression of Lf and Ly, although staining for the former often extends relatively more into the acini (Korsrud and Brandtzaeg 1982). It was surprising, therefore, to find co-expression of Lf and Ly in duct-like elements of only two pleomorphic adenomas. However, production of Ly seems to be prone to repression as suggested previously from studies both of various salivary gland carcinomas (Caselitz et al. 1981a) and monocyte-derived malignant processes (Isaacson et al. 1983). In addition, absence of lysozyme may be ascribed to methodological problems since this small molecule easily leaches from sections of ethanol-fixed material (Brandtzaeg 1981a). Our study was based on ethanol fixation to avoid masking of antigens present in secretory epithelium; the latter artefact may to some extent invalidate marker studies based on formalin-fixed routine pathological material, especially if pretreatment of the sections with proteolytic enzyme is omitted (Brandtzaeg 1982).

Lack of IgA in some of the SC-positive duct-like elements was probably explained by insufficient supply of dimeric IgA. In normal glands, dimeric IgA is provided by local J-chain-positive IgA immunocytes (Korsrud and Brandtzaeg 1980). Local IgA-producing cells were few in the pleomorphic adenomas; and the diffuse IgA-staining observed in the stroma probably represented serum-derived monomers which cannot be taken up by SC-expressing epithelium (Brandtzaeg 1981b).

In conclusion, marker studies of secretory activity in pleomorphic adenomas may provide functional information about the degree of glandular differentiation and may contribute to improved understanding of the histogenesis of these complex tumours.

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