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Lactoferrin and Lysozyme in Carcinomas of the Parotid Gland

A Comparative Immunocytochemical Study with the Occurrence in Normal and Inflamed Tissue *

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Summary. Lactoferriin and lysozyme, parts of the non-specific defense system, were studied in normal and diseased parotid glands, using the immunohistochemical PAP-method. 31 normal and inflamed glands were investigated. The presence of lactoferrin and lysozyme was demonstrated in the acinar cells and some duct cells. The amount of these substances was increased in obstructive parotitis. The 52 carcinomas showed a distinct distribution pattern for lactoferrin (positive cases: adenocarcinomas 5 of 8; cystadenocarcinomas: 3 of 5; adenoid cystic carcinomas 2 of 4; salivary duct carcinomas 2 of 3). Some of the carcinomas in pleomorphic adenomas were positive for lactoferrin. Squamous cell carcinomas and anaplastic carcinomas were constantly negative.

All carcinomas were negative for lysozyme. These observations are discussed with respect to their physiological and pathological significance.

Key words: Parotid gland carcinomas – Lysozyme – Lactoferrin – Immunocytochemistry

Introduction

Recently, substances involved in antibacterial defenses but which differ from immunoglobulins have been studied, in particular lactoferrin and lysozyme (Klockars and Reitamo 1975; Mason and Taylor 1977). Since lactoferrin and lysozyme are involved in antibacterial defenses connected with secretion, their presence was investigated in the salivary glands (Pinkus and Said 1977; Reitamo et al. 1977; Spicer et al. 1977; Mason and Taylor 1978). To date, no systematic

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study of their presence in salivary gland tumours and acjacent tissue has been undertaken.

We have applied the immunoperoxidase technique to a collection of 52 parotid cancers and 31 normal and inflamed parotid glands. Our intention was to make a comparative analysis of the distribution pattern of lactoferrin and lysozyme in the normal and inflamed glands as well as in the tumour tissue.

Material and Methods

31 normal and inflamed parotid glands were used for comparison. A collection of 52 carcinomas of the human parotid gland (Salivary Gland Register of the University of Hamburg) was analysed. Among this group there were 8 adenocarcinomas, 5 cystadenocarcinomas, 4 adenoid cystic carcinomas, 3 salivary duct carcinomas, 8 squamous cell carcinomas, 21 carcinomas in pleomorphic adenomas and 3 anaplastic carcinomas.

The tissue was prepared for light microscopical and immunohistochemical investigations by conventional techniques. Slides were stained conventionally by hematoxylin-eosin, PAS-reaction and astra blue.

The immunoperoxidase technique (the socalled PAP-technique; Sternberger et al. 1970) was used in the variation as described by Mason and Taylor (1978).

The method included the blocking of the endogenous peroxidase, incubation with rabbit anti-lactoferrin or anti-lysozyme (serum purchased from Dakopatts, Copenhagen), incubation with goat anti-rabbit serum, incubation with rabbit antiperoxidase-peroxidase complex, DAB reaction.

The controls for specificity involved omitting of the primary antiserum (negative results were obtained). The reaction for the two proteins in question were compared with reactions for other substances like CEA (Caselitz et al. 1980) and immunoglobulins.

Results

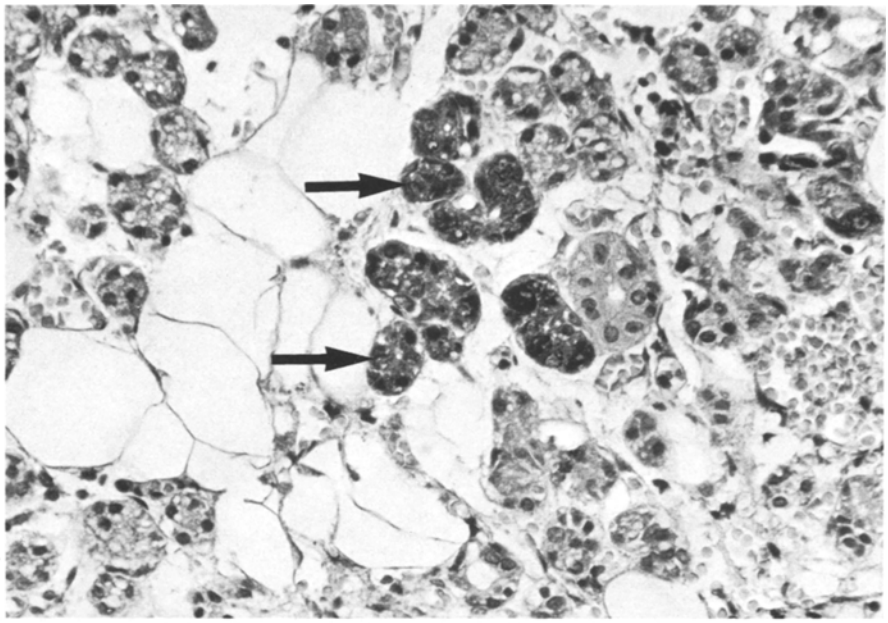
1. Normal Parotid Gland

Lactoferrin. This protein could be demonstrated in moderate quantities. The presence of lactoferrin was seen in some acinar cells which were scattered among numerous negative glandular cells. The duct cells were negative, in general. Staining of other components, like the myoepithelial cells, was not observed. The surrounding connective tissue compartments were regularly negative for lactoferrin with the exception of granulocytes and monocytes (Fig. 1). Rarely, lactoferrin was seen in the duct cells.

Lysozyme. This protein was demonstrated in the acini and some of the intercalated ducts. The intensity of staining was lower than for lactoferrin, but the distribution pattern was similar. Positive cells were scattered among negative ones. The granulocytes and some rare macrophages were also positive for lysozyme (Fig. 2).

2. Inflamed Parotid Gland

Inflamed glandular tissue, often found in the vicinity of the tumour, showed different amounts of lactoferrin and lysozyme.



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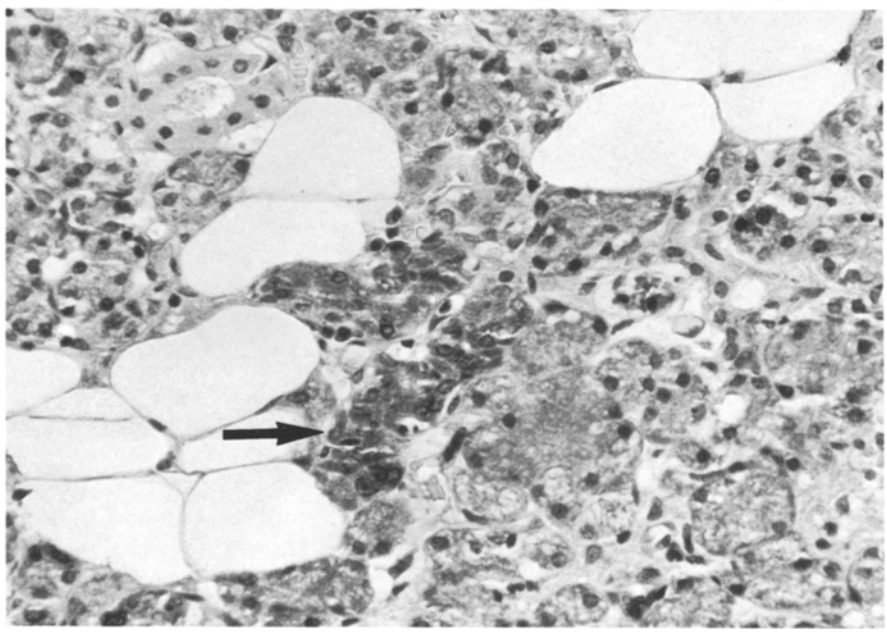

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Fig. 1. Normal parotid gland: Positively stained acini are seen among negative stained acini and ducts (*arrows*, center). Immunoperoxidase staining for lactoferrin. ×300

Fig. 2. Normal parotid gland: Positively stained acini among negatively stained acini and ducts (*arrows*, center). Immunoperoxidase staining for lysozyme. ×300

Lactoferrin. The presence of lactoferrin was clearly and intensely demonstrated in chronic obstructive parotitis. The cuboidal ductal elements which are obviously the substrate of this disorder were strongly stained for lactoferrin (Fig. 3). The most impressive staining was seen in the immediate neighbourhood of the tumour or its capsule. The glandular tissue at a distance from the inflamed part showed an intense staining of the acini, some of the intercalated ducts and, sometimes, of the larger ducts. Myoepithelial cells were negative for lactoferrin. Granulocytes displayed a positive pattern.

Lysozyme. The distribution of lysozyme was similar to that of lactoferrin. Some cuboidal duct cells were positive and some of the acini and intercalated ducts were positive as well. Sometimes the staining intensity of lysozyme was stronger than in the normal cases. Generally, the presence of lysozyme was not so intense as that in the normal gland.

3. Carcinomas of the Parotid Gland

Lactoferrin. The staining with anti-lactoferrin revealed a distinct pattern in the different carcinomas. The results are presented according to the histological type of the carcinomas.

Adenocarcinoma. Five of the eight tumours were positive for lactoferrin. The presence of this protein was seen intracytoplasmatically but also in the secretory products inside the lumina. Sometimes, lactoferrin was concentrated at the apical cell border (Fig. 5).

Cystadenocarcinoma. Three of the five cystadenocarcinomas were positive for lactoferrin. There were some single positive tumour cells as well as groups of positive tumour cells. The latter were generally arranged like primitive ducts. Sometimes positive and negative tumour cells were found in direct contact.

Adenoid Cystic Carcinoma. Two of four tumours were positively stained for lactoferrin. Generally, solid cell clusters were positively stained (Fig. 6).

Salivary Duct Carcinoma. Two of the three carcinomas were positive for lactoferrin. Some group of cells near the lumina were generally stained, whereas the clear cells at the periphery were negative (Fig. 7).

Carcinomas in Pleomorphic Adenomas. Lactoferrin could be demonstrated in two muco-epidermoid carcinomas (Fig. 8). The positive tumour cells were stained apically and intracytoplasmatically. They often formed duct-like structures. The squamous parts, however, were negative. The secretory products in the lumina were positive. The pattern of the adenoid cystic carcinoma in pleomorphic adenoma was similar to that seen in pure adenoid cystic carcinoma. Two salivary duct carcinomas were positive. Generally, the cells adjacent to the lumina were stained for lactoferrin.

In the case of two anaplastic carcinomas, lactoferrin was found inside the adenomatous part. The cells which generally formed primitive ducts were stained intracytoplasmatically.

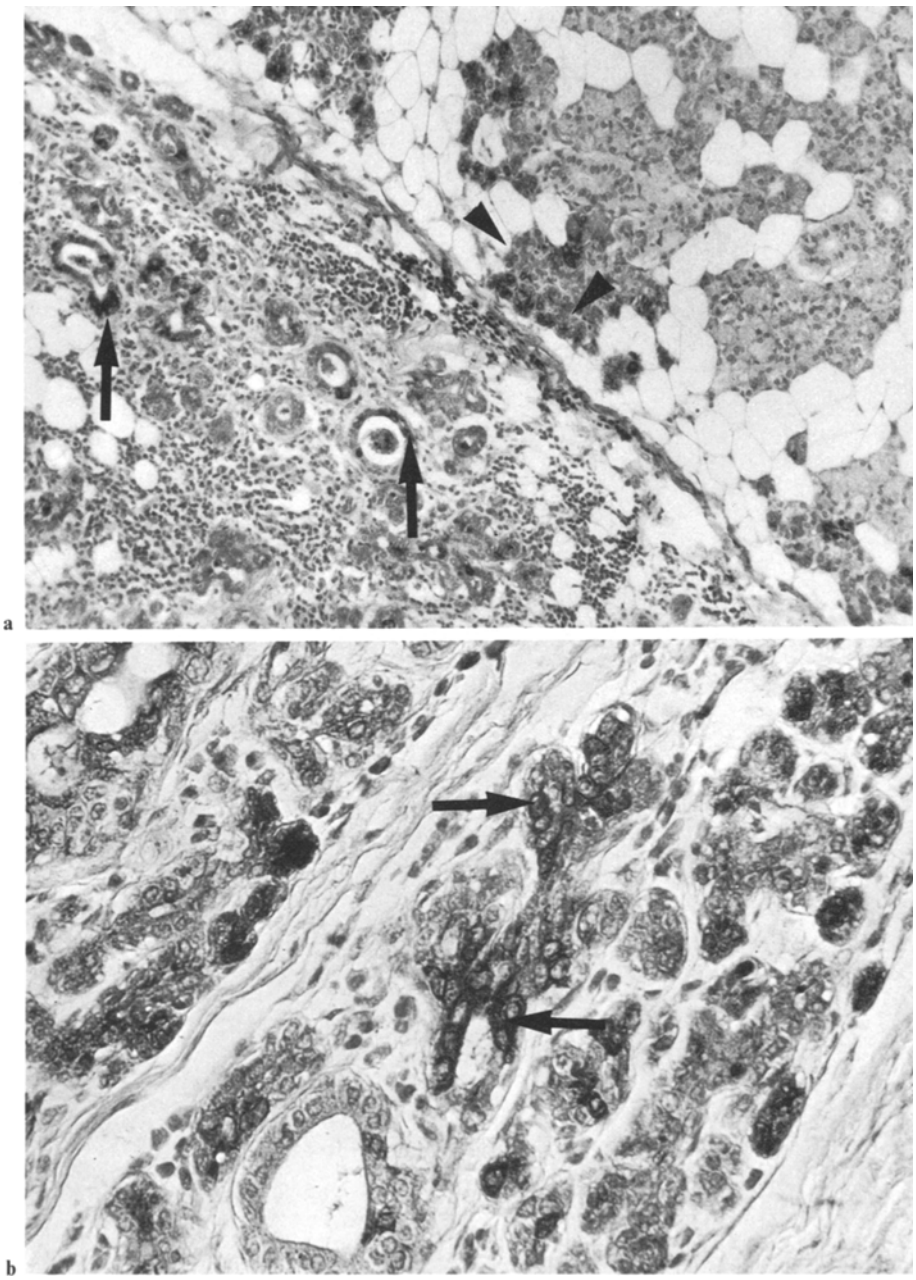
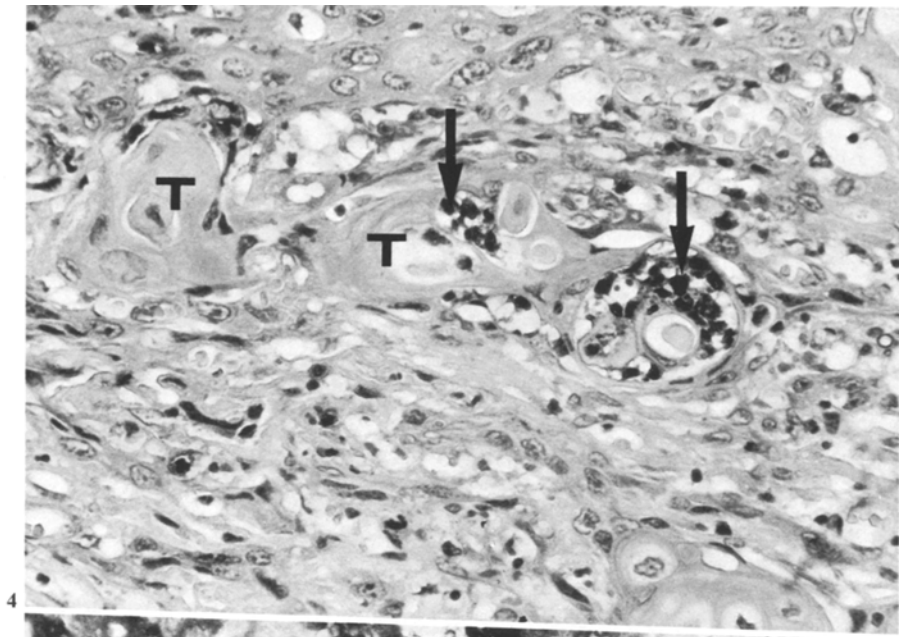


Fig. 3a, b. Chronic parotitis. **a** On the right upper normal parotid tissue is seen with some positive acini (*arrowheads*); on the left lower part, some positive cuboidal ducts (*arrows*) in between an infiltration of lymphoid cells. **b** Positively stained cuboidal cells (*arrows*); mild lymphoid infiltrate. Immunoperoxidase staining for lactoferrin. **a** ×120, **b** ×300



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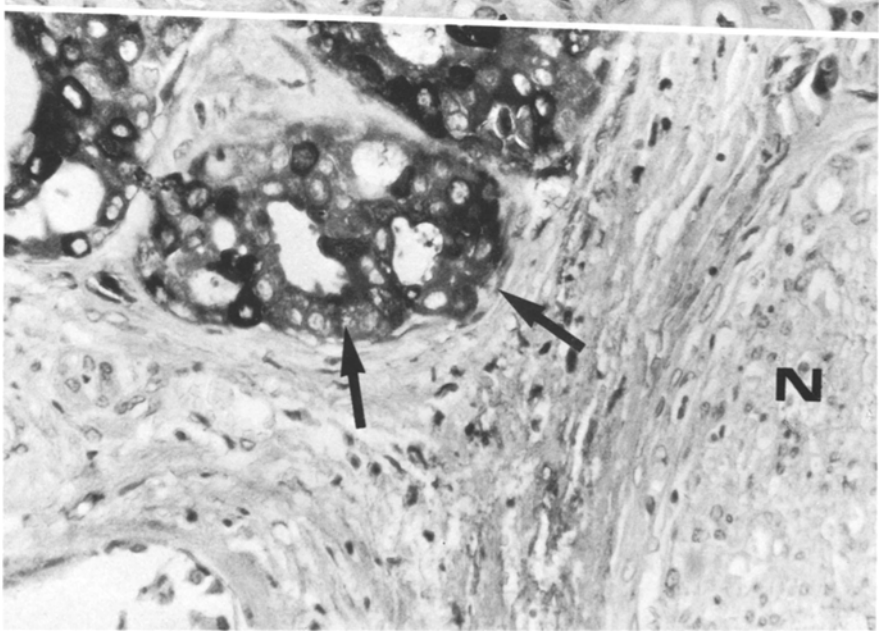

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Fig. 4. Squamous cell carcinoma: Negative tumour cells (*T*). In the necrotic areas infiltration of positively stained leukocytes (*arrows*). Immunoperoxidase staining for lysozyme. ×300

Fig. 5. Adenocarcinoma: Intensely stained tumour cells (*arrows*); at the right is a nerve (*N*). Immunoperoxidase staining for lactoferrin. ×300

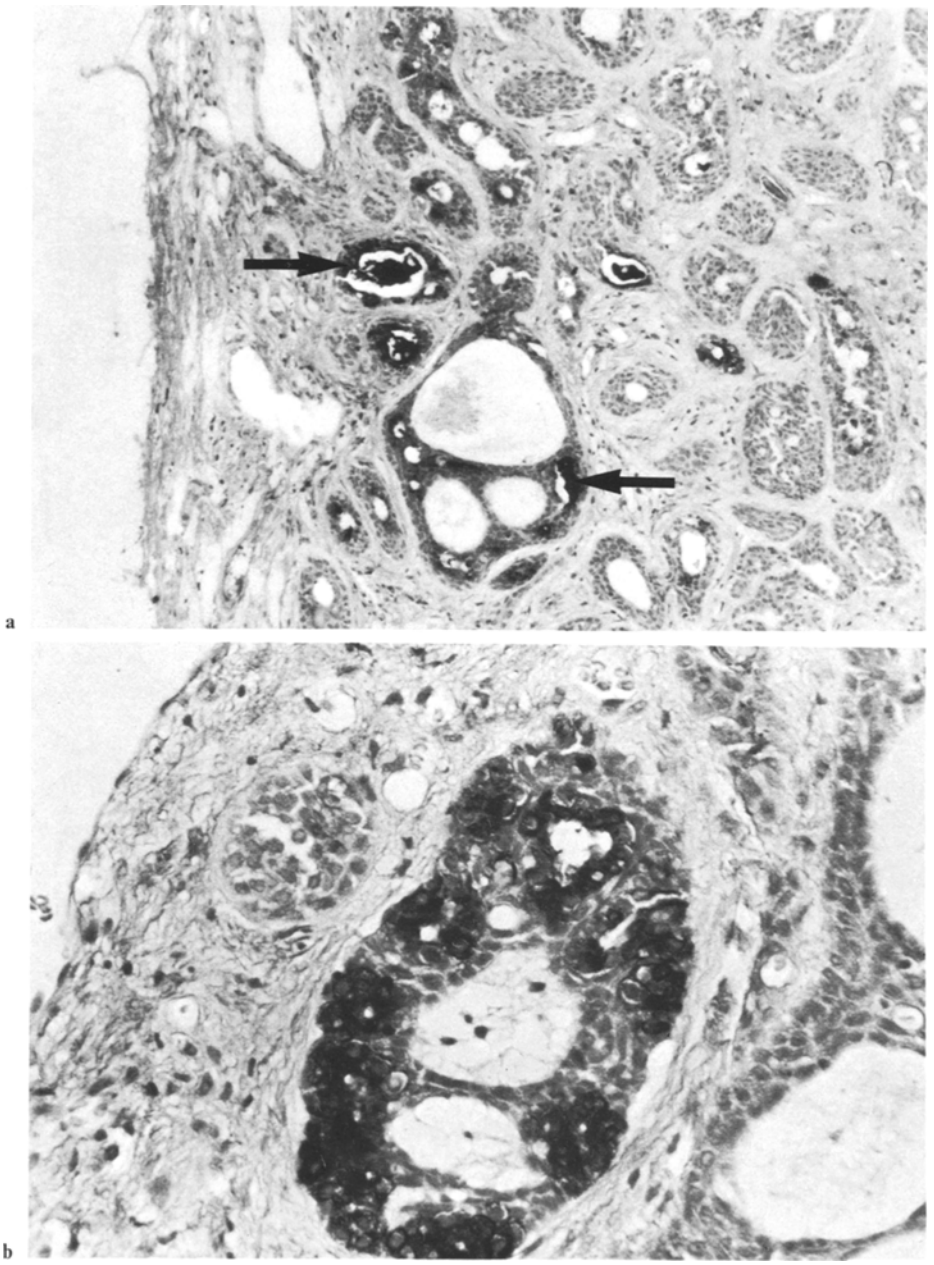

Fig. 6a, b. Adenoid cystic carcinoma. **a** Positive tumour cells around lumina (*arrows*). Positive material in the lumina. **b** Strongly positive tumour cells in the center. Immunoperoxidase staining for lactoferrin. **a** ×120, **b** ×300

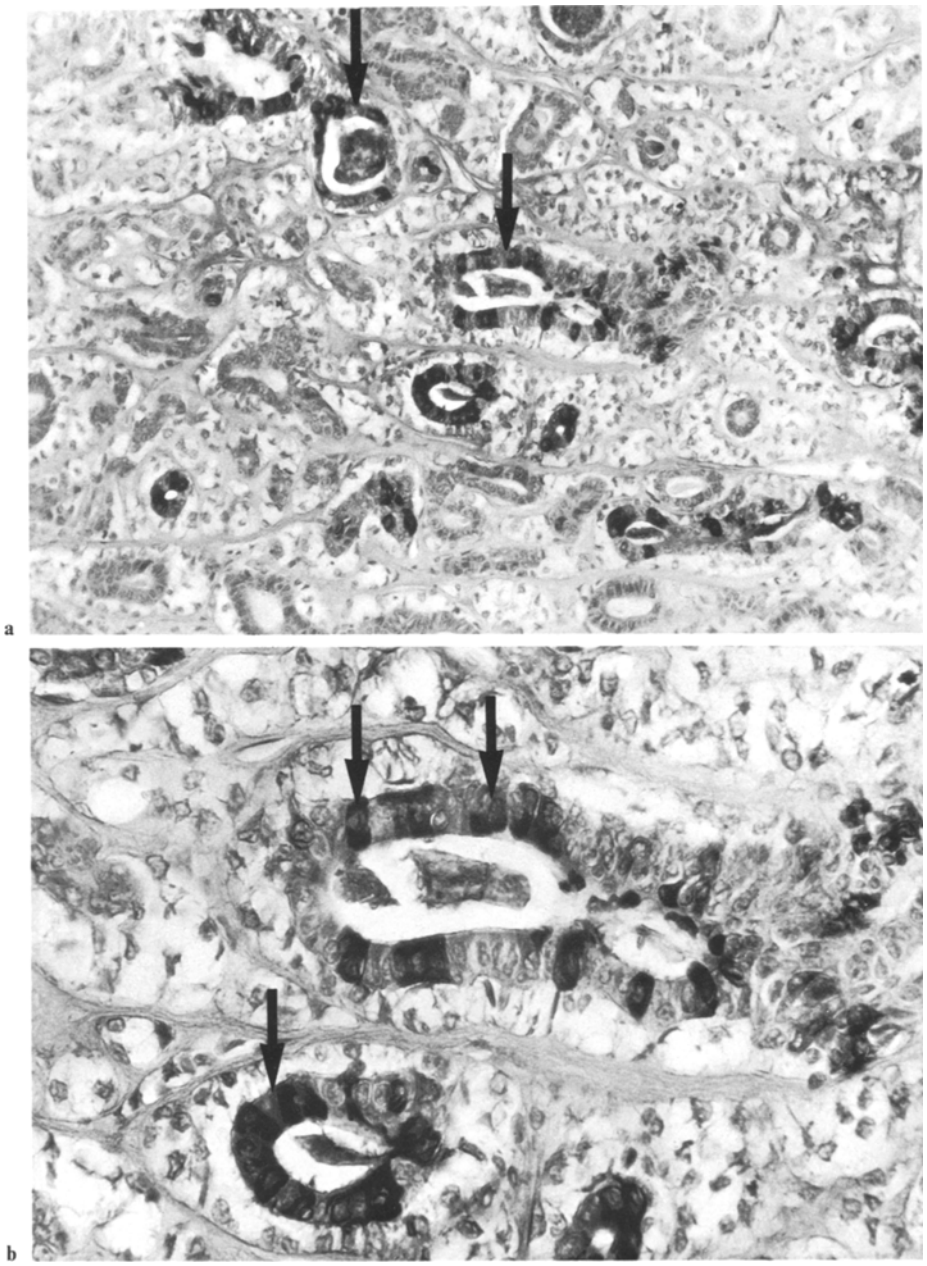

Fig. 7a, b. Salivary duct carcinoma. **a** Strongly stained tumour cells on the luminal side (*arrows*); negatively stained tumour cells at the periphery of the ducts. **b** Strongly stained tumour cells (*arrows*) at the side of lesser stained ones. Negative stained tumour cells at the periphery of the ducts. Immunoperoxidase staining for lactoferrin. **a** ×120, **b** ×300

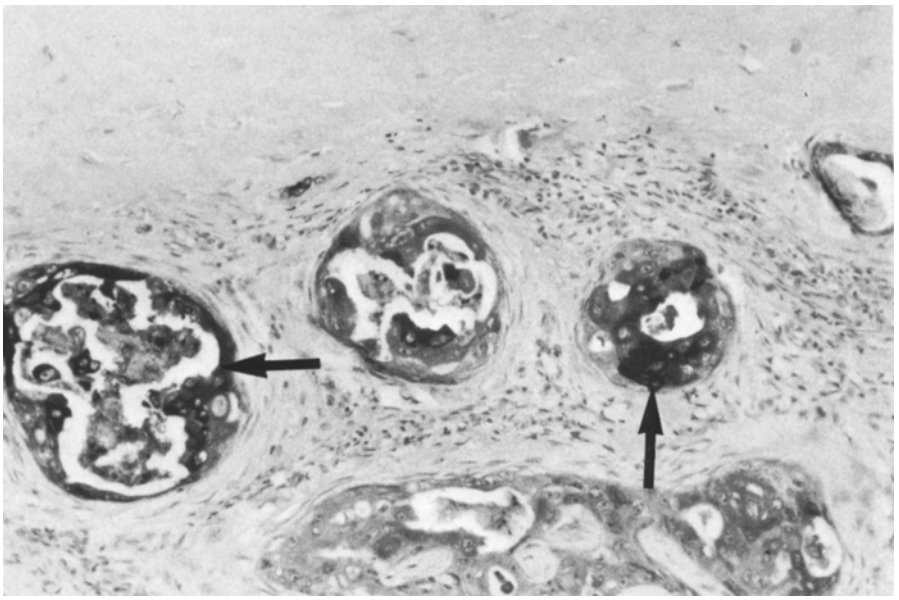

Fig. 8. Mucoepidermoid carcinoma in a pleomorphic adenoma. Intensely stained tumour cells (*arrows*) in the carcinomatous part of the pleomorphic adenoma. Immunoperoxidase staining for lactoferrin. ×120

Squamous Cell Carcinoma. No tumour cells were positive for lactoferrin. Sometimes, remnants of the parotid gland were found within the tumorous tissue. These remnants were positive for CEA, but not the tumour in itself.

Anaplastic Carcinomas. All tumour cells were negative for lactoferrin.

Lysozyme. All carcinomas, regardless of their type, were negative for lysozyme. Sometimes, lysozyme-positive granulocytes were found in the tumour (Fig. 4).

Discussion

Apart from immunoglobulins which are directed against defined antigens, there exists a non-specific defense system which may cooperate with the specific one. The two substances playing an important role are the lactoferrin and the lysozyme.

Lactoferrin is a glycoprotein of molecular weight of 76,000 dalton (Masson et al. 1969). After its identification in human milk, its bactericidal effect was attributed to its iron-binding capacities (Wilson and Miles 1975; Cottier 1980). In in vitro experiments the bacteriocidal effect of lactoferrin was abolished when iron was added to the media (Masson et al. 1966).

The presence of lactoferrin was established not only in human milk, but also in tears, urine and saliva (Wilson and Miles 1975). In recent years, lactoferrin elevation was demonstrated during the course of chronic recurrent parotitis

(Tabak et al. 1978). Since the introduction of suitable immunofluorescence and immunoperoxidase methods (Tourville et al. 1969; Reitamo et al. 1980), the distribution of lactoferrin has been demonstrated in human salivary glands (Reitamo et al. 1980), in the gastro intestinal mucosa (Isobe et al. 1979), in myoid cells (Mason 1977), in the mammary gland (Mason and Taylor 1978) and bronchial glands (Mason and Taylor 1978).

The systematic study of Reitamo et al. (1980) on different salivary glands in autopsy material showed good preservation of this antigen in paraffin embedded tissue. We used a similar method, but were restrained to formalin fixed tissue. The tissue specimens, however, were all prepared in a similar manner, so that the results could be compared among themselves. In agreement with the observations of Reitamo et al. (1980) we found few groups of acinar cells which stained positively for lactoferrin and some of the smaller ducts which were also positive. In addition, polymorphonuclear leucocytes stained positively.

Elevation of lactoferrin in human saliva during various chronic inflammatory disorders (Tabak et al. 1978) found its equivalent in an obvious augmentation of lactoferrin in the inflamed tissue adjacent to the tumours. In chronic obstructive parotitis, often found in the vicinity of neoplasms, the cuboidal duct cells stained strongly for lactoferrin. Even the normal gland adjacent to areas of parotitis showed a higher amount of lactoferrin than the gland at the periphery of involved tissue.

The origin of lactoferrin cannot be determined solely on morphological grounds. It may be possible that this protein is secreted by acinar cells, but the presence of lactoferrin in the ducts may also be explained by absorption (Reitamo et al. 1980).

The presence of lactoferrin in parotid carcinomas was not extensively studied as yet. Interestingly, lactoferrin could be demonstrated most intensely in adenocarcinomas. Although we cannot exclude the possibility that this protein might be involved in the defense system against tumours, it is probable that the presence of lactoferrin in the tumour cells is related to the production of the protein in the tumour in itself. The large amount of lactoferrin as revealed by the immunoperoxidase technique is an argument for the production by the tumour tissue itself, although this interpretation should be controlled by other methods than morphological analysis. The production of lactoferrin in adenocarcinomas and cystadenocarcinomas displays a link between acinar cells and the cuboidal cells seen in inflammation. These observations suggest that we indeed may interpret lactoferrin as a "marker" of glandular or acinar differentiation of the parotid tissue. This aspect of lactoferrin does not seem to be directly related to the role of the protein in the defense mechanism.

Adenoid cystic carcinoma was also positive for lactoferrin, but this protein could only be found in some parts of the tumour. Salivary duct carcinomas were positive in some parts, duct-like cells near the lumen stained positively. Obviously, the glandular character of salivary duct carcinomas seems to be demonstrated by the presence of this substance. In the heterogeneous group of the carcinoma in pleomorphic adenoma, five out of twenty-one carcinomas showed positive staining which was generally restrained to the more glandular differentiated forms. The two positive mucoepidermoid carcinomas showed the presence of lactoferrin in the mucous parts, whereas the squamous parts were

negative for lactoferrin. This observation was repeated in the squamous cell carcinomas, which were all negative. In contrast to carcinoembryonic antigen, which was found in squamous as well as in adenocarcinomas of the parotid gland (Caselitz et al. 1981), the presence of lactoferrin seems to be restrained to the glandular parts of the tumour. If one interprets these observations in the view of the scheme offered by Eversole (1971), one could assume that the presence of lactoferrin is restrained to that type of tumour derived from the intercalated duct progenitor cells. On the other hand, the tumour derived from the excretory duct reserve cells, like the squamous cell carcinoma are negative for lactoferrin.

In the literature, the analysis of the tissue distribution of lactoferrin has been restricted either to normal tissue (Mason and Taylor 1978) or to myeloproliferative disorders (Mason 1977). In different leukaemias, lactoferrin may serve as an indicator for the more mature leukaemic precursors (Mason 1977).

Lysozymes are small molecular weight basic proteins attaching to the mucopeptide complexes of the bacterial wall by the cleavage of the bond between the N-acetyl-muramic acid and the N-acetyl-glucosamine (Strominger and Tipper 1974; Wilson and Miles 1975; Spicer et al. 1977). The protein has a molecular weight of 15,000 dalton (Cottier 1980) and accelerates the lysis of gram-negative bacteria (Wilson and Miles 1975). In the nonspecific defence system it seems to have a role as an antibacterial agent on a variety of epithelial surfaces (Spicer et al. 1977; Cottier 1980). The enzyme was formerly detected by cytochemical methods (Speece 1964; Ghoos and van Trappen 1971), but recently, an easier approach to the study of lysozyme was made by the immunoperoxidase technique (Pinkus and Said 1977; Reitamo et al. 1977; Spicer et al. 1977; Reitamo 1978). Reitamo (1978) did extensive studies on lysozyme antigenicity and tissue fixation. He showed that the preservation of antigenicity was good when the tissue was fixed in formaldehyde or glutaraldehyde. Prolonged fixation, however, could influence the staining intensity. Our material was fixed in formaldehyde and preservation of this enzyme should be expected.

As a constituent of the primitive nonspecific defense system, lysozyme was found in mononuclear phagocytes (Reitamo et al. 1978; Klockars et al. 1979; Löning et al. 1980), in the proximal convoluted tubules of the kidney (Reitamo et al. 1978), in the ciliary layer of the trachea, the type II pneumocytes and in laryngotracheal glands (Spicer 1977), in gastric glands (Reitamo et al. 1979), in Paneth cells (Reitamo et al. 1978), in connective tissue, skin and breast (Reitamo et al. 1978). A special point of interest was the presence of lysozyme in the salivary glands. With immunofluorescence studies, Kraus and Mestecky showed the presence of lysozyme in the basal cells of the striated ducts (1971). Immunoperoxidase studies revealed a different pattern, probably due to the difference in antibodies and sensitivity.

In the minor salivary glands, lysozyme was generally found in the serous demilumes and intralobular ducts (Reitamo et al. 1977). Spicer et al. (1977) showed lysozyme in the parotid acini, in mucous glands of the submandibular glands, in lingual serous glands and in acinar cells of the lingual mixed serous

and mucous glands. Pinkus and Said (1977) found lysozyme in some parotid acinar cells. Reitamo (1978) detected staining of the serous units of the submandibular glands.

Our findings on a large collection of human salivary glands showed the presence of lysozyme in the acini and the intercalated ducts. The distribution pattern seemed to be comparable to that of lactoferrin. Lysozyme was seen in the granulocytes and in some of the macrophages. In the inflamed parotid gland, lysozyme was seen in the cuboidal cells.

Although lysozyme was extensively studied in myeloproliferate disorders (Pinkus and Said 1977), there exist only a few remarks on the presence of lysozyme in other tumours. Pinkus and Said (1977) studied carcinomas of the colon, breast, lungs and others, which were all negative for lysozyme, as were the two pleomorphic adenomas they studied.

In contrast to the distinct distribution of lactoferrin, lysozyme was not found in our collection of parotid gland tumours regardless of their differentiation. Independently of the tumour, lysozyme was detected in granulocytes and macrophages involved in the defense against the neoplastic process. They are possibly a marker of host resistance (Currie and Eccles 1975).

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