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Identification of Neoplastic Paneth Cells in an Adenocarcinoma of the Stomach Using Lysozyme as a Marker, and Electron Microscopy

Philipp U. Heitz and Werner Wegmann
Departments of Pathology, University of Basel and Kanton Basel-Landschaft, Switzerland

Summary. A large number of cells containing large eosinophilic granules in their supranuclear cytoplasm was observed in a well differentiated adenocarcinoma of the stomach and its metastases. These cells were identified as Paneth cells by electron microscopy and by their content of lysozyme. Lysozyme-immunoreactivity was well preserved after fixation of tumor tissue in liquid formaldehyde followed by postfixation in osmium tetroxide. Immunoreactivity at immunoelectron microscopy was confined to the large osmiophilic secretory granules. We conclude that morphologically and biochemically differentiated Paneth cells occasionally occur in neoplasms of the gastrointestinal tract.

Key words: Gastrointestinal tumors – Neoplastic Paneth cells – Lysozyme – Immunocytochemistry.

Introduction

The occurrence of Paneth cells in intestinal metaplasia of the gastric mucosa, in inflammatory disease of the colon, and in the colon mucosa adjacent to malignant tumors is well known (Lewin, 1969; Otto, 1974; Klockars et al., 1977). In contrast neoplastic Paneth cells have only occasionally been observed in human adenomas (Miyajima and Takeuchi, 1976), and in carcinomas of the small intestine (Stern and Sobel, 1961), an adenocarcinoma of Meckel's diverticulum (Scharfenberg and de Camp, 1975), in familial polyposis and in juvenile polyps (McColl et al., 1964; Gibbs, 1967), in adenomas (Lewin, 1968, 1969), and in some carcinomas of the colon (Holmes, 1965; Hardmeier, 1966; Gibbs, 1967). The cells were also present in a papilloma of the gallbladder (Kerr and Lendrum, 1936), and in a carcinoma of the nasal mucosa (Schmid et al., 1979). To our knowledge there is only one report in which the occurrence of Paneth cells in a gastric carcinoma is mentioned (Hardmeier, 1966).

Offprint requests to: Ph. U. Heitz, M.D., Department of Pathology, Schönbeinstraße 40, CH-4056 Basel, Switzerland

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To date the presence of Paneth cells in tumors was assumed on the basis of results obtained by using special stains (i.e. Masson's trichrome stain, the phosphotungstic acid hematoxylin method and Lendrum's phloxine tartrazine technique) but we are not aware of a report in which a specific marker was used. The existence of truly neoplastic Paneth cells is, therefore, not universally accepted (Otto, 1974).

Case History

A 63 year old male presented because of dysphagia. An endoscopic examination revealed a stenosing tumor at the cardia infiltrating the distal portion of the oesophagus. A transthoracic oesophago-gastrectomy was performed. Continuity was assured by oesophago-gastrostomy. The postoperative course was uneventful.

Material and Methods

Light- and Electron Microscopy. Biopsies and the surgical specimen were immersed in buffered liquid formaldehyde (4%) and embedded in paraffin. The following reactions were then carried out: H and E, Alcian blue-PAS, van Gieson's, Masson's trichrome and Lendrum's phloxine tartrazine stain.

Samples of formaldehyde-fixed tumor tissue were washed overnight in phosphate buffer (0.1 M, pH 7.2), postfixed with osmium tetroxide (1%) and embedded in Epon 812. Semithin sections were stained with Toluidine blue, ultrathin sections with uranyl acetate and lead citrate.

Immunocytochemistry. Deparaffinized sections (5 μm) of the tumor, of a lymph node metastasis, and of the acid secreting mucosa of the stomach were incubated for lysozyme using the unlabeled antibody enzyme method (Sternberger, 1979). Antibodies against human serum lysozyme raised in rabbits (Dakopatts, lot nr. 081/75) were used at dilutions of 1/500 to 1/2,000. The antibody was diluted with phosphate buffered saline (PBS, pH 7.2) containing human serum albumin (0.25% w/v) and sodium azide (15 mmol). Sheep anti-rabbit IgG 1/30 and soluble peroxidase-anti-peroxidase complexes (1/30) were applied as second and third layers respectively. The histochemical reaction for peroxidase was carried out using 3,3'-diaminobenzidine-tetrahydrochloride (DAB; 0.05% w/v) and hydrogen peroxide (0.01%) in 0.05 M Tris-HCl buffer (pH 7.6). After fixation with osmium tetroxide (1%) in phosphate buffered saline (pH 7.2) the sections were dehydrated and mounted.

Formaldehyde fixed tissue postfixed with osmium tetroxide was used for *electron microscopic immunocytochemistry.* The Epon was removed from *semithin sections* (2 μm) by a saturated solution of sodium hydroxide in absolute ethanol (Lane and Europa, 1965). The sections were then immersed in an aqueous solution of periodic acid (5%) for 5 min for bleaching (Baskin et al., 1979). After bleaching, the sections were rinsed in tap water (5 min) and in PBS (5 min). The immunocytochemical technique was then applied as described above. Post-embedding immunostaining for lysozyme was subsequently carried out on ultrathin sections cut serially to immunostained semithin sections. TBS (Tris-buffered saline, 0.05 M, pH 7.6) was used throughout the staining procedure for ultrathin sections. All non-immune sera added to the incubation solutions were decomplemented prior to use. The sections were viewed in a Philips EM 300 or in a Zeiss EM 9 electron microscope.

Controls. Biopsy samples of human duodenal mucosa were used as *control tissue.* They were quenched in melting isopentane (2-methylbutane) at −159.9° C, freeze-dried overnight in a thermoelectric freeze-drier at −40° C, vapor-fixed at 60° C for 3 h with formaldehyde and embedded in paraffin. Further specimens were immersed in vacuum distilled glutaraldehyde (3%; Serva) in PBS (pH 7.2) and embedded in paraffin.

Control reactions were carried out as follows:
a) anti-lysozyme antibodies diluted 1/1,000 absorbed with lysozyme (10 nmol) extracted from hen egg white (Serva) or lysozyme extracted from human urine (Worthington) as first layer
b) non-immune rabbit serum as first layer
c) TBS as first, second or third layer
d) omission of DAB or H_2O_2 from the incubating medium for the peroxidase reaction.

Results

Gross Findings and Light Microscopy. The specimen contained a polypoid tumor (largest diameter 4.5 cm). The well differentiated adenocarcinoma invaded the submucosa of the distal oesophagus and penetrated into the perigastric adipose tissue. A large number of cylindric tumor cells contained numerous eosinophilic granules in the supranuclear cytoplasm suggesting the presence of Paneth cells. These cells stained bright red in Masson's trichrome and Lendrum's phloxine tartrazine stains, and purple in the Alcian blue-PAS stain. In several areas mucin secreting cells and Paneth cells were present in the same neoplastic acinus or tubule. 10/15 regional lymph nodes contained metastatic tumor tissue in which Paneth cells could be observed.

Chronic atrophic gastritis with intestinal metaplasia and severe focal epithelial dysplasia was present in the mucosa of the stomach adjacent to the tumor. A large number of typical Paneth cells was seen in the metaplastic areas.

Electron Microscopy. Semithin sections displayed blue granules localized almost exclusively in the supranuclear cytoplasm of many tumor cells (Fig. 1a). At the ultrastructural level the preservation of tissue was less than perfect because it had been fixed in formaldehyde. Many secretory granules were found to be strongly osmiophilic (Fig. 1b). At high magnification there were marked differences of osmiophilia, and in moderately dense granules a strongly osmiophilic core, sometimes resembling myeline-like structures. There was no visible limiting membrane. In some cells granules could be seen to be localized within the profiles of rough endoplasmic reticulum. The mean diameter of the granules was found to be 1,530 nm (range: 1,020–2,200 nm). The mean diameter of the granules was estimated by measuring the width of 500 granule profiles taken at random. It was corrected in order to account for the fixation and sectioning artifact according to Baetens et al. (1976). The rough endoplasmic reticulum and the Golgi complex of the tumor cells were well developed. Granulated cells showed less numerous and shorter microvilli than mucin secreting tumor cells. In areas devoid of Paneth cells numerous goblet cells were seen. Intermediate forms of mucin secreting cells and Paneth cells were not observed.

Immunocytochemistry. A strong immunocytochemical reaction was present in many tumor cells with all dilutions of antibody used. It was seen to occur also in polymorphonuclear granulocytes and monocytes present in the tumor stroma. Tumor cells containing lysozyme-immunoreactivity were also observed in a lymph node metastasis (Fig. 2a–d).

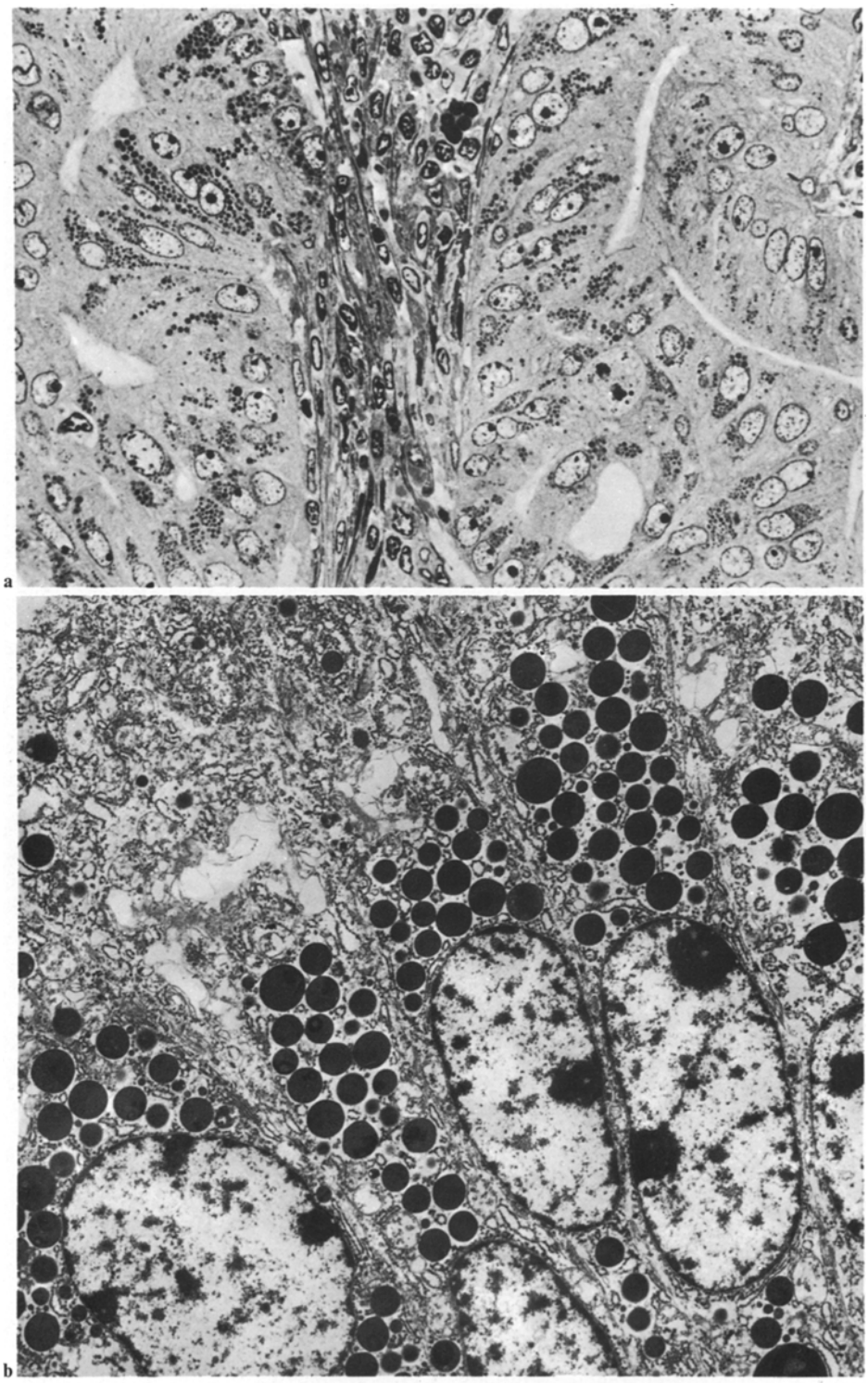

Fig. 1a and b. Adenocarcinoma of the stomach. **a** Large number of cytoplasmic secretory granules in tumor cells. Semithin section, Toluidine-blue ($\times 640$). **b** Large osmiophilic granules predominantly located in the supranuclear cytoplasm of tumor cells. Eccentric dense core in some secretory granules. Fixation in formaldehyde, postfixation in osmium tetroxide. Lead citrate and uranyl acetate stain ($\times 4{,}750$)

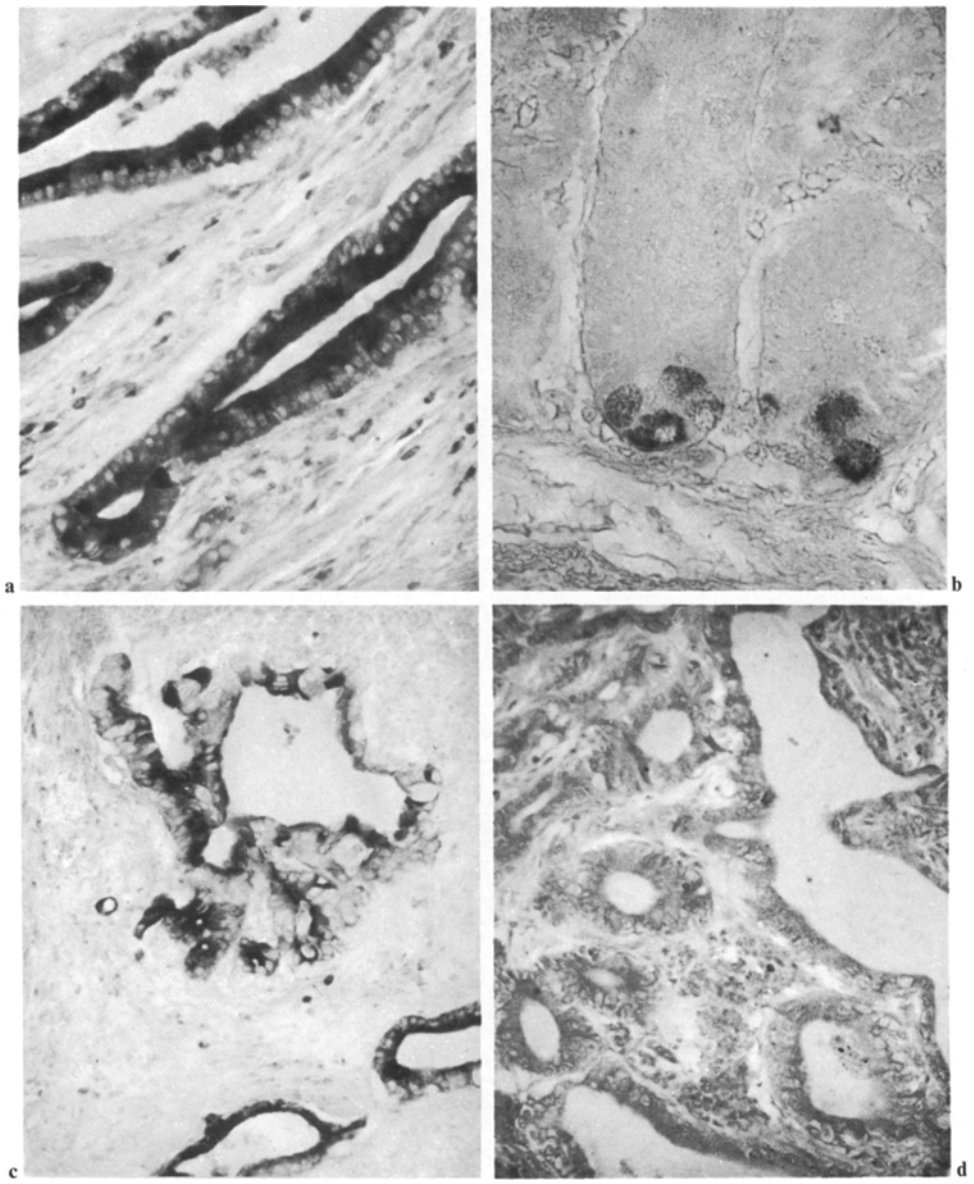

Fig. 2. a Adenocarcinoma of the stomach. Positive lysozyme-immunoreactivity in the apical cytoplasm of the majority of tumor cells. Positive reaction in polymorphonuclear granulocytes and monocytes of the tumor stroma (×213). **b** Lysozyme-immunoreactivity in Paneth cells at the base of duodenal crypts (×333). **c** Lysozyme-immunoreactivity in a lymph node metastasis of an adenocarcinoma of the stomach (×133). **d** Absence of lysozyme-immunoreactivity after preabsorption of anti-human lysozyme antibody with lysozyme extracted from human urine (×133). **a–d:** Unlabeled antibody enzyme method

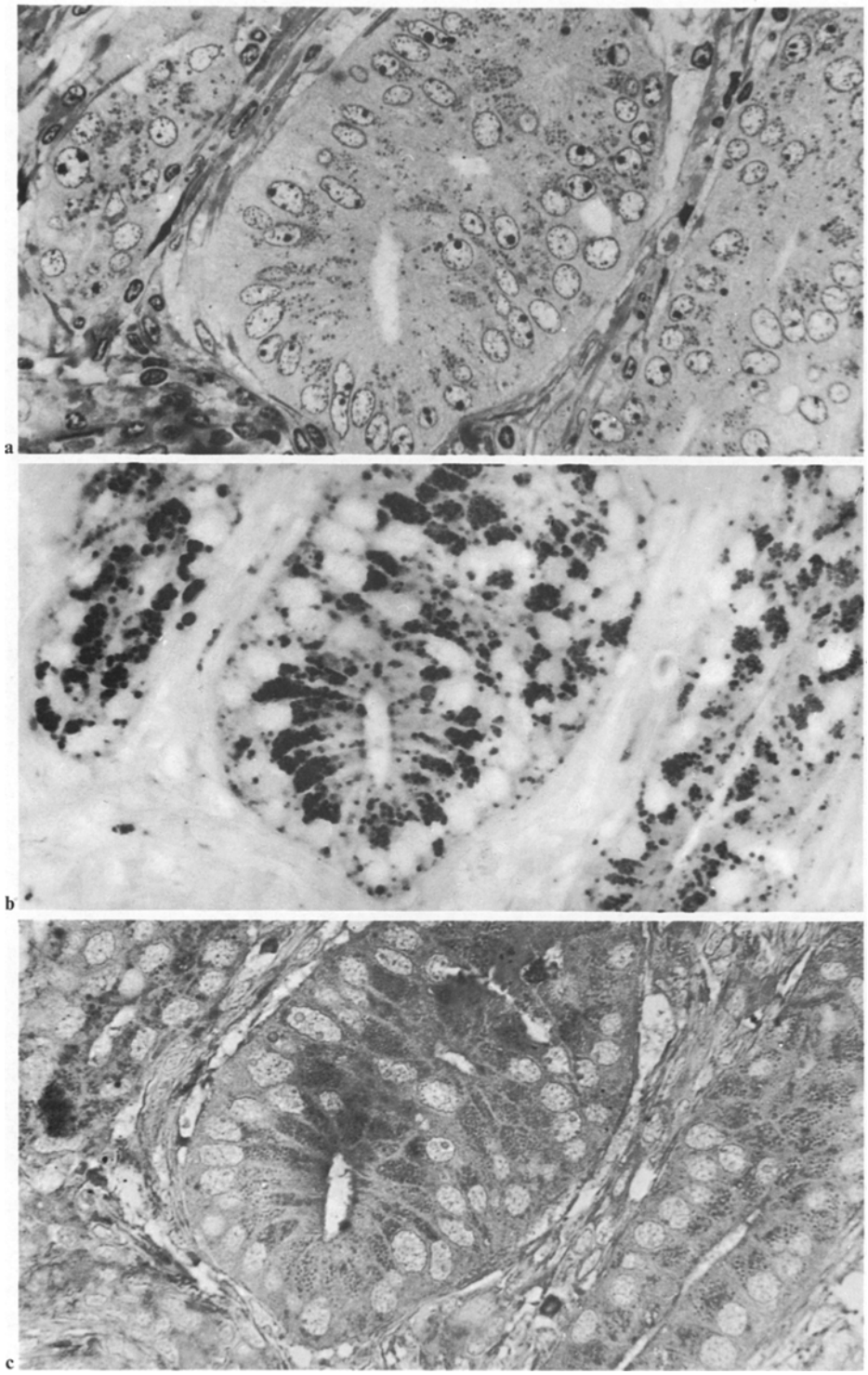

Fig. 3a–c. Adenocarcinoma of the stomach. Serial semithin sections. **a** Large cytoplasmic secretory granules in the majority of tumor cells. Toluidine-blue (×533). **b** Lysozyme-immunoreactivity is predominantly localized to supranuclear cytoplasmic secretory granules (×533). **c** Absence of reaction product after preabsorption of anti-human lysozyme antibody with lysozyme extracted from human urine (×533). Formaldehyde fixation, postfixation with osmium tetroxide. Unlabeled antibody enzyme method

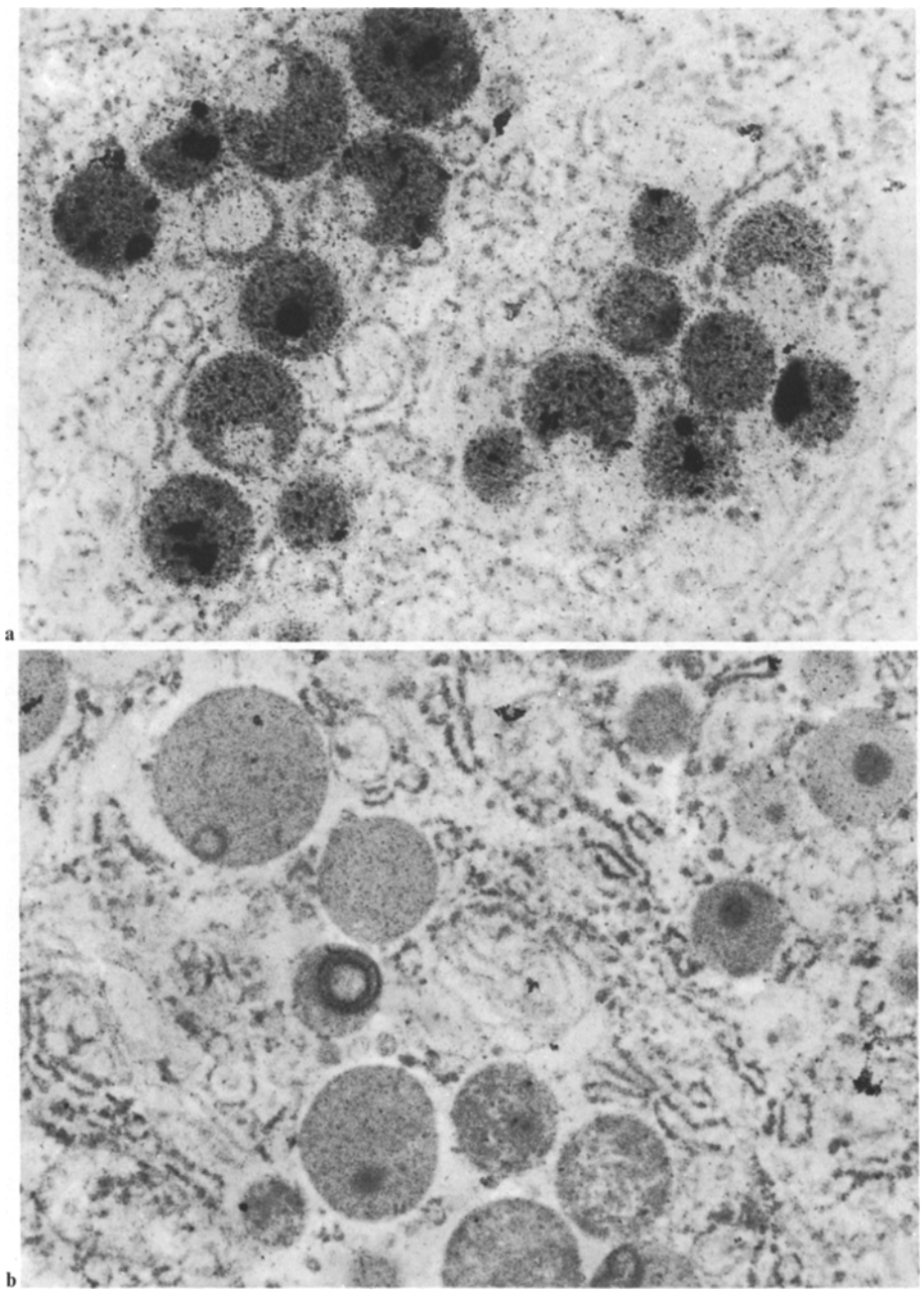

Fig. 4a and b. Thin sections of an adenocarcinoma of the stomach. **a** Electron dense reaction product on large cytoplasmic secretory granules some of which contain an osmiophilic dense core (× 15,950). **b** Absence of lysozyme-immunoreactivity after preabsorption of anti-human lysozyme antibody with lysozyme extracted from human urine (× 15,950). Formaldehyde fixation, postfixation with osmium tetroxyde. Unlabeled antibody enzyme method

In semithin sections the immunostaining was limited almost exclusively to the supranuclear part of tumor cells (Fig. 3b). In ultrathin sections the immunoreactive material was confined to large secretory granules (Fig. 4a).

Control reactions were invariably negative (Fig. 2d, 3c and 4b) except after preabsorption of the anti-lysozyme antibodies with lysozyme extracted from hen egg white. In the normal human duodenal mucosa the immunocytochemical reaction was confined to granular cells at the basis of crypts of Lieberkühn (Fig. 2b) Cells containing lysozyme immunoreactivity were also seen in metaplastic gastric mucosa adjacent to the tumor.

Discussion

We have demonstrated the presence of Paneth cells in a well differentiated adenocarcinoma of the stomach by immunocytochemical staining for lysozyme.

Paneth cells are normally found almost exclusively at the base of the intestinal crypts of Lieberkühn in many species, including man, but not in the cat and dog (Otto, 1974; Sandow and Whitehead, 1979). They are characterized by eosinophilic granules located in the apical portion of the cell. Electron microscopy reveals a polar protein secreting exocrine cell containing an abundant rough ergastoplasmic reticulum, a prominent Golgi apparatus and large osmiophilic secretory granules (diameter 1–3 μm). In man, these granules consist of a basic protein core intermixed with neutral mucosubstances, and of acid mucosubstances (Spicer et al., 1967). The basic protein has been identified as lysozyme (E.C. 3.2.1.17) by substrate film methods (Speece, 1964; Ghoos and Vantrappen, 1973), by biochemical characterisation of a secretory granule fraction (Deckx et al., 1967), and by immunocytochemical techniques at the light microscopic (Klockars and Osserman, 1974; Mason and Taylor, 1975; Peters and Vantrappen, 1975) and electron microscopic level (Erlandsen et al., 1974). Recently, the presence of immunoglobulins of the A and G classes has been disclosed in Paneth cells by immunocytochemistry (Rodning et al., 1976). The association of immunoglobulins and lysozyme may have important functional implications (for review see Montero and Erlandsen, 1978; Sandow and Whitehead, 1979).

Lysozyme can be considered as a marker for Paneth cells. In addition, lysozyme has been localized recently in a subpopulation of mucus-producing cells found in small intestinal specimens from normal rats and from patients with inflammatory bowel diseases (Montero and Erlandsen, 1978).

Lysozyme immunoreactivity can be assumed to be specific for the following reasons: a) the antibodies were used at dilutions of up to 1/2,000, b) the reaction was entirely negative in all control reactions, except after preabsorption of the primary antibody with lysozyme extracted from hen egg white, c) a positive reaction was obtained after preabsorption of the antibody directed against human serum lysozyme with 10 nmol of lysozyme extracted from hen egg white. The extent of cross reaction between these two phylogenetically remote lysozymes exhibiting over 50% differences in their sequences is known to be rather small (Arnon, 1977), and d) Paneth cells were selectively immunostained in

normal human duodenum and in intestinal metaplasia of gastric mucosa adjacent to the tumor. The positive reaction of polymorphonuclear granulocytes and monocytes is not an argument against the specificity of immunocytochemistry because the presence of lysozyme in these cells is well known (Pryzwansky et al., 1978).

It is of interest that lysozyme-like immunoreactivity was preserved after fixation of tissue samples in formaldehyde followed by osmium tetroxide.

We cannot entirely exclude the presence of mucous-producing tumor cells containing lysozyme as described by Montero and Erlandsen (1978). However, there is no evidence of mucin production by the tumor cells containing large osmiophilic granules and displaying lysozyme immunoreactivity in our material.

We conclude, therefore, that neoplastic Paneth cells occasionally occur in malignant tumors of the human gastrointestinal tract. As the cells were also observed in a lymph node metastasis they may be considered to be truly neoplastic. Furthermore it may be assumed that Paneth cells observed in this tumor were morphologically and biochemically differentiated. We are not able to give an explanation for the occurrence of Paneth cells in the well differentiated carcinoma of this patient. This apparently rare phenomenon may be related to the genesis of intestinal metaplasia of the stomach. In the latter, differentiation of Paneth cells from a precursor cell of the mucosa is frequently observed. It is conceivable that precursor cells present in a well differentiated adenocarcinoma of the stomach or intestine may be stimulated to differentiate into Paneth cells by an unknown stimulus. At present, it is not known if the presence of neoplastic Paneth cells in a carcinoma has any relationship to its proliferation rate or to its ability to spread to the lymph nodes or to distant organs.

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Note Added in Proof

After submission of this manuscript Shousha reported a papilary adenocarcinoma of the colon containing Paneth cells as well as mixed goblet-Paneth cells. However, the figures shown are not entirely convincing because Paneth cell granules were not shown to contain lysozyme. We observed the occurrence of Paneth cells in a tubulo-villous adenoma of the colon after immunostaining for lysozyme.

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