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Gastric Marker Proteins

Purification and Immunohistological Demonstration of the Chief Cell Esterase

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Summary. An immunogenic acid stable esterase of gastric origin was purified from normal gastric juice. Specific antibodies were obtained by solid immunoadsorbents and were used for indirect immunoenzyme histology on gastric resection specimens. This esterase proved to be a cell specific marker for gastric chief cells. Different patterns of chief cell alterations in gastritis and cancer were observed.

Key words: Gastric esterase – Chief all marker – Immunoenzyme histology – Gastritis – Cancer

Introduction

Our first study on immunogenic "marker substances" of normal human gastric mucosa was performed with the aid of heteroimmunesera, immunoelectrophoresis (IEP) and agar gel electrophoresis, followed by histochemical staining techniques. These studies resulted in the discovery of a multitude of hitherto unknown substances which were arbitrarely labelled according to their electrophoretic mobilities and staining properties until definite biochemical and histological characterization could be undertaken (Rapp et al. 1964).

One of the first substances identified was an immunogenic carboxylesterase with beta/gamma mobility in IEP. This substance, which seemed to be a major component of gastric mucosa, was labelled VI A. Subsequent studies have shown that VI A was absent or decreased in saline extracts of gastric carcinoma and tumor adjacent tissue (Aronson et al. 1965; Rapp et al. 1966). VI A proved to be organ specific by immunodiffusion studies and was demonstrated as a

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secretory component in acid gastric juice (Rapp et al. 1970). In the gastric juice of patients with atrophic gastritis, intestinal metaplasia, pernicious anemia, Billroth II resections and in most gastric cancer patients, VI A was absent or decreased (Rapp et al. 1970, 1973). Upon partial purification VI A was characterized as an acid and heat stable non-specific carboxylesterase with an pH optimum at pH 6.0 and with an estimated molecular weight of 140,000 Daltons (Rapp and Lehmann 1972). Immunochemically related esterase activities were also observed in the rabbit and guinea pig (Kuhlmann and Rapp 1970).

In this study we report the definite histological localization of this major gastric marker protein in chief cells of human gastric mucosa, by the indirect immunoperoxidase technique using specific antibodies raised by highly purified esterase. We have shown that this immunogenic and antigenic esterase is an useful specific marker substance for gastric chief cells in health and disease. Hence its designation as chief cell esterase (CE).

Material and Methods

Gastric Juice (GJ)

GJ of normals was collected by nasal and oral intubation after Pentagastrin stimulation following overnight fasting. Acid GJ obtained during a 1 h-secretion period was pooled, filtered on Kleenex and centrifuged at 70,000 × G for 30 min at 4° C. The supernatant of GJ was neutralized with 1 n NaOH and stored at −20° C until use.

Purification Procedure

DEAE-ion exchange chromatography. The neutralized supernatant of GJ was concentrated 50–100 fold by Visking dialysis bags (10 mm) in a negative pressure concentration unit (Elukon, Ionic, Heidelberg) and dialysed against a 0.01 M $Na_2HPO_4 - NaH_2PO_4$ buffer, pH 7.0 (phosphate buffer) overnight. All purification procedures were performed at 4° C if not indicated otherwise. Concentrated GJ (40 ml; 10 mg/ml protein) was applied to a DEAE-cellulose column (20 g Servacel, Type 32, Serva, Heidelberg) of 20.0 × 2.5 cm, previously equilibrated with the 0.01 M phosphate buffer at pH 7.0. Discontinuous elution at constant pH 7.0 and at room temperature was performed with the 0.01 M phosphate buffer initially and then, with rising NaCl concentration in 0.01 M phosphate buffer at 0.04, 0.08, 0.1 and 0.2 M and finally with saturated NaCl buffer solution. Elution was monitored at 280 µm. Fractions of 10 ml were collected and pooled according to the elution patterns. Pools of eluted material at 0.01 and 0.04 M were concentrated approx. 50 fold to approx. 10.0 and 2.0 mg/ml protein and finally dialysed against physiological buffered saline (PBS).

Molecular exclusion chromatography. Gel filtration was performed with AcA 22 (LKB) in a Pharmacia column of 5 × 86 cm equilibrated with PBS at 4° C. The concentrated DEAE fractions obtained from the DEAE-chromatography at 0.01 M (5.4 ml; 70 mg protein) were applied for downward elution. PBS was used as eluant. A hydrostatic pressure of 40 cm H_2O was applied and a flow rate of approx. 3 ml/h/cm^2 was maintained. Elution was monitored at 280 µm and fractions of 10 ml were collected. Eluted fractions were pooled according to the elution patterns and concentrated to approx. 4.5 mg/ml protein. The gel column was characterized with Dextran blue and K_{av}-values were calculated.

Preparative Polyacrylamide Gel Electrophoresis (PAGE)

It was performed in a horizontal slab system as described in detail previously (Rapp and Lehmann 1976). The dimension of the hard glass cell was 18 × 9 cm (PAGE unit, Ionic, Heidelberg). The gel contained Tris-citric acid buffer of 0.15 M, pH 9.0. A 10.0% separation gel (18 cm length)

and 3.5% spacer gel (1 cm length) were used. Tris-boric acid of 0.175 M and pH 9.0 was used as electrode buffer solution. Samples of 10 mg protein obtained from the molecular exclusion chromatography at K_{av} 0.6 (peak 2) were mixed with sucrose and applied for separation. Electrophoresis was performed at 4° C for 4 h at 350 volt and 55 mAmp. At the end of the separation the bromphenolblue tracking had traversed 18 cm towards the anode. Gel strips of 3 mm were cut, extracted, eluted, concentrated and dialysed. Eluted fractions were pooled according to the esterase staining patterns after an analytical PAGE and concentrated to approx. 0.2 mg/ml.

Analytical Techniques. Gel diffusion techniques were performed in 0.05 M Na-Veronal buffer, pH 8.2 in 1% Agarose. Enzyme electrophoresis was performed in the same buffer (Rapp and Lehmann 1972).

Analytical PAGE was performed as vertical slab gel electrophoresis with the Ortec Model 4,200 and the 4,200 pulsed constant power supply (Allen et al. 1969) and gel system No 2 (Maurer 1971) were used. Proteins were stained with Coomassie Brilliant Blue R 250, glycoproteins were stained with periodic acid Schiff (PAS) reagent. Acid mucins were stained with Alcian blue at pH 1.0 and 2.5 (Wardi-Michas 1972).

Precipitates in gel diffusion techniques were stained with amido black and esterase activities were revealed with betanaphthyl acetate and Fast Blue salt as described by Rapp and Lehmann (1972). Protein was determined by the Lowry method (Lowry et al. 1951) using bovine serum albumin (Behringwerke, Marburg) as standard.

Antisera and Antibodies. Antisera were produced in 4 rabbits using purified samples of CE. Rabbits were immunized s.c. with 100 µg protein in complete Freund's adjuvant and boosted with the same amount i.m. 3 times at intervals of three weeks. Rabbits were bled and antisera were stored at −20° C.

Purified antibodies were obtained by immunoadsorption. Solid immunoadsorbents were made according to Cuatrecasas (1970) using Sepharose 6 B and PBS extracts of normal fundal mucosa (4 mg protein per ml Sepharose). Samples of 5 ml of antisera were applied to a 9 × 1.5 cm immunoadsorbent column and absorbed antibodies were eluted with 3 M NaSCN, dialysed against PBS by Sephadex G 25 gel filtration and concentrated to 2 mg/ml protein and stored at −70° C until use.

Antisera and antibodies were tested in gel diffusion techniques and stained for protein and esterase activity. Antisera were compared with other gastric marker substances already described: pepsinogen group I and II, slow moving protease (Rapp and Lehmann 1976), major gastric glycoprotein (obtained from Dr. Schrager, Wigan, England) and neutral mucosubstances of the surface epithelium (Rapp et al., unpublished).

Immunoenzyme Histology. The modified ethanol-acetic acid fixation procedure and paraplast embedding was used as described (Wurster et al. 1978). Gastric specimens obtained by surgery for gastric and duodenal ulcer ($n=140$) and for cancer ($n=40$) were used. From each resection specimen 12 tissue blocks from representative areas of duodenum, antrum and corpus were obtained. Serial paraplast sections of 4 to 8 µ were made, deparaffinized and stained routinely with haematoxylin eosin, PAS and Alcian blue at pH 2.5 and 1.0. Endogenous peroxidative activities were blocked as described (Wurster et al. 1978). The indirect immunoenzyme technique using peroxidase labelled anti-rabbit IgG (Behringwerke, Marburg) at $^1/_{20}$ working dilution was applied. Purified anti-CE antibodies were used at 0.010 mg/ml protein concentration as first layer, washed in PBS and followed by the peroxidase labelled antirabbit IgG. Technical procedures and the criteria for specificity have been described by us in detail (Wurster et al. 1978). For classification of gastric cancer the nomenclature of Laurén (Laurén 1965) was used.

Results

Purification of Chief Cell Esterase

When neutralized GJ was applied to DEAE-cellulose chromatography, the bulk of CE as demonstrated by agar gel enzyme electrophoresis and immunodiffusion

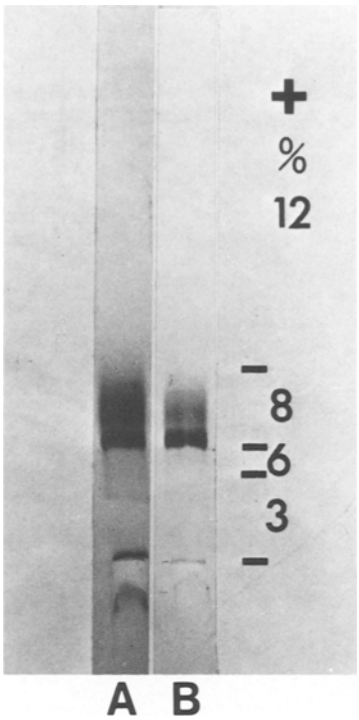

Fig. 1 A and B. Electrophoresis of purified chief cell esterase (0.2 mg/ml). **A)** Esterase staining with beta-naphtylacetat and Fast Blue B Salt at pH 7.0, analytical PAGE. **B)** Protein staining with Coomassie R 250, analytical PAGE

techniques was eluted at 0.01 and 0.04 M. When both DEAE fractions were further applied to molecular exclusion chromatography a total of 4 peaks were eluted. CE was found in the second peak at K_{av} 0.6. A further purification of CE was achieved by preparative PAGE. Typical esterase activity and immunoreactive material was eluted in the cathodic gel strips No 4 and 5 of the 10.0% separation gel. In analytical PAGE purified CE gave rise to two bands which stained for protein and esterase in the 8% gel zones (Fig. 1), whereas in agarose electrophoresis only one esterase band was demonstrated in the beta zone. When purified CE was tested in gel diffusion with specific antisera directed against other gastric marker proteins no immunochemical relationship was observed.

Antisera and Purification of Antibodies

Four rabbits immunized each with a total of 400 µg protein of purified CE gave rise to antisera which contained precipitating antibodies directed against CE obtained from fundal extracts, acid and neutralized gastric juice. In IEP the typical precipitin pattern with beta/gamma mobility was observed. Identical reactions were observed with purified antibodies obtained by solid immunoadsorbents. No other precipiting antigen/antibody systems were observed when a standard battery of saline extracts of major human organs, plasma, and body fluids were studied in double gel diffusion techniques. All precipitin lines

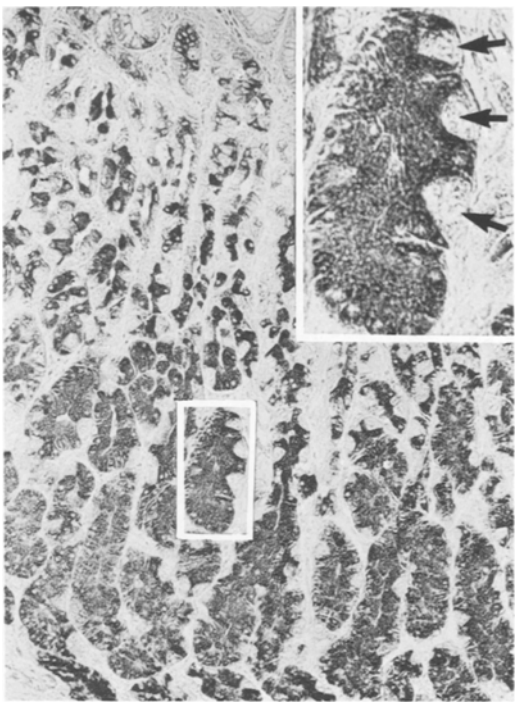

Fig. 2. Normal fundal mucosa stained for chief cell esterase using purified antibody (0.01 mg/ml protein) and peroxydase labelled anti-rabbit IgG. Parietal cells (arrows inset) do not stain. Note positive cells in the luminal part of the glands, resembling neck cells. × 100 (× 1,000)

obtained by anti-CE antisera and antibodies did stain for protein and esterase activity.

Immunohistochemical Demonstration of Chief Cell Esterase.

Normal Gastric Mucosa. By application of specific antibodies, CE was invariably demonstrated in chief cells and lower neck cells of normal fundal mucosa. The staining was found throughout the cytoplasm (Fig. 2). Chief cells staining with CE were most numerous in the lower portion of the fundal glands. Their number decreased in the upper portion of the glands, where CE positive cells resembled neck cells of columnar shape. The immunological contrast of CE staining was excellent and proved to be specific according to various criteria. Stroma and connective tissue did not stain.

No CE staining was observed in the surface and pit cells of the whole stomach, neither in parietal cells (Fig. 2, inset) nor in pyloric cells. However, some single cells or glands situated amidst pyloric glands showed CE staining. This was observed mainly in specimens resected for duodenal or gastric ulcer disease. Duodenal, epithelial and Brunner cells did not stain. In some specimens, however, resected for duodenal ulcer disease, single CE positive cells were observed between the Brunner cells or near the basement membrane of the glands. When tissue sections of major human organs were tested with CE antibodies no staining was observed.

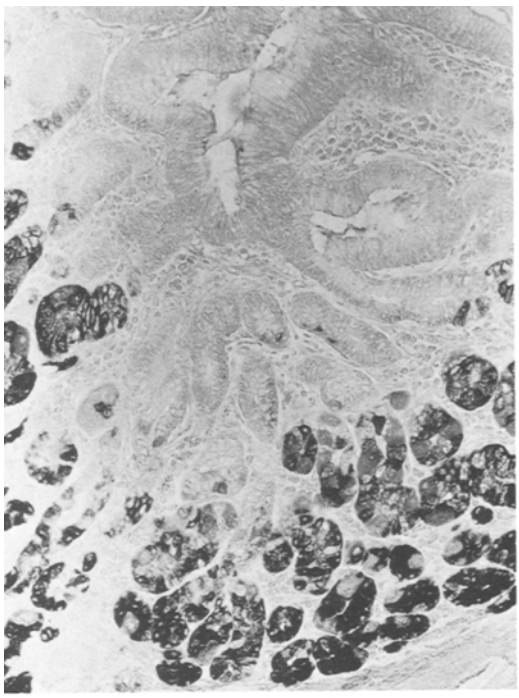

Fig. 3. Fundal mucosa, chief cell atrophia due to foveolar hyperplasia. Note different degree of CE staining in chief cells. Indirect immunoperoxydase staining. ×100

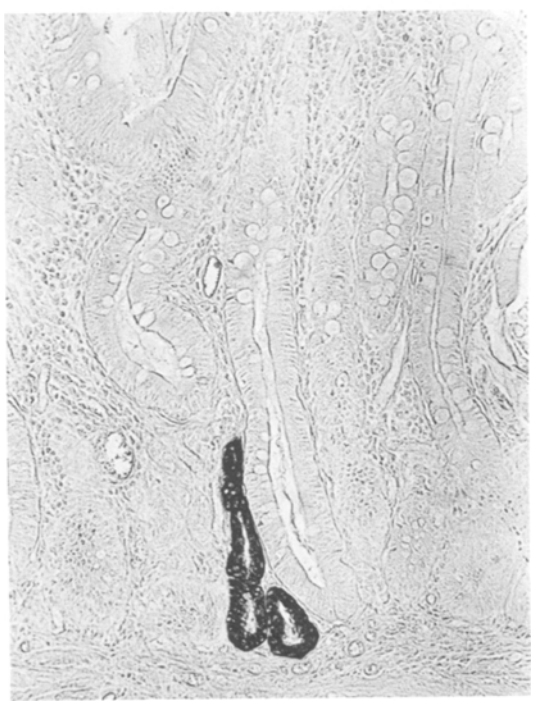

Fig. 4. Fundal mucosa from specimen with heavy intestinal metaplasia. Only small area of cystic dilated glands staining for CE. Indirect immunoperoxydase staining. ×100

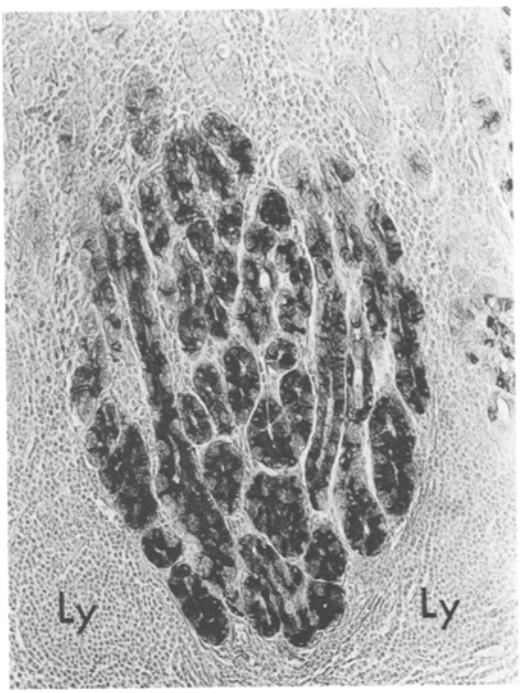

Fig. 5. Focal atrophia of chief cells caused by lymphocellular infiltration (*Ly*). Indirect immunoperoxydase staining. ×100

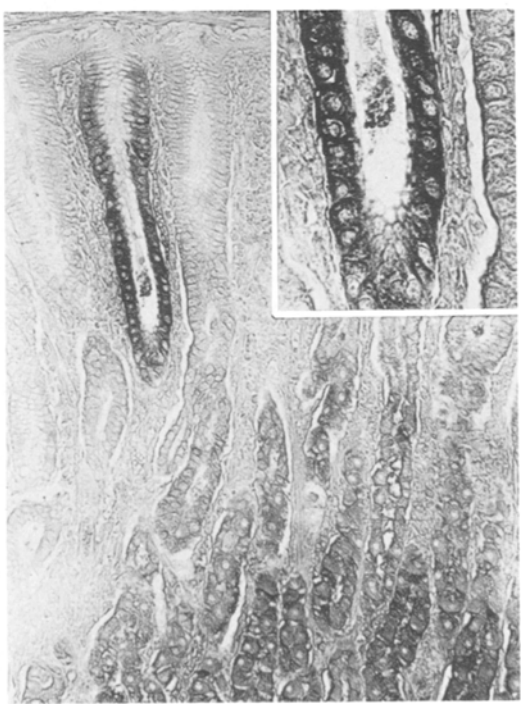

Fig. 6. Cancer adjacent fundal mucosa with CE positive pit cells. Indirect immunoperoxydase staining. ×100 (×400)

Gastritis and Gastric Cancer. In gastritis (associated with cancer or not) and in ulceration various degrees of focal or diffuse chief cell deletion were observed. In combination with classical staining techniques and other marker systems we have described various morphological alterations which were associated with gastric chief cell deletion: 1) loss by genuine atrophy, 2) displacement by foveolar hyperplasia (Fig. 3), 3) substitution by intestinal metaplasia (Fig. 4), 4) displacement by lymphofollicular proliferation or T-cell infiltration (Fig. 5), 5) displacement or destruction by plasma cell or PMN cell infiltration.

The following atypical patterns of gland and chief cell configuration were observed: 1) cystic dilatation of chief cell glands, 2) hypertrophy of single chief cells, 3) CE-staining of atypical fundal pit cells (Fig. 6). These atypias were mostly observed in fundal mucosa adjacent to cancers.

Discussion

The systematic study of gastric marker substances using specific immunochemical methods (Rapp et al. 1964) was undertaken with the aims 1) to identify specific gastric cells, 2) to evaluate cell function and deletion clinically by cell specific secretion products in gastric juice or in plasma, 3) to elucidate the histogenetic relationship of normal and pathological gastrointestinal cell types in dysplasia, metaplasia and cancer.

Esterase activities in normal and diseased human gastric mucosa have been demonstrated mainly in chief cells (Chessick 1953; Correa et al. 1963), but also in parietal cells, surface and pit cells, intestinal metaplasia (Stoffels et al. 1972) and cancer (Planteydt and Willighagen 1965) by the application of histochemical staining techniques.

By agar gel enzyme electrophoresis of fundal extracts we demonstrated at least five distinct esterase activities with different electrophoretic mobilities, two of them (VII A and VI A) being immunogenic but not immunochemically related (Rapp et al. 1964). As the most important of these activities (VI A) was deleted in gastric cancer cells further efforts were undertaken to characterize this substance. By specific antisera we have shown that this major esterase was organ specific for the gastric fundus and was not related to other gastric proteins such as acid proteases (Rapp and Lehmann 1976) or intrinsic factor (Rapp et al. 1971). Of clinical importance was the decrease or absence of this enzyme in gastric juices of patients with a variety of gastric diseases, as revealed by enzyme electrophoresis and quantitative immunodiffusion (Rapp et al. 1970, 1973). In preliminary studies using indirect immunofluorescence and cryostat sections we were not able to locate this enzyme definitively (Rapp et al. 1970; Kuhlmann et al. 1970). Progress was achieved only recently by a modified fixation procedure for paraplast tissue sections and by the use of immunoadsorbed highly specific antibodies (Wurster et al. 1978).

On the basis of previous purification studies (Rapp and Lehmann 1972) we used a modified purification procedure which consisted in three steps and resulted in small quantities of extremely well purified CE.

Upon immunization and immunoadsorption specific antibodies were obtained which reacted only with CE. In analytical PAGE the CE gave rise to different enzyme bands. For this reason we have to consider the esterase as a microheterogeneous set of several electrophoretically distinct components. In agar gel enzyme electrophoresis CE was observed mainly as a large band in the beta region. Differences between analytical PAGE and agar gel enzyme electrophoresis are due to the higher separation capactiy of the PAGE system used.

By indirect immunoenzyme histology using purified antibodies and ethanol-acetic acid fixed paraplast sections of gastric tissue the esterase was located in the chief cells of the fundus and designated chief cell esterase. The fact that the CE was also found in columnar cells of various sizes resembling lower neck cells can be considered as a further proof for the histogenetic relationship between these cell types, the neck cell being considered as the precursor cell of parietal and chief cells. The CE positive neck cells could thus be the "intermediate cell type between major chief cells and undifferentiated stem cells" as suggested by Rubin et al. (1968). Some glands or single cells situated admidst pyloric glands and duodenal Brunner cells did contain CE. The presence of contaminating antibodies directed against these cells can be excluded, as the overwhelming mass of these cells did not stain with the CE antibodies. It has already been noted (Ito 1967) in the cat and in man (Rubin et al. 1968) that some mucus cells of the pylorus do contain electron dense zymogen granules which resemble those of the fundal chief cells. We can therefore assume that some CE positive chief cells or at least immunochemically related cells do exist in the pylorus and in the duodenum. This finding might be of importance for the pathogenesis of gastric and duodenal ulcer disease.

In the present study CE proved to be a valuable marker for the chief cell mass in normal and pathological conditions. As already observed in previous studies on water soluble extracts of gastric cancer tissue (Rapp et al. 1964) CE was deleted in all gastric cancers, notwithstanding the different cancer cell types. Esterase activities already described in cancer tissue (Planteydt and Wellighagen 1965) must belong to a different system of esterases.

Deletion of the chief cell mass was observed mostly in cancer adjacent fundal mucosa and in gastritis of variable origin. This deletion was associated with different pathological changes such as foveolar hyperplasia, metaplasia, lymphofollicular and inflammatory infiltration.

The chief cell deletion reported in this study explains the results of previous measurements of the same esterase in gastric juices of patients with various pathological conditions (Rapp et al. 1970, 1973). Though still undefined as to its precise physiological function CE can be considered to be a useful cell specific tool for simultaneous histological and laboratory evaluation of the gastric chief cell mass and the function of its component cells.

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