

Immunohistochemical Studies on Human Gastric Mucosa

Procedures for Routine Demonstration of Gastric Proteins by Immunoenzyme Techniques*

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Summary. Two different fixatives were applied to human gastric mucosa for the study of antigenic marker substances. The first consists of 96% ethanol and 1% acetic acid (EA method), the second of 4% formaldehyde, 0.5% picric acid and 0.25% glutaraldehyde (FPG method). Samples of resected gastric specimens were fixed, dehydrated and cleared in benzene and embedded in paraplast. The morphology of gastric tissue was well preserved by both methods and permitted the simultaneous application of classical staining procedures and the immunoenzyme peroxidase technique for the demonstration of antigenic substances. The following marker substances could be demonstrated: Pepsinogen I and II group, surface epithelial antigen, parietal cell antigen, chief cell antigen, antral mucous cell antigen, carcinoembryonic antigen, goblet cell antigen and common site antigen of leucocytes. Various factors responsible for nonspecific reactions, such as endogeneous peroxidase activity and protein-protein interactions were studied. The latter were circumvented by the use of highly purified antibodies or immunoglobulin fractions. The EA method proved to be the method of choice for future routine application of combined classical histology and immunoenzyme histology in gastric and intestinal diseases.

Key words: Immunohistology — Human gastric mucosa — Gastric antigens.

Zusammenfassung. Zwei Fixierungsverfahren wurden für das Studium von antigenen „Marker“ auf die menschliche Magenschleimhaut angewandt. Die erste Methode bestand in 96% Äthanol und 1% Eisessig (Methode AE),

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die zweite in der Kombination von 4% Formaldehyd, 0.5% Pikrinsäure und 0.25% Glutaraldehyd (Methode FPG). Proben von resezierten Mägen wurden fixiert, dehydratisiert und nach Behandlung in Benzol in Paraplast eingebettet. Die Gewebsmorphologie des Magens war in beiden Methoden gut erhalten und erlaubte die simultane Anwendung klassischer Färbetechniken und immunenzymhistologischer Verfahren zur Darstellung antigener Substanzen in der menschlichen Magenschleimhaut. Die folgenden „Marker“-Substanzen konnten in beiden Verfahren nachgewiesen werden: Pepsinogen I und II, Deckepithelantigen, Belegzellantigen, Hauptzellantigen, Mucusantigen der Schleimzellen des Antrums, carcinoembryonales Antigen, Becherzellantigen und „common site“ Antigen der Leukozyten. Verschiedene Faktoren, die für unspezifische Reaktionen verantwortlich sind, wie endogene Peroxydasen, Protein-Protein Interaktionen wurden untersucht und letztere durch die Anwendung hochgereinigter Antikörper oder Immunglobulinfraktionen umgangen. Die AE Methode erwies sich als Methode der Wahl für die zukünftige Routineanwendung kombinierter klassischer Histologie und der Immunenzymhistologie im Bereich der Magen- und Darmerkrankungen.

Introduction

Immunohistochemical study of human intestinal tissue is a prerequisite for increased knowledge of functional histology and immunopathology in intestinal diseases. New antigenic marker substances can be used in this way on the basis of their molecular specificities. However, optimal histological studies of intestinal mucosal antigens requires the simultaneous application of immunohistochemical and conventional histological staining procedures to serial sections obtained from the *same* block of intestinal tissue. For this purpose the immunoenzyme (IE) technique, especially the peroxidase labelled antibody method is of distinct advantage over classical immunofluorescence. Cytological details can be determined with more ease and certainty in a conventional light microscope without the fading phenomena seen with fluorescent labels. We have also shown, in a series of unpublished observations that the IE techniques are the method of choice for application of the Texture Analysing System (Leitz, Wetzlar, FRG).

Problems of endogenous staining caused by tissue enzymes, of nonspecific staining (NSS) due to protein-protein interactions of immunochemically unrelated substances in tissues and in immune-sera, remain. Undesired staining (USS) may occur due to crossreactivity of enzyme labelled antibodies with tissue immunoglobulins, or by rheumatoid factorlike activities in gastric plasma cells. During fixation and paraffin embedding one has also to consider the immunochemical peculiarities of the antigenic substances under study, chemical alteration of the tissue responsible for nonspecific interaction sites, the degree of diffusion and binding affinities of the antibodies used and the influence of these methods on morphology, as demonstrated by classical histochemical staining techniques.

In this paper we describe two modifications of the original method of Sainte-

Marie (1962) by using ethanol-acetic acid (method EA) or formaldehyde, picric acid and glutaraldehyde (method FPG) as fixatives. These two methods have already been successfully applied in IE techniques, to mouse and rat liver (Kuhlmann, 1975, 1976). In the last two years more than 10 human gastric marker proteins have been studied routinely with these methods (Wurster et al., 1976 a, b). As this is the first of a series of papers on immunohistochemistry of human gastric markers we also describe the technical procedures for obtaining and preparing gross resection specimens of the stomach and our methods and criteria for testing the specificity of antibodies and the IE reaction.

Material and Methods

Tested proteins are listed in table no. 1. Five typical "autoantigens" were tested, defined by autoantibodies in pathological human sera and usually present in normal human and animal mucosa. Organspecific gastric proteins included pepsinogen I and II group (PgI and PgII), gastric epithelial antigen (E-Ag), Esterase antigen of the chief cells (EVI A), a glycoprotein of the antral mucous producing cells, classical carcinoembryonic antigen (CEA) as defined by Gold (1965) and CEA common site antigen (CSAg) (Primus et al., 1977) known under various descriptive terms such as NCA (von Kleist et al., 1972) NGP (Mach et al., 1972) and CCA-III (Primus et al., 1977). Further, a recently identified goblet cell antigen (Rapp et al., 1977) and the immunoglobulins IgG, IgA and IgM produced by gastric plasma cells, were included in this study.

Preparation of Immun sera and Antibodies. For the demonstration of human autoantigens, patients sera were used at 1/2 and 1/10 dilution in physiological buffered saline (PBS). These sera contained autoantibodies with titers ranging from 1/50 to 1/200.

Antisera from the rabbit directed against human IgG, IgA and IgM, with heavy chain specificity, were purchased from Behringwerke (Marburg, Germany). All other antisera were raised in rabbits or in goats as described in the original publications (see Table 1). Immunoglobulin fractions of antisera directed against gastric organ specific proteins were obtained by DEAE-chromatography at pH 7.0. Antisera were dialysed and the DEAE cellulose was equilibrated with a 0.01 M phosphate buffer, pH 7.0. Globulin fractions were eluted at initial molarities of 0.04 M and 0.08 M. Fractions were concentrated by vacuum ultrafiltration using Visking dialysis bags (Serva, Heidelberg) and were dialysed against PBS.

Antibodies directed against CEA and CSAg were obtained from goat anti-CEA serum by immunoabsorbent columns. Normal human serum, 0.6 M perchloric acid extract of human lung tissue and of liver metastases from an adenocarcinoma of the colon were coupled to CNBr-activated Sepharose 4B, according to the method of CUATRECASAS (1970). They were eluted with 3 M NaSCN. Anti-CEA serum was first absorbed routinely by a plasma immunoabsorbent column. Common site antibody (CSAb) directed against the CEA-common site antigen was eluted from the lung column. CEA-antibodies were finally obtained by the CSAb exhausted serum after adsorption and elution from the liver metastasis CEA column.

Horseshadish peroxidase conjugated human, rabbit and goat IgG were obtained from MILES-YEDA Ltd. and used at 1/20 working dilutions in PBS. Optimal dilution of the first and second antibody reagents were determined by chessboard titration using appropriate tissue sections, and choosing the highest dilution which resulted in optimal "immunological contrast" of the typical protein under study but giving rise to a minimum of NSS and USS of connective tissue, muscularis mucosae and stroma.

Immunochemical control of monospecificity of globulin fractions and of antibodies was performed in highest protein concentration available (immunoglobulin fractions 10 to 40 mg/ml protein; antibodies 1 to 1.5 mg/ml) in double gel diffusion, counter current electrophoresis and two-dimensional immunoelectrophoresis using 1.0% agarose (Serva, Heidelberg), 0.05 Na-Veronal-buffer, pH 8.2. Gels were washed in PBS, dried and stained with amido black or Coomassie R250.

Table 1. List of antigenic markers used in this study (each marker system will be described in subsequent publications)

Antigenic markers	Specific IgG or antibody	µg/ml ^a	Localization	Reference
Parietal cell autoantigen	serum 1/10		parietal cells	Jourdan, 1976
Mitochondrial autoantigen	serum 1/10		parietal cells and chief cells	
Smooth muscle autoantigen	serum 1/2		Ø	
Reticulin autoantigen	serum 1/2		Ø	
Nuclear autoantigen	serum 1/2		Ø	
Immunoglobulins				
IgG	R IgG ^b	22	plasma cells	
IgA	R IgG	83	plasma cells	
IgM	R IgG	143	plasma cells	
Epithelial antigen	R IgG	500	surface epithelium of fundus and antrum	Kuhlmann et al., 1970
Pepsinogen I	R AB ^c	250	chief cells, neck cells	Rapp et al., 1976
Pepsinogen II	G AB	183	chief cells, antral mucus cells	
Chief cell antigen	R AB	30	chief cells	Rapp et al., 1972
Antral mucus cell antigen	R IgG	500	antral mucus cells, Brunner's cells	Rapp et al., in preparation
Carcinoembryonic antigen	G AB	100	CEA pos. tumor cells dysplastic cells	Gold et al., 1965
Common site antigen	G AB	20	leucocytes, monocytes	Primus et al., 1977
Goblet cell antigen	R IgG	500	goblet cells of intestinal metapl.	Rapp et al., 1977/78

^a Protein concentrations of immunoglobulin fractions or antibodies in mg/ml

^b R IgG = Immunoglobulin IgG obtained from rabbit antiserum by DEAE

^c R AB = Rabbit (R) or goat (G) antibodies purified by solid immunoadsorbent from antisera

The purity of immunoglobulin fractions were tested by analytical flat disc electrophoresis according to ALLEN with the Ortec apparatus, using discontinuous voltage gradients and gel system no. 2 (Maurer, 1971). The classes of antibody containing immunoglobulins or of the antibodies were identified by reversed immunoelectrophoresis using anti-immunoglobulin sera (Miles-Yeda Ltd.). Protein concentrations were determined by the Folin method (Lowry et al., 1951).

Gastric Tissues. Gastric specimens were obtained by surgery for ulcer or cancer resections. After resection the stomach was opened along the larger curvature, stretched out in a polyethylene bag. The bag was sealed and laid on crushed ice, mucosa up. At the pathological department at least 12 samples of gastric tissue were taken along the larger and smaller curvature and from macroscopically apparent lesions. Tissue specimens were trimmed with a razor blade into pieces of 0.5 × 0.5 × 0.5 cm. Sites of excision were marked with black numbers on white water resistant

cardboard. The resected specimen was then photographed for documentation and for topographically defined extraction procedures. The remaining resected specimen was then stored at -20°C in the polyethylene bag until used for further immunochemical and biochemical studies.

Tissue Fixation and Processing. Tissue blocks from gastric mucosa were fixed following the ethanol-acetic acid method or the formaldehyde-picric acid-glutaraldehyde method (Kuhlmann, 1975). Formaldehyde, freshly prepared from paraformaldehyde (Merck, Germany) and vacuum distilled glutaraldehyde with a purification index of P.I. = 0.1–0.2 were used throughout this study.

The ethanol-acetic acid method (EA) consisted of: (a) fixation at 4°C in 96% ethanol (from absolute ethanol) containing 1% acetic acid for 15 to 24 h; (b) dehydration in 6 changes of absolute ethanol, 20 min each at 4°C and followed by 6 changes of absolute ethanol, 20 min each at room temperature; (c) clearing in 2 changes of benzene, 1 h each; (d) transfer into paraplast (Sherwood Inc., USA), 4 baths of 1 h each, followed by embedding.

The formaldehyde-picric acid-glutaraldehyde method (FPG) was employed at 4°C : (a) fixation in 4% formaldehyde and 0.5% picric acid in 0.2 M cacodylate pH 7.2 for 1 to $1\frac{1}{2}$ h, followed by 4% formaldehyde and 0.5% picric acid supplemented with 0.25% glutaraldehyde in cacodylate for 1 to $1\frac{1}{2}$ h; (b) frequent rinsing with 0.2 M cacodylate buffer, pH 7.2 during 1 h, then washing overnight with at least 6 changes of the buffer solutions; (c) dehydration in 70% ethanol until supernatant was clear, followed by 95% ethanol for 30 min and 6 changes of absolute ethanol, 20 min each; (d) continuation of dehydration at room temperature with 3 changes of absolute ethanol, 20 min each; (e) clearing in benzene and embedding in paraplast as described above.

Immunocytochemical Procedures. All procedures were carried out at room temperature. 5 to 7 μm thick sections were cut, mounted on acetone cleaned slides, deparaffinized in xylene and passed from absolute ethanol into PBS. Prior to incubation with antibodies, endogenous peroxidases

Table 2. Results of nonspecific staining (NSS)

Factors responsible for NSS	Incubation step		Mucosal cells	Muscularis + muscularis mucosa connective tissue	Stroma	Plasma cells	Leuco-cytes Mono-cytes
	first layer	second layer					
Endogenous peroxidase							
EA	Ø ^a	Ø	0	0	0	0	0
FPG	Ø	Ø	0	0	0	0	+
Conjugated anti-IgG							
EA	Ø	Ab-P ^b	(+)	+	+	+	0
FPG	Ø	Ab-P	0	++ ^d	0	0	0
Protein-protein nonimmune IgG							
100 µg/ml							
EA	IgG ^c	Ab-P	+	+++	++	++	
FPG	IgG	Ab-P	0	++	0	0	
10 µg/ml							
EA	IgG	Ab-P	(+)	(+)	+	+	
FPG	IgG	Ab-P	0	+	0	0	

^a Ø = PBS

^b Ab-P = peroxidase conjugated anti-rabbit or goat IgG

^c IgG = nonimmune purified immunoglobulin from the rabbit and goat

^d Muscularis mucosae only

in tissue sections were inhibited by treatment with 1% H_2O_2 in PBS for 60 min. Slides were then rinsed in PBS. The indirect staining method was employed throughout. Incubations were first performed with unlabelled immunoglobulin fractions or with isolated antibodies for 20 min, then with peroxidase labelled anti-globulins. Non reacting proteins were eliminated by 3 successive washings for 5 min each in PBS after each incubation step.

Peroxidase activity was revealed by incubation in 3,3'-diaminobenzidine (Merck, Germany), 0.5 mg/ml in 0.2 M Tris-HCl buffer, pH 7.2 containing 0.01% H_2O_2 (Graham and Karnovsky, 1966). After washings in cacodylate buffer, slides were osmicated for 1 to 2 min in 0.1% OsO_4 when necessary, then dehydrated and mounted under coverglass.

Immunocytoological Controls. Endogenous tissue peroxidases were revealed by direct reaction of deparaffinized sections in Graham and Karnovsky's medium without prior incubation in immunological reagents. The inactivation of tissue endogenous peroxidases was controlled by the same procedure, but after H_2O_2 treatment.

NSS resulting from protein-protein interaction was tested by different concentrations (0.5, 0.1 and 0.01 mg/ml protein) of purified rabbit and goat IgG (Miles-Yeda Ltd.) and of DEAE fractions (0.04 M, pH 7.0) of normal rabbit and goat serum used as the initial reagent. USS resulting from cross-reacting antibodies of conjugated antiglobulins was tested with PBS as first reagent followed by the conjugate (Table 2).

Specific, defined immunocytoological staining was proved by reaction of sections with immunsera fractions/antibodies which were absorbed by solid immuno-adsorbents containing the specific antigen. Organ specificity of antibodies used was tested by human organs fixed and embedded by the two techniques. Other controls, (comparison, using the mucosa of the body and antrum as well as normal and pathological gastric mucosa) were performed.

Routine histology was performed with H.E., PAS and alcian blue staining at pH 1.0 and pH 2.5. Stained sections were examined under bright field illumination and selected areas were photographed using Ilford 18 DIN, PANF and Agfachrom professional 50 L, 18 DIN.

Results

Application for Practical Use

Method EA needs 15 to 24 h for fixation and a further period of 8 h for final processing of specimens. It can thus be performed in two working days. Because fixation can be prolonged up to 48 h, the EA procedure can be started before weekends. Method FPG necessitates 3 h for fixation, an overnight period with changes of buffer and subsequently 6 to 8 h for embedding. Hence this method can be performed within 2 days.

Hematoxylin-eosin staining (Fig. 1): Tissue shrinkage of parenchymal and interstitial cells is more intensive in EA than in FPG and classical routine formalin fixative. Contrast and resolution of nuclear membrane and chromatin are better in EA, whereas in FPG chromatin shows clumping. The basophilic staining of the cell nucleus is more intense in FPG. Cytoplasm and zymogen granules are more homogeneously distributed in EA with low eosinophilic staining, whereas in FPG marked granular distribution and strong eosinophilic staining were observed.

PAS staining: In FPG, surface epithelium, neck cells, goblet cells, duodenal Brunner cells and goblet cells in intestinal metaplasia stain more intensely than in tissue treated by EA.

Alcian blue staining material in goblet cells in intestinal metaplasia resulted in similar staining with both methods.

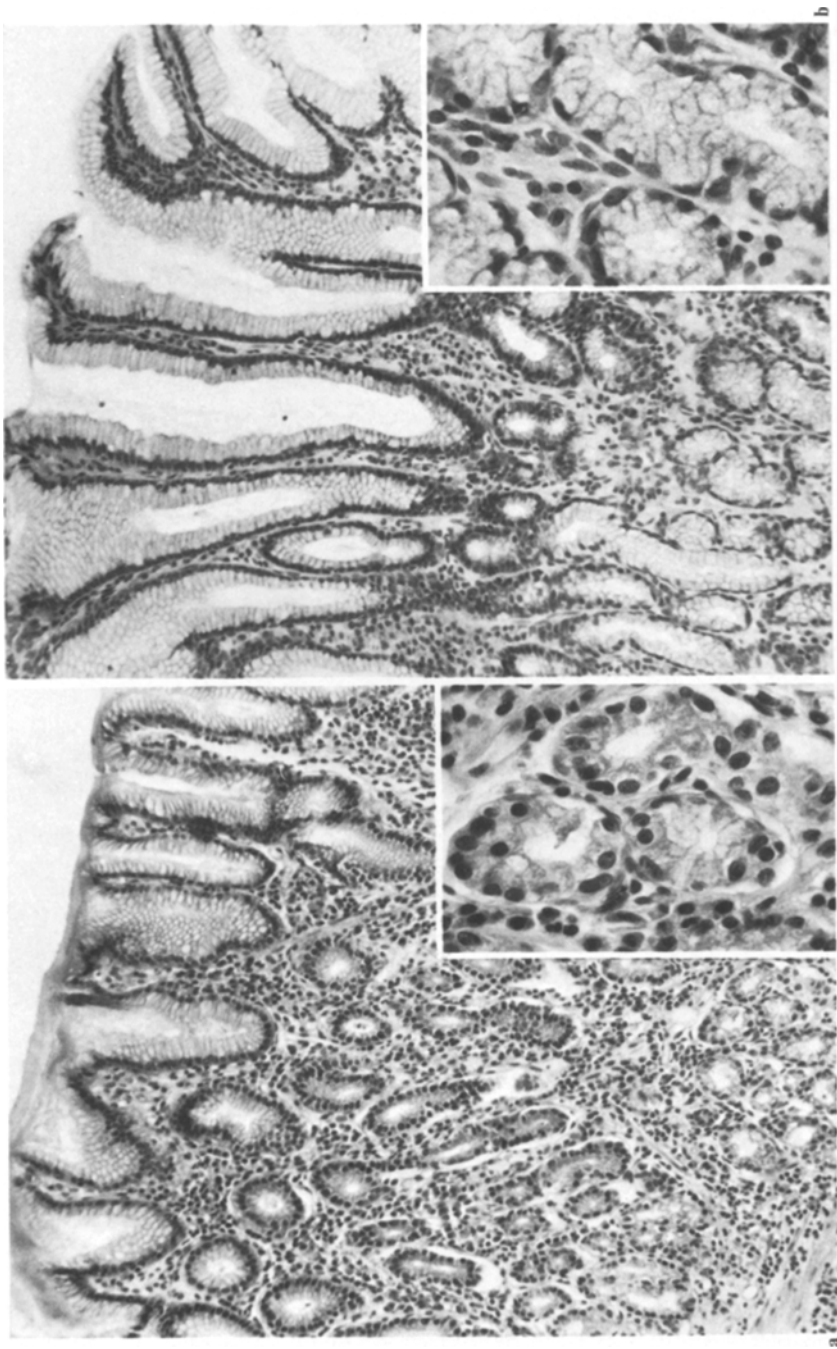


Fig. 1 a and b. Human stomach, antrum. Comparison of H.E. staining. Original $\times 540$. **a** Ethanol, acetic acid fixation method. Note tissue shrinkage when compared with Figure 1 b. **b** Formaldehyde-pyric acid-glutaraldehyde fixation method

Connective tissue, muscularis mucosae and smooth muscle fibers showed comparable staining in both methods.

Nonspecific Staining (Table 2)

Endogenous peroxidase activity was observed in leucocytes and monocytes before and after inhibition with H_2O_2 in sections obtained by method FPG. No endogenous peroxidase activity was observed in method EA.

NSS by conjugated antiglobulin reagent at working dilution of 1/20 was observed in the muscularis mucosae and in connective tissue in both methods. In EA weak NSS staining was observed in the stroma, at the cell bases of gastric cells and in the cytoplasm of plasma cells. Some batches of conjugated antigoat globulin produced minimal staining of the cell nucleus of gastric and interstitial cells. In tissue sections obtained by the FPG method NSS was confined to the muscularis.

NSS resulting from protein-protein interactions as tested with purified rabbit and goat IgG was less in FPG than in EA. In the EA method NSS by IgG was observed at high protein concentration in all gastric tissue compartments and cells with the exception of the mucus layer and the cytoplasm of the surface lining cells. At low protein concentration low NSS was confined to the muscularis and stroma.

In the FPG method NSS by IgG was limited to the muscularis, to leucocytes and monocytes of the stroma. Surprisingly low NSS was observed when whole normal human plasma was used. When normal rabbit and goat immunoglobulin fractions were used NSS comparable to IgG was observed.

Defined Specific Staining of Gastric Constituents

Among the five systems of "autoantigens" only the parietal cell and mitochondrial antigen could be demonstrated by both methods. The other autoantigens of smooth muscle, reticulin and nuclear material no longer reacted when the tissue was treated by both methods. Parietal cell antigen was observed in the parietal cells (Fig. 2) whereas mitochondrial antigen was found in parietal and chief cells. In FPG parietal cell antigen was less intense and showed a more granular cytoplasmic pattern whereas in EA the staining was much stronger and homogeneous (Fig. 2).

Immunoglobulins of the classes IgG, IgM and IgA were best demonstrated by method EA which resulted in a more diffuse cytoplasmic staining. By method FPG a more granular cytoplasmic distribution pattern was obtained. A typical example of IgA staining is given in Figure 3. USS of IgG and IgA was observed in the stroma of some specimens.

Pepsinogen group I and II as produced by the chief and neck cells of the corpus stained in a homogeneous cytoplasmic pattern in tissue treated by EA (Fig. 4a). Method FPG resulted in a more coarse, granular intracytoplasmic

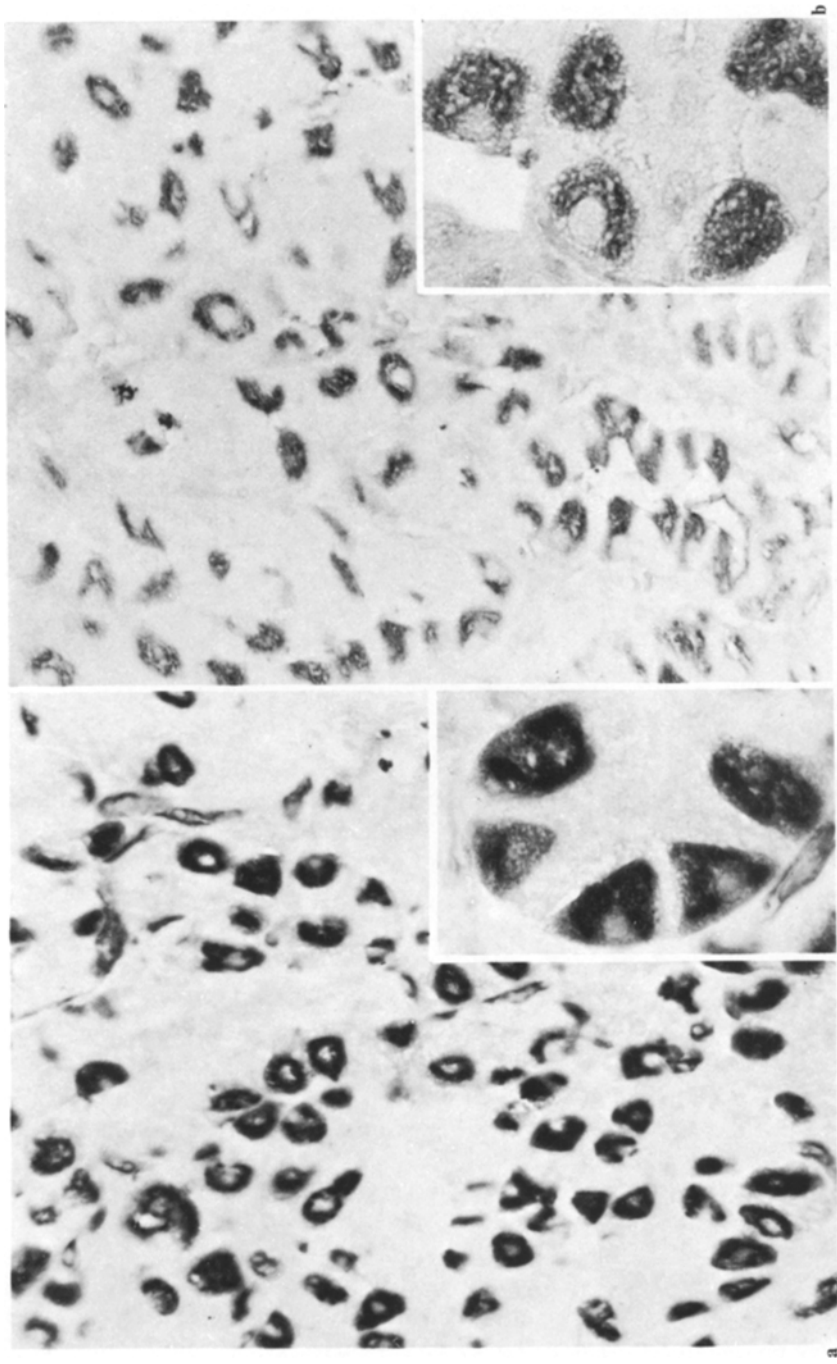


Fig. 2a and b. Demonstration of parietal cell antigen with human serum containing autoantibodies directed against parietal cells. Stomach, fundus. Staining of parietal cells. Original $\times 250$. Inset: $\times 540$. **a** Ethanol, acetic acid fixation. **b** Formaldehyde-picroic acid-glutaraldehyde fixation method

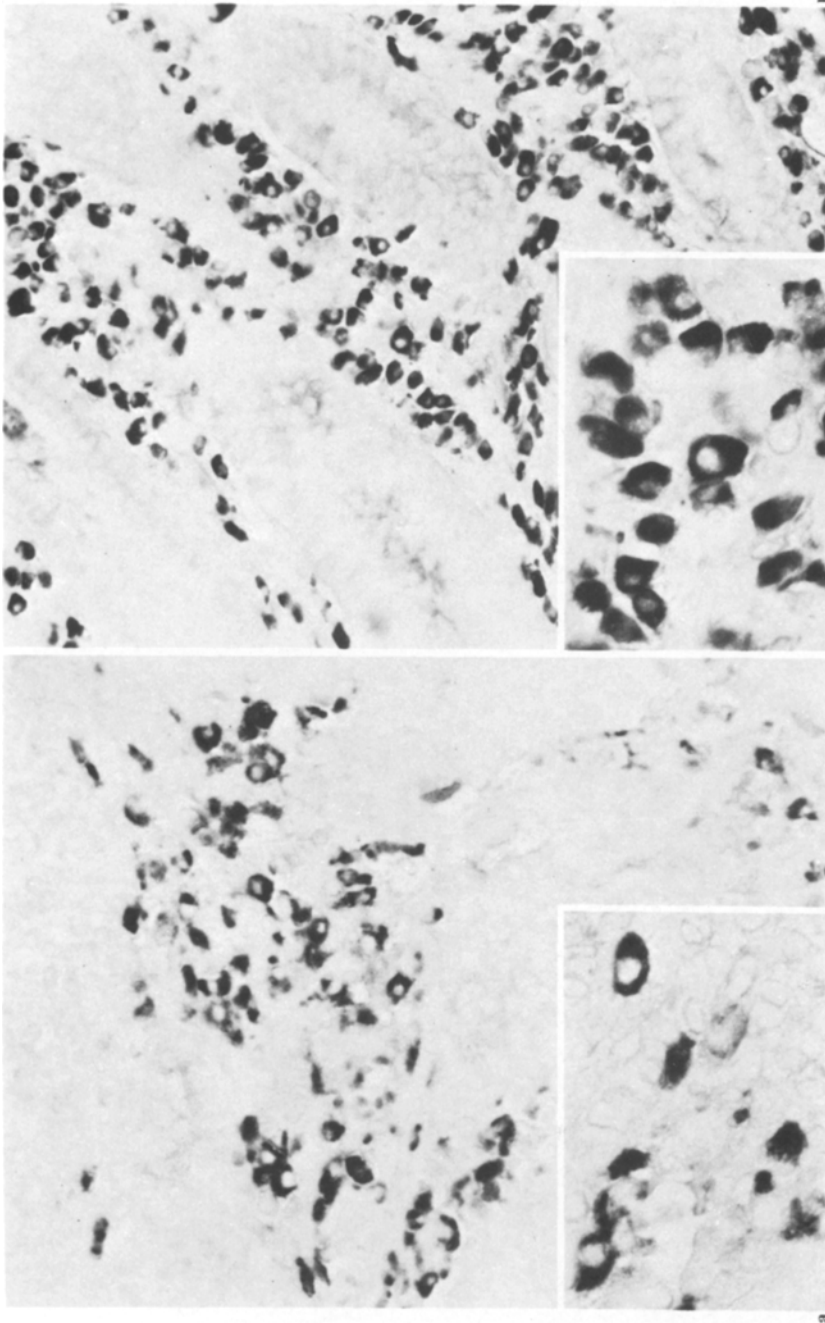


Fig. 3a and b. Demonstration of IgA (heavy chain specific) producing plasma cells in stroma of fundal mucosa in gastritis. Original $\times 250$. Inset: $\times 540$. **a** Ethanol, acetic acid fixation. Note minimal background staining of gastric cells. **b** Formaldehyde-picric acid-glutaraldehyde fixation method. Note less pronounced nonspecific staining as compared to Figure 3a

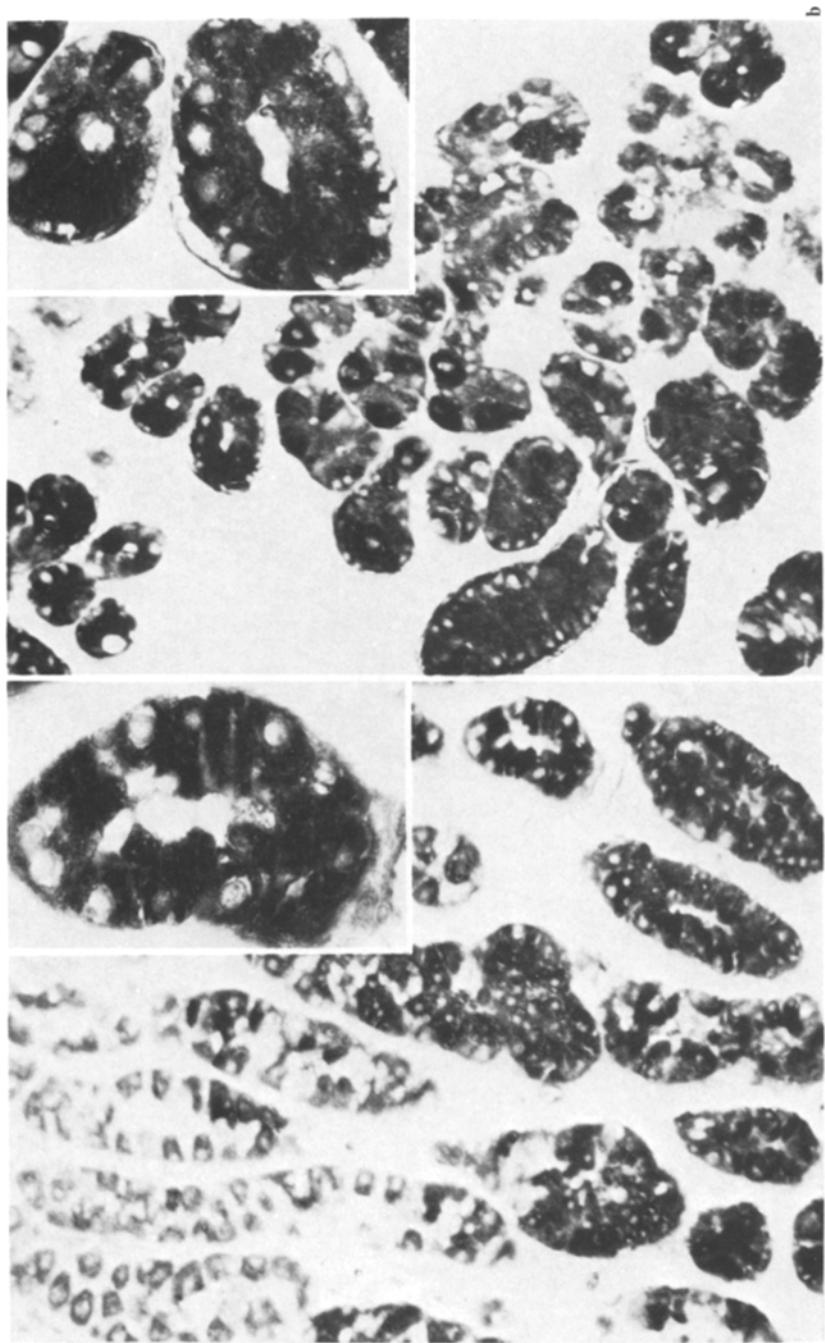


Fig. 4a and b. Demonstration of pepsinogen I group in chief and neck cells of fundal mucosa. Original $\times 540$. **a** Ethanol, acetic acid fixation. Slight nonspecific staining of stroma. Diffuse specific staining of cytoplasm. **b** Formaldehyde-picric acid-glutaraldehyde fixation method. Specific granular staining of cytoplasm

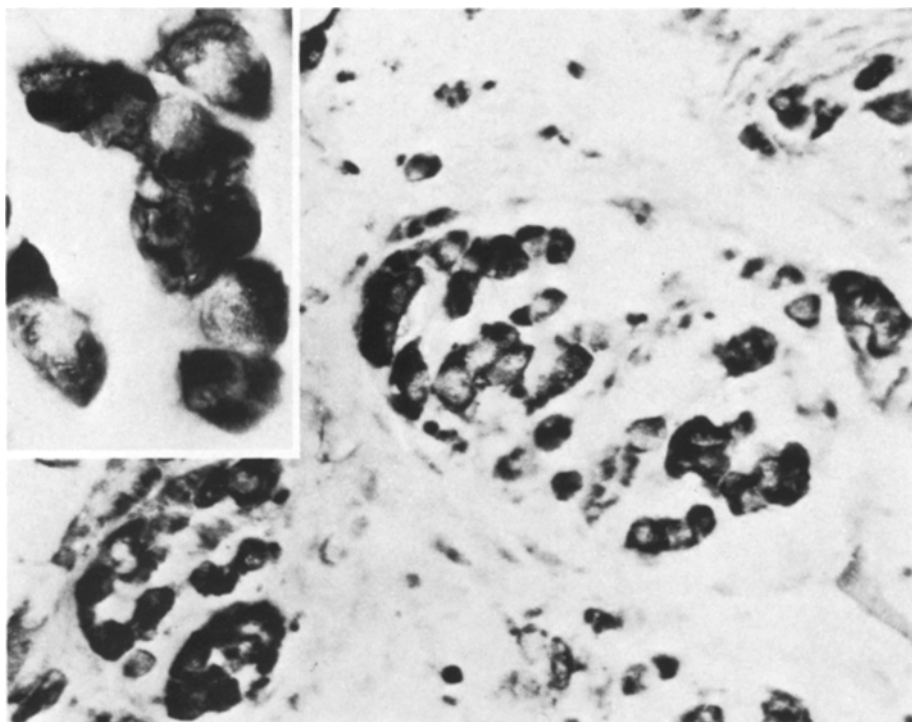


Fig. 5. Demonstration of carcinoembryonic antigen (CEA) in signet ring cell carcinoma. Original $\times 160$. Inset: $\times 540$

pepsinogen distribution pattern (Fig. 4b). Pepsinogen II produced by antral cells was equally well demonstrated by both methods.

Chief cell esterase antigen VI A was shown by both methods in chief cells of the corpus. Method FPG resulted in a more granular pattern of this antigenic enzyme.

Surface epithelial cell antigen was demonstrated with both methods in epithelial and neck cells, the EA method resulting in a brighter staining.

A perchloric acid stable glycoprotein was found with both methods in the mucus producing antral cells and in Brunner's glands of the duodenum.

The best method for the demonstration of classical CEA in CEA-positive gastric tumor cells and in atypical cells was the method EA (Fig. 5). CEA staining in tissue sections obtained by method FPG was less brilliant. Common site antigen was best demonstrated in tumour cells and leucocytes by method EA. Method FPG resulted in a much weaker staining of common site antigen and interference of endogenous peroxidases was observed.

Discussion

Enzyme conjugated antisera have been used on cryostat or paraffin-embedded tissue sections with great success for the demonstration of low molecular weight endocrine peptides. Far more problems are encountered when proteins of high

molecular weight and complex immunochemical structures are demonstrated in immunohistochemistry. For any protein or other high molecular weight substance specific fixation procedures have to be chosen to avoid loss of antigenic reactivity.

By this study we have demonstrated that appropriate fixation and embedding methods can be used successfully for combined immunoenzyme histochemistry and classical staining procedures on serial paraffin sections of human gastric tissue. Various cell specific gastric protein markers as well as immunoglobulins were well preserved in their antigenic structure and in their original cell environment. The application of classical staining techniques to serial sections of the same block of tissue demonstrated a well preserved morphological texture of the gastric tissue as compared to routine formalin fixatives. Immuno-histological preparations were obtained which are permanent and which can be examined and photographed under bright field illumination. In this respect we consider the IE techniques as useful alternatives for immunofluorescent methods with their well-known disadvantages, such as fading, indistinct morphology and time consuming photography.

One major problem in the use of peroxidase conjugated antibodies resides in peroxidative activities, e.g. in macrophages, neutrophils, eosinophils, erythrocytes and even in intestinal cells, which react with the histochemical substrate (DAB-H₂O₂) giving rise to strong staining (for further references see Robinson et al., 1975). These difficulties were overcome in the EA method only, by treatment of sections with concentrated H₂O₂ prior to immunocytochemical incubation.

NSS, caused by protein-protein interactions and depending on the type of fixation procedure used is a major problem in immunohistochemistry: as stated by Ploem (1975), antigen-antibody binding "are in essence not different from the so called non-specific protein-protein interaction" occurring between immuno-chemically unrelated and inactive proteins. We have shown that NSS is dose dependant and can be overcome by the use of purified antibodies obtained from solid immuno-adsorbent columns. An increase of specificity and a decrease of protein-protein interactions was obtained in this way. We anticipate that further progress will be achieved using purified anti-IgG antibodies for enzyme conjugation (Kuhlmann, 1977).

USS was observed with peroxidase labelled immunoglobulin from the goat which reacted with the cytoplasm of plasma cells. Whether this reaction is due to cross-reacting antibodies of the conjugated globulins or to rheumatoid factor like immunoglobulins in gastric plasma cells remains to be examined.

A particular problem in fixation techniques resides in the susceptibility of the structure and arrangement of antigenic determinants on the molecules under study. Both fixatives and embedding techniques employed in this study conserved the antigenic structure of most gastric marker proteins with exception of the less "hardy" autoantigens of smooth muscle, reticulin and of nuclear origin.

A large part of our effort in immuno-histochemistry must be devoted to the immunochemical characterization of antibodies and of their specificity, cross-reactivity and avidity. During this study we have observed that the IE method is 50–100 times more sensitive than the most sensitive crossover electroimmuno-

diffusion technique. For this reason even a purified antibody should be tested at the highest protein concentration available in these techniques. In subsequent studies we plan to apply radio-labelling of antibodies and autoradiography in gel diffusion prior to IE. Analytical methods employed for control of mono-specificity of antibodies should be in the same range of sensitivity as the IE methods.

The advantage of the EA method, our method of choice, is its suitability for routine application, the weak eosinophilic staining of the cytoplasm and the well preserved nuclear structures upon HE-staining. In the EA method suppression of endogenous peroxidases were achieved by substrate inhibition. Most gastric protein markers mentioned in this study were well preserved by the EA fixative and resulted in a "fair immunological contrast" upon IE staining. The disadvantage of the EA method as compared to the FPG method was a nonspecific staining due to nonimmunological protein-protein interactions in all tissue compartments at the high protein concentration of the first reagent. This resulted in a more or less undesired background staining. However, this could be overcome by the use of low concentrations of purified antibodies.

The FPG method is for the moment less appropriate for routine application unless automatic devices can be used. The great advantage of the FPG method is a low NSS, giving rise to an excellent "immunological contrast". In gastric tissue treated with the FPG method we observed a coarse granular pattern of cytoplasm, zymogens, mucosubstances and nuclear structures in both IE and in classical staining. Some antigenic proteins such as CEA, CSAg and immunoglobulins showed less staining when compared with the EA method. The disadvantage of the FPG method consists in retaining considerable amounts of peroxidative activities in various cell types. The EA and FPG method proved to be far superior in respect to their low NSS, antigenic preservation and localization when compared with gastric tissue fixed with buffered and unbuffered formalin (unpublished).

Various organ-specific or disease related antigenic markers have been identified during the last ten years and highly specific antisera/antibodies are available. Most of these markers can be demonstrated by either of the two methods described in this paper using the IE techniques. Future research work has to foster on fixatives or combination thereof which will conserve the advantages of both methods described in this paper.

The immunochemical identification, purification and immunohistochemical demonstration of gastric antigenic marker substances can be considered to be a multidisciplinary comprehensive approach which results in a better understanding of the structure and function of an organ. Since immunological methods display an extreme sensitivity and specificity, immunogenic and antigenic substances can be studied in molecular, genetic, histological and clinical reference systems, thus filling the gap between classical morphology, molecular biology and clinical data. We anticipate that this comprehensive approach will be useful for the study of other human organs.

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