

Immunochemical studies of the endocrine cells of the gastrointestinal tract

II An immunoperoxide technique for the localization of secretin-containing cells in human duodenum

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SYNOPSIS Endocrine cells containing secretin have been identified in the epithelium lining human duodenum by direct and indirect immunoperoxidase techniques using immune sera raised against pure natural secretin. The techniques were applied to sections of carbodiimide-fixed tissue embedded in polyethylene glycol. Some sections, stained by a modified indirect technique, were processed for electron microscopy; secretin-containing granules were present but ultrastructural preservation was too poor to be of value. The potential advantages of a peroxidase technique over fluorescein-conjugated antisera are discussed.

Many cells in the epithelium lining the gastrointestinal tract have been shown to have an endocrine function. The localization and characterization of the hormones secreted have been determined by a number of techniques which include bioassay and radioimmunoassay of gut extracts, correlated cytochemical and electron microscope studies, and the application of specific immunochemical procedures. Although these endocrine cells have been intensely studied using all of these techniques, it is only recently that the usefulness of specific immunochemical procedures has become fully appreciated. The use of specific antisera directed against the various hormones present in the gastrointestinal tract will undoubtedly play an important role in establishing the precise localization of particular hormones in individual cells.

Gastrin-containing cells have already been the subject of much research using fluorescent immunochemistry (McGuigan, 1968; Polak, Coulling, Doe, and Pearse, 1971c; Bussolati and Canese, 1972). However, other members of the gastrointestinal endocrine cell population initially proved difficult to study in this way since the fixatives in common use apparently affected the antigenic determinant sites of the hormone molecules. It was not until Kendall, Polak and Pearse (1971) showed that the antigenicity of some gastrointestinal hormones was

retained in tissue fixed in other fixatives, including carbodiimide, that immunochemical methods could be used to localize hormones other than gastrin.

The introduction of immunoperoxidase techniques provided a useful alternative to fluorescent methods for localizing hormones as the end product can be visualized at both light and electron microscope levels. Gastrin-containing cells have already been localized at the light microscope level (McGuigan and Greider, 1971; Piris and Whitehead, 1974; Robinson and Dawson, 1975) and at electron microscope level (Greider, Steinberg, and McGuigan, 1972) using these techniques.

This paper reports the localization and characterization of secretin-containing cells in human duodenum by the application of immunoperoxidase techniques to carbodiimide-fixed tissue.

Material and Methods

Samples of duodenal mucosa obtained from two patients who came to surgery for carcinoma of the pancreas were fixed for three hours at 4°C in freshly prepared 10% 1 ethyl-3(3 dimethyl-amino-propyl)-carbodiimide hydrochloride (Sigma) buffered to pH 7.2 with 0.12 M Millonig's phosphate buffer containing 3% sucrose. After fixation the tissue was washed in three 20-minute changes of 0.12 M Millonig's phosphate buffer containing 5% sucrose. The tissue was then dehydrated in a graded

ethanol series, soaked in 100% polyethylene glycol 1000 (Fluka AG) for two one-hour changes in a vacuum oven at 40°C, embedded in fresh 100% polyethylene glycol, and hardened at 5°C in a refrigerator.

Sections were cut at 5µm, floated on a 5% glycerol solution for one hour, and picked up on egg albumin-coated slides. Excess glycerol was drawn off with tissue paper and the slides were dried on a warming tray at 45°C for at least 30 minutes (Mazurkiewicz and Nakane, 1972).

PREPARATION OF IMMUNE SERA

The antiserum (a gift from Dr K. D. Buchanan, Department of Medicine, Queen's University of Belfast) was raised in New Zealand white rabbits against a conjugate of pure natural secretin and ovalbumin. The specificity of the antiserum was checked by the double gel diffusion method of Ouchterlony and by immunoelectrophoresis. It showed no detectable cross-reaction with gastrin, intestinal or pancreatic glucagon. The globulin fraction was obtained by precipitation with cold 40% saturated ammonium sulphate following the procedures outlined in a previous paper (Robinson and Dawson, 1975). The protein content of the globulin fraction was determined by the Biuret method and the fractions were stored undiluted at -20°C until required.

IMMUNOLOGICAL STAINING

After a brief wash in physiologically buffered saline (PBS), sections were stained by either the direct or indirect technique.

In the direct method, sections were exposed for 30 minutes at room temperature to peroxidase-conjugated antiseoretin antibodies, diluted 1:10 with PBS and prepared by the technique outlined by Avrameas and Ternynck (1971). After incubation, sections were washed for 45 minutes in several changes of PBS and then stained for peroxidase activity using 5 mg of 3,3'-diaminobenzidine tetra-HCl (DAB) dissolved in 10 ml of 0.05 M Tris/HCl buffer, pH 7.6, containing 0.01% freshly prepared hydrogen peroxide (Graham and Karnovsky, 1966). Sections were stained for 5 minutes at room temperature. After washing in several changes of PBS, sections were mounted in phosphate-buffered glycerol.

In the indirect method, sections were first flooded with unconjugated rabbit antiseoretin antibodies, diluted 1:8 with PBS for 30 minutes at room temperature and then, after a 45-minute wash in several changes of PBS, incubated for a further 30 minutes at room temperature in peroxidase-labelled swine antirabbit IgG (Dakopatts) diluted 1:10 with

PBS. After washing, peroxidase activity was visualized by incubation for 5 minutes in a DAB medium prepared as before. Sections were then washed in PBS and mounted in phosphate-buffered glycerol.

Control sections were carried out in each experiment and included the following: (1) incubation in antiseoretin antibodies before application of peroxidase-conjugated antiseoretin antiserum; (2) incubation in swine antirabbit IgG before the application of peroxidase-conjugated swine antirabbit IgG; (3) incubation in antiseoretin antibodies to which excess pure natural secretin had been added followed by incubation in peroxidase-conjugated swine antirabbit IgG; (4) incubation in irrelevant conjugated antibodies. In addition, to localize endogenous peroxidase activity some sections were incubated in the DAB medium only.

For photography, sections were counterstained in either 1% methylene blue, 1% light green, PAS, or 1% osmium tetroxide for 30 seconds. Although osmium tetroxide increased the background staining, it proved to be the most useful counterstain for photography.

ELECTRON MICROSCOPY

Some sections, cut at 8µm and stained by the indirect method using incubation times of 60 minutes to ensure complete penetration of the sera, were processed for electron microscopy using the technique described by Mazurkiewicz and Nakane (1972). After immunochemical staining the sections were postfixed for one hour in 3% glutaraldehyde buffered to pH 7.2 with 0.12 M Millonig's phosphate buffer, washed in 0.12 M Millonig's phosphate buffer, and then exposed to 1% osmium tetroxide buffered to pH 7.2 with Millonig's phosphate buffer for 30 minutes. After a brief rinse in distilled water, sections were dehydrated in ethanol and impregnated with Araldite resin. Embedding was accomplished by inverting a gelatine capsule filled with fresh resin over the appropriate area and polymerizing the resin at 60°C for 48 hours. Cured blocks were separated from the slides by immersion in liquid nitrogen. After trimming, thin sections were cut on an LKB III ultratome, mounted on copper grids, and viewed initially without additional heavy metal staining and subsequently double stained with uranyl acetate and lead citrate in a Phillips EM 300 electron microscope.

Results

Staining of sections by both the direct and indirect techniques clearly demonstrated the presence of cells containing secretin. Labelled cells were localized

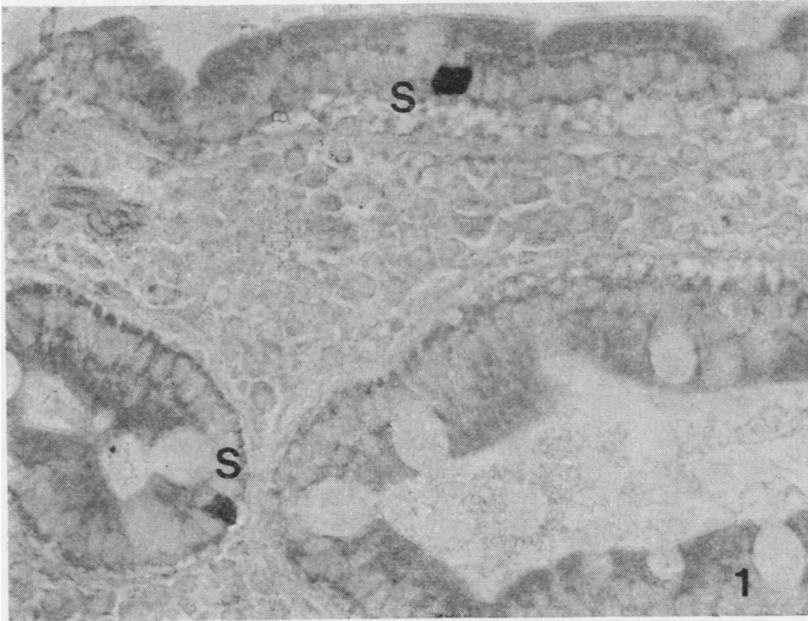


Fig 1 *Indirect immunoenzyme preparation showing secretin-containing cells (S) in lower region of a villous and in a gland. Section counterstained in 1% osmium tetroxide. × 455*

mainly in the epithelium lining the lower portions of villi and upper regions of the crypts (fig 1). A few positive cells were found near the apex of the villi and in the deeper parts of the crypts (fig 2).

The cells were predominantly pyramidal in shape and the same cell was often in contact with both the

basal lamina and lumen (fig 1). The reaction product for peroxidase label was always localized in the basal half of the cell.

No positively stained cells were found in the mucosal epithelium in sections which had been incubated in antibody solution containing excess



Fig 2 *Direct immunoenzyme preparation showing a secretin-containing cell (S) near the apex of a villous. Section counterstained in 1% osmium tetroxide. × 665*

natural secretin, in irrelevant peroxidase-conjugated immune sera, or in the DAB substrate only. Staining was absent or very much reduced in intensity in sections pre-treated with the appropriate unconjugated immune sera before incubation in conjugated serum.

Sections stained by the modified indirect immunoperoxidase procedure and processed for electron microscopy failed to produce interpretable results. Granules giving a peroxidase reaction were visible, but ultrastructural preservation was so poor that it was impossible to determine with accuracy which particular cells contained the reaction product. Their possible identity could be deduced only from the position within the tissue. Further work is in progress to improve ultrastructural preservation.

Discussion

The majority of recent researches aimed at establishing which particular gastrointestinal endocrine cell contains which polypeptide hormone have used specific immunochemical staining procedures with fluorescein-conjugated antisera in direct or indirect techniques (gastrin: McGuigan, 1968; Bussolati and Pearse, 1970; enteroglucagon: Polak, Bloom, Coulling, and Pearse, 1971a; secretin: Bussolati, Capella, Solcia, Vassallo, and Vezzadini, 1971; Polak, Bloom, Coulling, and Pearse, 1971b; vasoactive intestinal polypeptide, gastric inhibitory polypeptide, and motilin: Pearse and Polak, 1974). Fluorescein-conjugated antisera give excellent results at light microscopic level but have certain disadvantages: preparations are not permanent, histological detail of surrounding tissue is not easily studied without restaining, a high quality fluorescent microscope is required, and, most important, since fluorescein is not electron dense, linked electron microscope studies must be done on sequential serial sections (Bussolati and Canese, 1972). For these reasons we have preferred the use of peroxidase-conjugated antisera, when the end result is visible in the light microscope as a brown black deposit, histological counterstains can be applied, and, since the end product is also electron dense, the same sections should be available for preparing electron microscopic ultrathin sections which will also show antiserum localization.

A variety of immunoperoxidase procedures has already been applied to studies on the distribution of hormones occurring in the adeno-hypophysis and pituitary glands. These studies have used either direct and indirect immunoperoxidase techniques (Mazurkiewicz and Nakane, 1972) or the peroxidase antiperoxidase procedure (PAP) (for a review see Moriarty, 1973). Of the polypeptide hormones

occurring in the gastrointestinal tract only gastrin has been identified at the electron microscope level using an immunoperoxidase technique (Greider *et al*, 1972). Attempts by several independent groups of workers to localize other gastrointestinal hormones using immunoenzyme techniques have so far been unsuccessful. The failure is perhaps due partly to problems encountered in retaining the antigenicity of some polypeptide hormones in fixed tissue. Since the antigenicity of several of these hormones is not retained in tissue preserved in fixatives in normal use, the specimen is generally fixed in carbodiimide (Kendall *et al*, 1971). Although ultrastructural preservation is reasonable in specimens fixed in carbodiimide and processed for routine electron microscopy, we found that carbodiimide-fixed and polyethylene glycol-embedded sections processed for electron microscopy using the method developed by Mazurkiewicz and Nakane (1972) produced uninterpretable results. The ultrastructure of the tissue was altered to such a degree that meaningful observations could not be made. The application of the PAP method may prove more successful as this can be carried out directly on ultrathin sections mounted on grids. Until such research is completed, the only available method of determining the ultrastructural appearance of cells which stain for adsorbed peroxidase activity at the light microscope level is the application of sequential sectional procedures similar to those developed by workers using immunofluorescent techniques (Bussolati and Canese, 1972).

At the light microscope level immunoperoxidase techniques are a useful alternative to immunofluorescence as the results are permanent and prepared slides can be examined under bright-field illumination. Although parallel immunofluorescent studies were not undertaken, the results obtained using immunoperoxidase techniques compare very favourably with those reported by researchers using fluorescein-conjugated antisera (Bussolati *et al*, 1971b; Polak *et al*, 1971). The distribution of positive cells within the duodenal mucosa, their shape, and the basal localization of the secretory product agree with the results reported by these workers.

As cells containing endogenous peroxidase were encountered only rarely in the duodenal mucosa, and since their morphology could easily be distinguished from that of cells containing adsorbed peroxidase, double staining of the preparation with 4-Cl-1-naphthol and DAB was not required (Robinson and Dawson, 1975).

Pearse and Bussolati (1970), Polak *et al*, 1971c; Polak, Stagg, and Pearse (1972), Polak, Pearse, van Noorden, Bloom, and Rossiter (1973) have all

shown that immunofluorescent preparations can have a diagnostic value. The successful development of immunochemical staining techniques based on peroxidase-conjugated antisera will now allow a more direct comparison between conventional haematoxylin and eosin preparations and immunochemically stained slides of the same specimen. In addition, if the various gastrointestinal hormones can be localized successfully by the application of peroxidase-antiperoxidase methods, since this technique in certain circumstances is more sensitive than radioimmunoassay and can detect small concentrations of antigen (Moriarty, Moriarty, and Sternberger, 1973), it may be possible if early changes in distribution and levels of hormone take place to observe these in immunoenzyme preparations before morphological characteristics of malignancy become evident in conventional histological preparations.

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