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Carcinoid granule extract contains antigenic determinants common to peptide hormone-producing cells and endocrine tumours

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Summary. Some soluble proteins, such as the chromogranins, are found in nearly all peptide hormone-producing cells. Little is known about their functional role, although they may act as enzymes or represent structural proteins. In the present study we have isolated granules from an ileal carcinoid tumour and raised antibodies to protein constituents within them. The antiserum proved to be useful for the immunohistochemical demonstration of peptide hormone-producing endocrine tumours in general and for the demonstration of most peptide hormone-producing cells.

Key words: Carcinoid granule – Antigenic determinants – Peptide hormone-producing cells – Endocrine tumours

Introduction

Secretory granules in peptide hormone-producing cells contain not only peptide hormones but also soluble and insoluble proteins. Some soluble proteins, such as the chromogranins (O'Connor et al. 1983; Angeletti 1986; Lassmann et al. 1986; Rindi et al. 1986), synaptophysins (Navone et al. 1986; Gould et al. 1986) and cystatins (Barrett et al. 1984), appear to be present in high concentrations. Many of these granular proteins occur in virtually all different peptide hormone-producing cell types in the body. Both the chromogranins and the cystatins are among these universally distributed granular proteins (O'Connor et al. 1986; Lassmann et al. 1986; Rindi et al. 1986; Navone et al. 1986; Gould et al. 1986). Little is known of their functional significance. Some of the granular proteins may be enzymes involved in peptide hormone precursor processing; others may play a role in hormone storage; still others may represent structural (matrix) proteins. Recent evidence suggests that some of the chromogranins may themselves be processed by proteolytic cleavages to yield fragments with possible biological activity (Tatemoto et al. 1986; Eiden 1987; Huttner et al. 1988; Simon et al. 1988). In the present study we isolated granules from an ileal carcinoid tumour and raised antibodies to protein constituents within them. The antiserum proved useful for the immunohistochemical demonstration of peptide hormone-producing endocrine tumours in general and for the demonstration of most peptide hormone-producing endocrine cells in the species studied.

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Materials and methods

Production of antiserum. Liver metastases (approx. 1 g) of a tachykinin- and 5-hydroxytryptamine (5-HT)-producing midgut carcinoid were freed of connective tissue, minced and hand-homogenized for a few minutes with a Potter-Elvehjem homogenizer (Teflon pestle) in 20 vol 0.35 M sucrose. These and all subsequent manipulations were carried out at 0° C. The homogenate was centrifuged at 1000 *g* for 20 min in a refrigerated centrifuge (Sorvall Superspeed RC 2-B) and the supernatant was then passed through a series of MF Millipore filters (47 mm diameter) with diminishing pore size: 8000, 5000, 3000, 1000, 800 and 650 nm. The filtrate was centrifuged at 45000 *g* for 30 min (MSE ultracentrifuge). The sediment thus obtained was dispersed in redistilled water, dialysed against redistilled water for 24 h and lyophilized. The freeze-dried material was suspended in 0.9% saline (200 µg/ml) and homogenized to provide a suspension consisting of soluble as well as insoluble granular components.

Two rabbits were immunized by intracutaneous injection of the suspension. Two hundred micrograms of the material in 1 ml 0.9% saline was emulgated with 1 ml Freund's complete adjuvant. Injections were made intradermally at multiple sites on the back of each animal. After 6 weeks antibodies against endocrine cell granule constituents (EGC) could be demonstrated by the fact that tumour cells in paraffin sections of formalin fixed midgut carcinoids (see below) were intensely immunostained. Booster injections were given (200 µg material in Freund's incomplete adjuvant) 6 and 12 months after the priming dose with a consequent improvement of titre. In preliminary tests one antiserum (code no. 7825) was found to produce strong immunostaining of a variety of endocrine cell types. This antiserum was used in the present study (see Table 1).

Tissue specimens. Normal specimens from various parts of the gastrointestinal tract (antrum and corpus of the stomach, duodenum, jejunum, ileum and colon), from the pancreas, from the pituitary (anterior and intermediate lobes), from the thyroid and from the adrenal medulla were collected from guinea-pig, pig and man. Hu-

Table 1. Characteristics of the primary antisera used for immunocytochemistry

Antigen	Code no.	Raised against	Working dilution			
			Species	Immuno-fluor-escence	PAP-staining	Source
ACTH	1	Pure porcine ACTH 1–39	Rabbit	1:80	1:640	Own
Endocrine granule constituent (EGC)	7825	Extract of granules from a substance P-producing mid-gut carcinoid type	Rabbit	1:80	1:160	Milab (Malmö, Sweden)
Gastrin	M8818	Synthetic gastrin 17	Guinea-pig	1:160	–	Milab
GIP		Pure porcine GIP	Rabbit	1:640	1:5120	Dr. T. O'Dorisio Columbus OH, USA
Glicentin	R64	Pure porcine glicentin	Rabbit	1:800	1:1600	A. Moody, Novo Res. Lab. Bagsvaerd, Denmark
Glucagon	7811	Pure porcine pancreatic glucagon	Rabbit	1:1280	1:2560	Milab
Glucagon	M8708	Pure porcine pancreatic glucagon	Guinea-pig	1:160	–	Milab
Insulin	M8101	Pure porcine insulin	Guinea-pig	1:160	1:320	Milab
Neurotensin	B44	Synthetic bovine neurotensin	Rabbit	1:640	1:5000	Milab
PYY	M8704	Synthetic porcine PYY	Guinea-pig	1:160	1:640	Milab
Secretin	5585	Pure porcine secretin	Rabbit	1:160	1:2560	
Serotonin	YC/45	Serotonin-albumin conjugate	Mouse	1:160	–	Sera lab Crawley Down, UK

GIP = Gastric inhibitory polypeptide; PYY = peptide YY; ACTH = Adrenocorticotrophic hormone

man specimens were obtained at surgery and porcine specimens were obtained from a local slaughter-house. The specimens were frozen in a mixture of propane and propylene, cooled to the temperature of liquid nitrogen, and freeze-dried. They were then fixed by exposure to formaldehyde vapour for 1 h at 80° C or to diethylpyrocarbonate (DEPC) vapours for 4 h at 55° C. Alternatively, specimens were routinely fixed in Bouin's solution. Small specimens from human ileum were immersed overnight in a mixture of 1% glutaraldehyde and 3% formaldehyde in 0.075 M phosphate buffer, dehydrated and embedded in Epon (Polarbed 812, Polaron Equipment, Watford, UK). Ultrathin (50 nm), non-osmicated sections were collected on Formvar-coated gold grids.

Specimens from 53 endocrine tumours were examined: 4 medullary thyroid carcinomas, 15 bronchial carcinoids, 7 gastric, 6 ileal, 3 appendiceal and 4 rectal carcinoids, 9 pancreatic endocrine tumours (2 gastrinomas, 1 glucagonoma, 1 PPoma, 3 insulinomas and 2 without demonstrable peptide content) and 5 phaeochromocytomas. The specimens were obtained at surgery or at autopsy and either frozen, freeze-dried and vapour-fixed as above or routinely fixed in 10% formalin or Bouin's solution. All specimens were embedded in paraffin and sectioned at 6 μm thickness. In addition, small specimens from two phaeochromocytomas were processed for electron microscopy as above.

Immunocytochemistry. Deparaffinized sections were processed for the immunocytochemical demonstration of EGC using the indirect immunofluorescence (Coons et al. 1955) or the immunoperoxidase (peroxidase-anti-peroxidase PAP) technique (Sternberger 1974). In the indirect immunofluorescence technique the sections are exposed to the specific peptide antiserum in the appropriate dilution overnight at +4° C. After thorough rinsing in phosphate buffer they are incubated in fluorescein isothiocyanate (FITC)-labelled anti-rabbit (or anti-guinea-pig) IgG for 30 min at room temperature. After another rinsing in phosphate buffer the sections are mounted in phosphate-buffered glycerin and examined with a fluorescence microscope equipped with filters selected to give peak excitation at 490 nm.

In the PAP procedure the sections are exposed to the peptide antiserum overnight at +4° C. After rinsing in phosphate buffer the sections are incubated in unlabelled goat (or swine, or sheep) anti-rabbit or anti-guinea-pig IgG for 30 min at room temperature followed by incubation for 30 min with PAP complex. This is followed by incubation with a solution of 0.05% diaminobenzidine hydrochloride and 0.01% hydrogen peroxide in Tris buffer (pH 7.6) for 15 min. The sections are mounted in Permount and examined with a light microscope. Simultaneous or sequential double immunostaining (Sundler et al. 1987) was used to identify EGC-immunostained cells with respect to their peptide hormone or 5-HT content. For simultaneous double immunostaining we used the EGC antiserum in combination with peptide hormone antibodies produced in guinea-pig [raised against insulin, gastrin, glucagon or peptide YY (PYY)] or a monoclonal antibody (raised against 5-HT). For details on the antibodies used, see Table 1. Second antibodies (i.e. antibodies to rabbit or guinea-pig IgG) were labelled with FITC or tetramethylrhodamine isothiocyanate. Sequential double immunostaining was performed as described previously (Tramu et al. 1980). Briefly, sections were first exposed to one peptide antiserum and to FITC-labelled IgG antibodies. The slides were examined and photographed. They were then exposed to potassium permanganate for 15–30 s for removal of antibodes. The

completeness of the antibody elimination was tested by application of FITC-labelled IgG antibodies. Sections devoid of immunofluorescence were processed for the demonstration of the second peptide and again photographed.

Immunogold staining for electron microscopic demonstration of EGC was carried out according to Roth et al. (1978). After preincubation with 1% human serum albumin the ultra-thin sections were incubated with a 1:50 dilution of EGC antiserum overnight at 4° C. After extensive rinsing in phosphate-buffered saline (PBS), the sections were incubated for 1 h with goat anti-rabbit IgG-gold (10 nm) solution diluted 1:20 (Peninsula Europe, St. Helens, UK) at room temperature and rinsed in PBS. The sections were contrasted with uranyl acetate and lead citrate, and examined in a JEOL 200 transmission electron microscope. Control sections were incubated with normal rabbit serum.

Results

The EGC antiserum stained numerous cells in the adenohypophysis, thyroid C cells, endocrine cells in the stomach, endocrine cells in the small and large intestines, pancreatic endocrine cells and adrenal medullary cells in the species examined (Fig. 1). Although the antiserum gave positive immunostaining with all fixatives used the staining intensity and the number of immunostained cells differed; generally DEPC vapour fixation was superior to formaldehyde vapour fixation and to routine formalin or Bouin fixation. The results are summarized in Table 2.

In the pancreatic islets, the glucagon cells were invariably intensely immunoreactive (Fig. 2a, b). In the guinea-pig virtually all islet cells were EGC immunoreactive, including the insulin cells. Also in human islets the insulin cells were EGC immunoreactive, although less so than the glucagon cells. In the pig the insulin cells did not diplay EGC immunoreactivity. Numerous endocrine cells in the acid-producing mucosa of the stomach displayed EGC immunoreactivity of varying degree in all species. The majority of them are probably identical with the so-called ECL cells, which is the major endocrine cell population in this location (Håkanson et al.

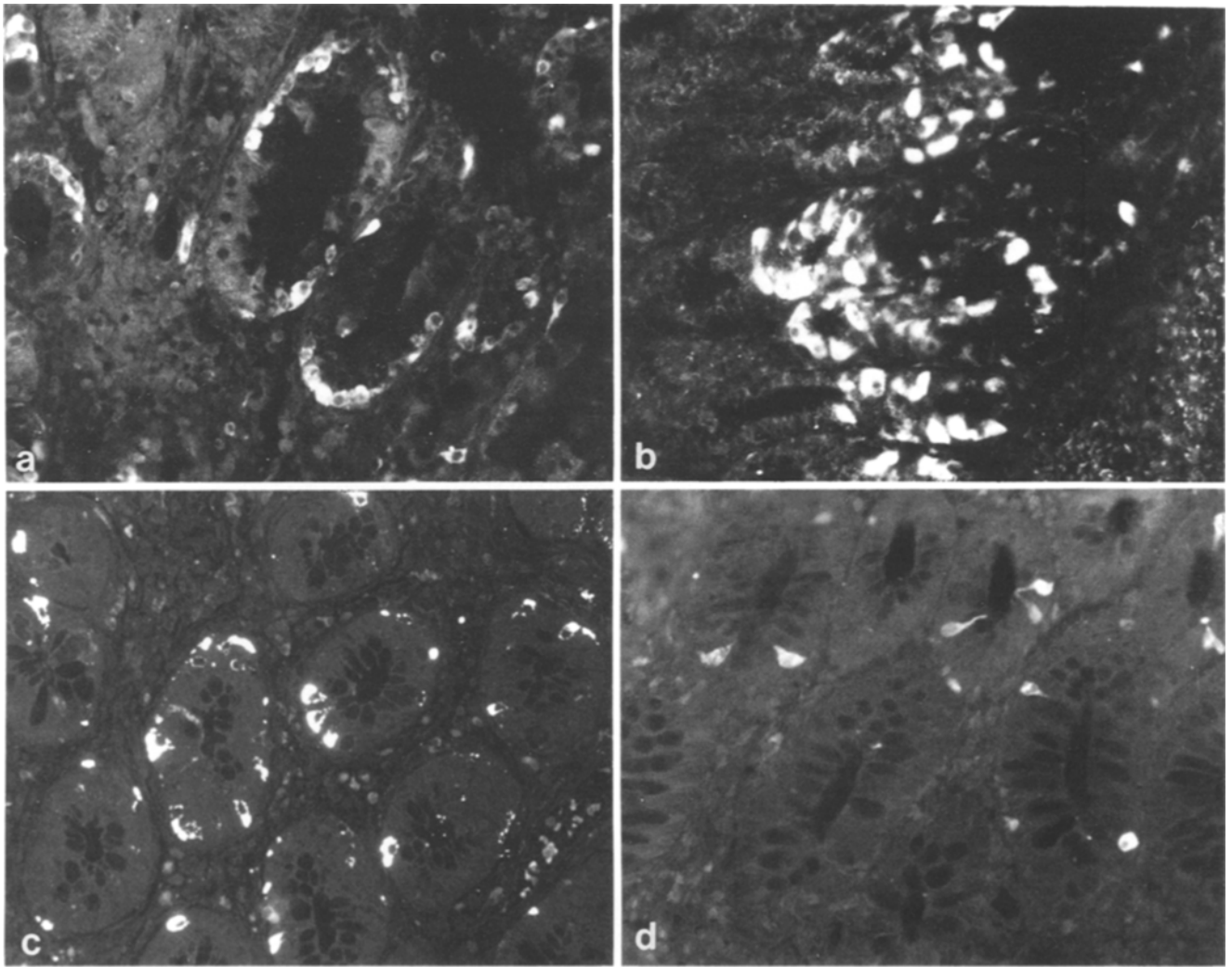

Fig. 1. a Human fundus. Patient with atrophic gastritis and associated ECL cell hyperplasia. Numerous endocrine cells, sometimes forming chains basally in the glandular epithelium, display EGC immunofluorescence. **b** Antrum from the same patient immunostained for EGC (mucosal surface to the right). Numerous endocrine cells including the gastrin cells are immunoreactive. **c** Human ileum. Numerous endocrine cells display EGC immunofluorescence. **d** Human colon. Scattered endocrine cells, some of which are equipped with characteristic apical processes, display EGC immunofluorescence. × 200

Table 2. Intensity of EGC immunostaining of peptide hormone-producing endocrine cells in various tissues of guinea-pig, pig and man

Specimen	PF fixation	DEPC fixation
Guinea-pig		
Pituitary	+	++
Thyroid	++	+++
Corpus	+++	+++
Antrum	+++	+++
Duodenum	+++	++
Jejunum	+	++
Ileum	+	++
Colon	+	++
Pancreas	+	++
Pig		
Pituitary	+	+++
Thyroid	0	0
Corpus	++	+++
Antrum	++	++
Duodenum	0	+
Jejunum	+	+++
Ileum	0	+
Colon	+	++
Pancreas	+	+++
Adrenal medulla	+	++
Man		
Pituitary	+	+++
Thyroid	+	+
Corpus	++	++
Antrum	++	+++
Duodenum	+	+++
Jejunum	+	+++
Ileum	+	+++
Colon	++	+++
Pancreas	++	++
Adrenal medulla	+++	+++

Results are given from tissues fixed either by exposure to formaldehyde (PF) or diethylpyrocarbonate (DEPC) vapours

+ Week immunostaining; ++ moderate immunostaining; +++ intense immunostaining

1986; Simonsson et al. 1988). In the antrum, the bulk of EGC-immunoreactive cells were identical with the gastrin cells and the enterochromaffin cells. In the intestine EGC-immunoreactive cells occurred scattered in the epithelium from the duodenum down to the rectum. In the small intestine the immunoreactive cells predominated in the crypts, although single cells occurred also in the villus epithelium. As shown by double immunostaining the EGC-immunoreactive cells included enterochromaffin cells (Fig. 2c, d), GIP-, CCK-, neurotensin- and secretin-immunoreactive cells in the small intestine and enterochromaffin cells in the colon. Interestingly, the glicentin/PYY cells did not stain with the EGC antiserum (Fig. 2e, f).

Virtually all cells of the adrenal medulla in the species examined displayed EGC immunoreactivity. In the pituitary the EGC-immunoreactive cells included the ACTH cells and MSH cells.

Cells in most of the tumours examined displayed EGC immunoreactivity of varying intensity. The results are summarized in Table 3 and shown in Fig. 3.

Table 3. EGC immunostaining of endocrine tumours

Tumour diagnosis	Number of EGC-immunoreactive tumours/ total number of tumours
Medullary thyroid carcinoma	3/4
Bronchial carcinoid	13/15
Gastric carcinoid	5/7
Ileal carcinoid	6/6
Appendiceal carcinoid	2/3
Rectal carcinoid	4/4
Pancreatic endocrine tumour	8/9
Phaeochromocytoma	5/5

EGC immunostaining at the electron microscopic level revealed at least two different endocrine cell types in the human ileum. One was an enterochromaffin cell type with characteristic pleomorphic, often elongated, electron-dense granules. Gold particles were loaded on the granules; only few gold particles were located outside the granule core (Fig. 4a). Another endocrine cell type that reacted with the antibody had typical large electron-dense, round granules (Fig. 4b). These cells belong to the so-called L cell category. The L cells are known to comprise two different cell populations, one storing neurotensin and another storing glicentin/PYY (Böttcher et al. 1986). The EGC-immunoreactive L cells are probably identical with the neurotensin cells, since they were found to be EGC immunoreactive at the light microscopic level, while the glicentin/PYY cells were immunonegative. The two phaeochromocytomas examined electron microscopically contained cells with numerous highly electron-dense, slightly pleomorphic secretory granules. The granules were intensely EGC immunoreactive as revealed by the accumulation of gold particles (Fig. 4c).

Discussion

During the last few years a number of markers for peptide hormone-producing cells and tumours have been described, and antisera against such markers have become available. Markers that demonstrate endocrine cells collectively aid in the diagnosis of endocrine tumours (Klöppel 1986; Morohoshi et al. 1987). Among such markers are enzymes such as neuron specific enolase (Polak et al. 1984) and aromatic L-amino acid decarboxylase (Lauweryns and van Ranst 1988), and enzyme inhibitors such as cystatin. Other markers probably represent matrix proteins, such as chromogranins (O'Connor et al. 1983; Rosa et al. 1985; Fischer-Colbrie et al. 1985, 1986; Grube et al. 1986; Hagn et al. 1986; Iacangelo et al. 1986; Lassmann et al. 1986; Rindi et al. 1986; Kimura et al. 1988), the 7B2 polypeptide of pituitary origin (Falgueyret et al. 1987), the plasma proteins, tetranectin (Christensen et al. 1987) and parvalbumin (Endo et al. 1984), the protein gene product (Rode et al. 1985; Day and Thompson 1987), the islet cell antigen (Krisch et al. 1986; Bordi et al. 1988) and synaptophysin (protein p38) (Navone et al. 1986; Gould et al. 1986; Chejfec et al. 1987; Buffa et al. 1988).

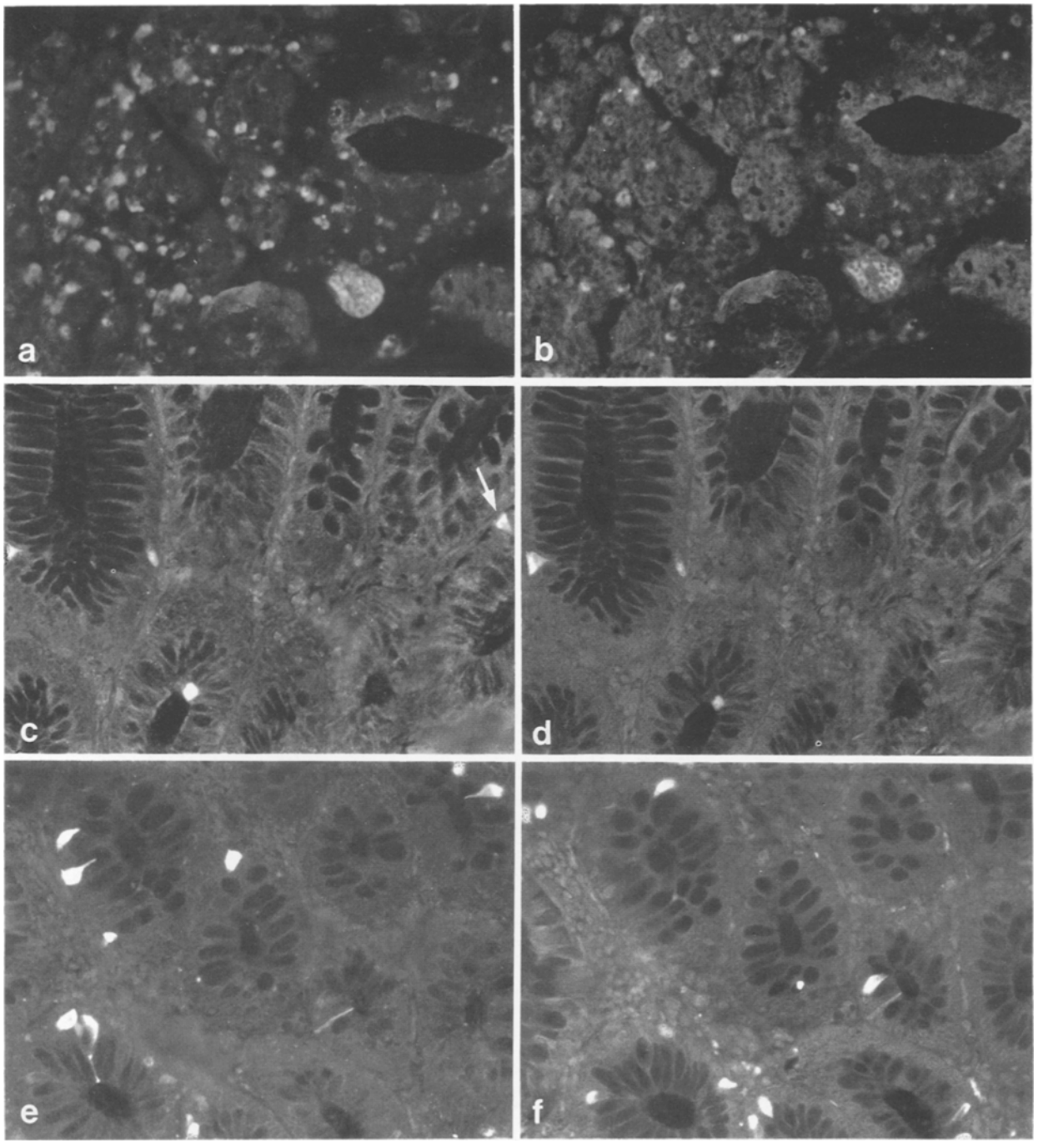

Fig. 2a–f. Human pancreas. Double immunostaining for EGC (**a**) and glucagon (**b**). Human rectum. **c** Section first immunostained with the EGC antiserum and, after elution of the antibodies, restained for serotonin (**d**). Note co-existence of EGC and serotonin in the enterochromaffin cells. A single EGC-immunostained cell (*arrow*) does not contain serotonin. **e** Human rectum. Section first immunostained with the EGC antiserum and then restained for glicentin. **f** EGC-immunoreactive cells are distinct from those storing glicentin. **a–d** ×150; **e, f** ×200

In the present study we used an antiserum raised against proteins extracted from the granules of a midgut carcinoid tumour, hence the name endocrine cell granule constituents (EGC). This tumour derives from 5-HT-containing enterochromaffin cells and it was to be expected therefore that the antibodies raised should demonstrate midgut carcinoids and ileal enterochromaffin cells. In addition, however, the antibodies demonstrated a great variety of endocrine cells in other parts of the gastrointestinal tract and also pancreatic islet cells, thyroid C cells and adrenal medullary cells. In electron microscopic studies of endocrine cells in the small intestine it was found that the immunostaining was confined to the secretory granules of the immunoreactive cells. The EGC antibodies demonstrated several types of peptide-hormone-producing endocrine tumours in addition to midgut carcinoids. This raised the question whether the ECG antiserum might contain antibodies against chro-

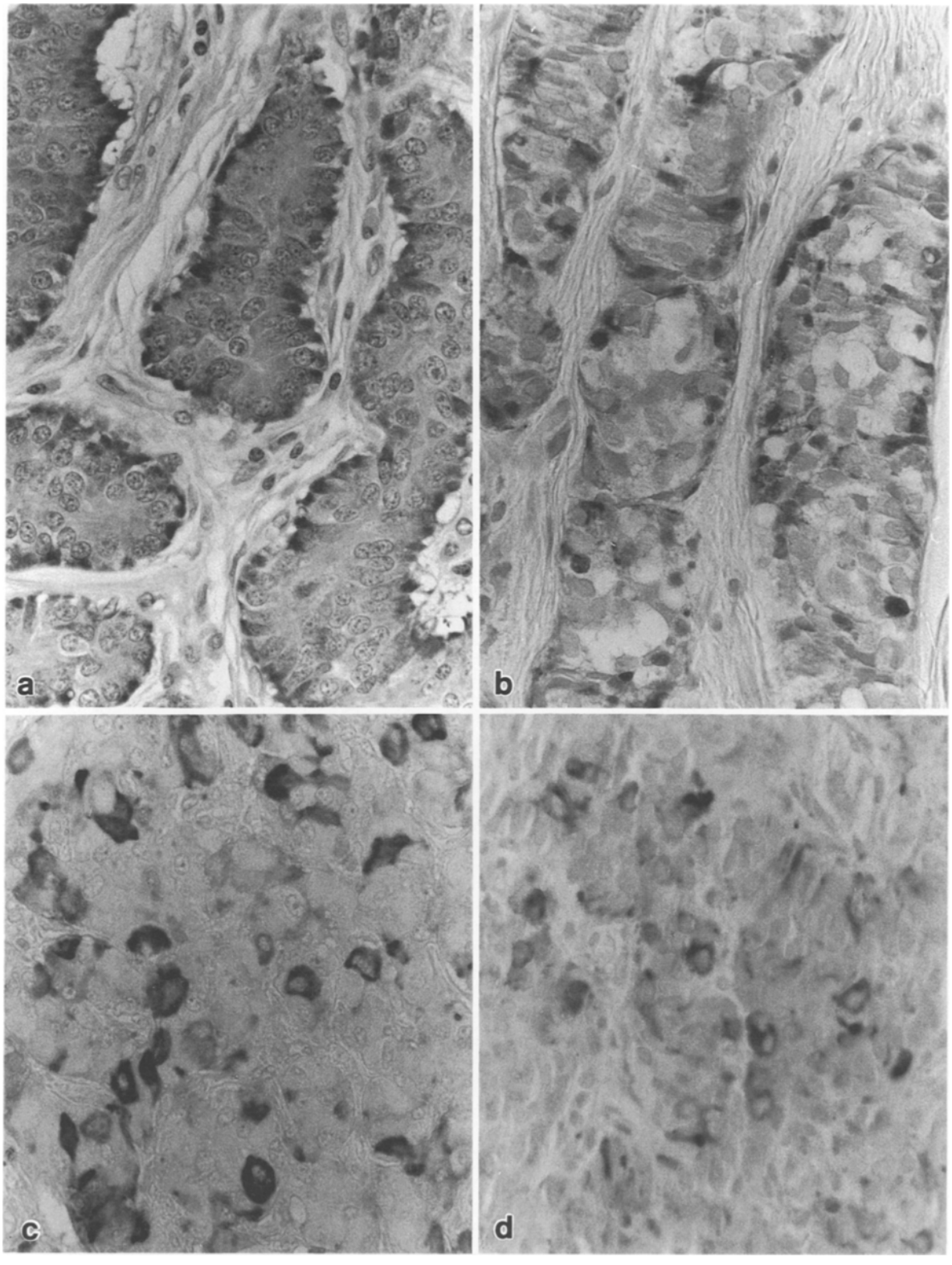

Fig. 3. EGC-immunoreactive cells in endocrine tumours (PAP technique): **a** Ileal carcinoid, **b** appendiceal carcinoid, **c** phaeochromocytoma, and **d** pancreatic gastrinoma. In the carcinoids (**a**, **b**) immunoreactive cells predominate in the periphery of tumour cell cords. In the phaeochromocytoma and the gastrinoma immunoreactive cells occur scattered in the tumour tissue. × 350

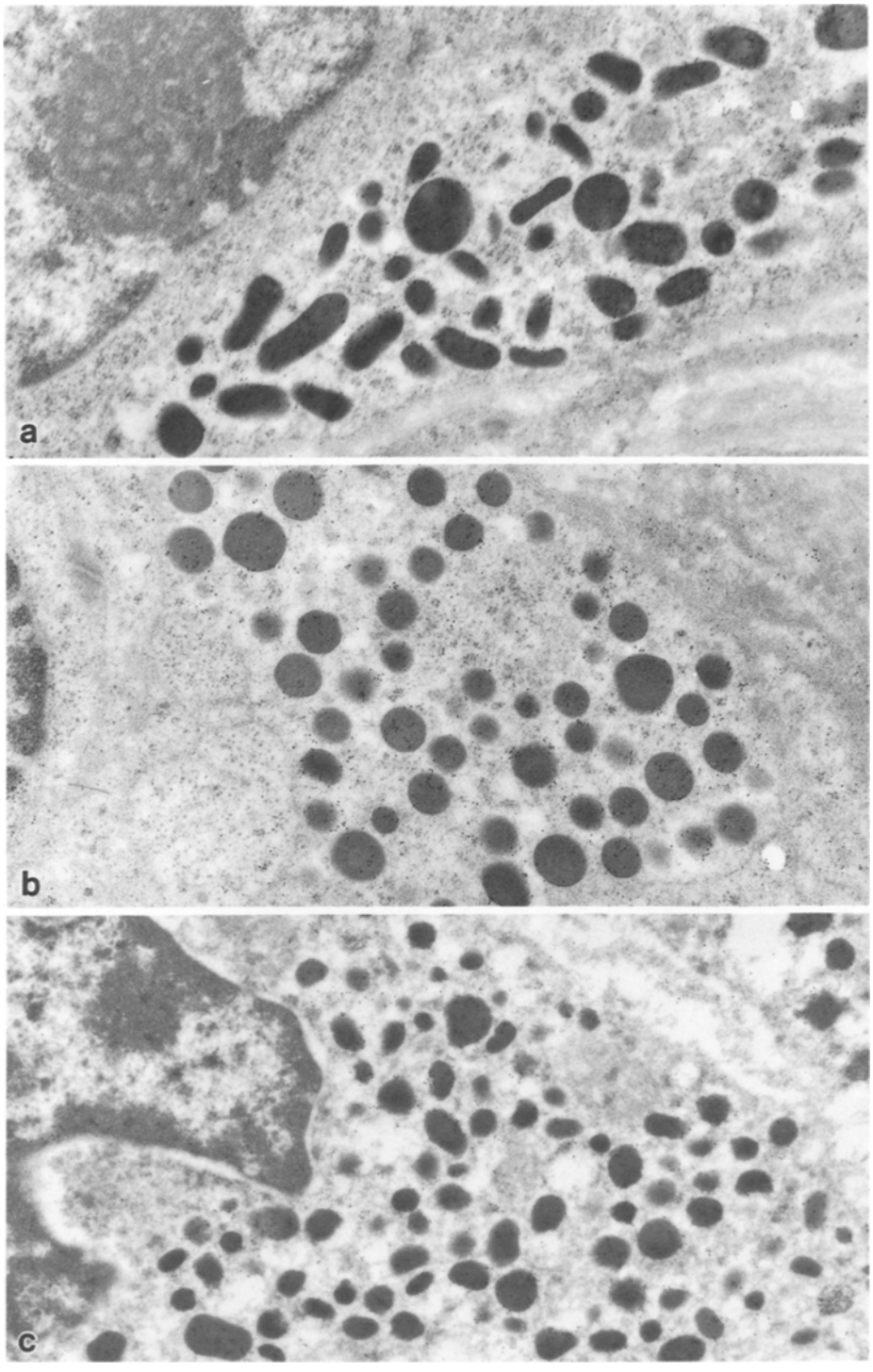

Fig. 4. Immunogold staining for EGC in enterochromaffin cell, characterized by pleomorphic, highly electron-dense granules (**a**), in an L cell, with large, round, homogeneously electron-dense granules (**b**), in human ileum, and in human phaeochromocytoma cell (**c**). Note accumulation of gold particles over the secretory granules. ×29000

mogranins. In fact preliminary results suggest that one antibody population in the antiserum is directed against chromogranin A (D.T. O'Connor, personal communication). These findings are supported by the present results and those of Vyberg et al. (1988), who compared the EGC antiserum with antisera against other endocrine cell and tumour markers in a study of endocrine tumours and found a remarkable similarity between the staining pattern produced by the EGC antiserum and that of chromogranin A antibodies. Whether there are also antibodies directed against other granule components has yet to be determined.

In conclusion, the results obtained with the EGC antiserum indicate that, like chromogranin antibodies, it can be used for the collective demonstration of peptide hormone-producing cells and for the diagnosis of tumours arising from such cells.

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