Virchows Arch [Cell Pathol] (1981) 36:207–218

Virchows Archiv B

Motilin Cells Investigated by the Use of Region-Specific Antisera

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Summary. The immunocytochemical localisation of motilin in endocrine cells of the mammalian gut has been investigated by the use of a range of region-specific antisera to both the C- and N-terminal parts of the peptide molecule.

The possibility of motilin being co-stored with serotonin (5-HT) in a subpopulation of enterochromaffin cells was also studied by the use of specific antibodies to 5-HT. Motilin-like immunoreactivity was found exclusively in the mucosal APUD cells of the small intestine in all species investigated. Further characterisation of these cells indicated that antibodies reacting with both the C- and N-terminals immunostain a population of nonargentaffin (argyrophil) cells. In addition, a minor subpopulation of EC cells was immunostained with N-terminal directed antibodies only.

In view of these results it is possible that the non-enterochromaffin cells detected by antibodies directed against both the N- and C-terminals of the motilin molecule are the source of the 22 amino acid motilin originally described. In contrast, the subpopulation of enterochromaffin cells detected only by N-terminal directed antibodies may be the source of a related peptide, yet to be identified. However, the possibility cannot be ruled out that the antigenic site on the peptide molecule may be altered when motilin is co-stored with variable quantities serotonin.

Key words: Immunocytochemistry – Motilin – APUD cells – Neuroendocrine system – Molecular forms serotonin.

Dedicated to Professor Hans-Werner Altmann on the occasion of his 65th birthday

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0340-6075/81/0036/0207/$02.40

Introduction

Motilin, discovered in 1971 (Brown et al. 1971), was subsequently used, in both natural and synthetic forms, for the production of antisera, leading to the investigation of its distribution and cellular localisation and its possible physiological role.

Results obtained in various laboratories by the use of different antibodies revealed several apparent discrepancies. For instance, there were reports of the release of motilin by intraduodenal infusions of alkali in dogs (Dryburgh and Brown 1975) and by infusions of acid in man (Mitznegg et al. 1978; Tai and Chey 1978). There is also a question as to whether motilin or a motilin-like peptide is present exclusively in endocrine cells of the gastrointestinal tract (Pearse et al. 1974; Polak et al. 1975b; Forssmann et al. 1976; Polak et al. 1976; Tobe et al. 1976; Heitz et al. 1978a, b; Polak et al. 1978; Helmstaedter et al. 1979; Seino et al. 1979; Polak and Buchan 1979) or whather it is, in addition, present in nervous tissue (both central and peripheral) (Yanaihara et al. 1978; Chey and Lee 1980; Fox et al. 1980). The reported circumscribed distribution of motilin within the small intestine has been challenged by the recent finding of a motilin-like immunoreactive material throughout the entire width and length of the gut. Further, there is considerable evidence that motilin and serotonin (5-HT) may be stored in variable quantities in the same APUD cells (Pearse et al. 1974; Polak et al. 1975; Pearse 1976; Heitz et al. 1978a).

These, often contradictory, findings have been, in part, attributed to the use of antisera which recognise different portions of the motilin molecule and have led to the suggestion that motilin may exist in blood and tissue in more than one molecular form. As it appears, at present, that most of the regulatory peptides occur either as members of structurally similar peptide "families" or in multiple molecular forms (Rehfeld et al. 1974; Rehfeld 1978; Dimaline and Dockray 1978; Fernstrom et al. 1980), it seems probable that motilin may also occur in more than one form. Indeed, recent work, using a variety of region-specific antisera to motilin, has revealed the presence of two molecular forms in blood and tissue from several mammalian species including man (Polak and Buchan 1979; Bloom et al. 1979; Yanaihara et al. 1980; Christofides et al. 1981). We report here the use of a series of region-specific antisera, to both natural and synthetic motilin, in order to localise, by immunocytochemistry, the origin of motilin in canine, porcine and human intestine.

Material and Methods

a) Tissue

Macroscopically and microscopically normal full thickness samples (2 × 2 cm) of fresh small intestine (n = 11) were collected from man (during Whipple's procedure) (n = 3), dog (n = 4) and pig (n = 4).

b) Fixation

Each sample was bisected. Half of each sample was immediately quenched in Arcton at −156° C, subsequently freeze-dried overnight and fixed in para-benzoquinone vapour (Pearse and Polak 1975). The material was then embedded in paraffin wax under vacuum. The other samples were fixed by immersion in modified Bouin's solution (75% saturated picric acid, 25% formaldehyde

and 1% glacial acetic acid, added immediately prior to use) for 3 h at 4° C. After dehydration in alcohol and xylene, the tissue was embedded in paraffin wax under vacuum.

c) Immunocytochemical Procedure

Prior to immunostaining, 5 micron and serial 3 micron sections from all samples were de-waxed by immersion in xylene (2 min) followed by petroleum ether (2 min) and allowed to air dry.

Indirect Immunofluorescence Method (Coons et al. 1955). The first layer of diluted primary antibody (Table 1) was applied for 12–18 h at 4° C. The sections were then washed in 0.01 M phosphate-buffered saline (PBS) (pH 7.2) and a second layer of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit globulin (Miles) was applied for 1 h at room temperature at a dilution of 1:150. The sections were again washed in PBS and mounted in a solution of PBS/glycerine (1:9).

Unlabelled Antibody Enzyme Method (Peroxidase Anti-Peroxidase (PAP) (Sternberger 1972). Sections were pretreated for 30 min each with 0.3% hydrogen peroxide to remove endogenous peroxidase activity, followed by normal goat serum (dilution 1:30) to block possible background staining. The first layer antisera were then applied for 12–18 h at 4° C. The second layer of unconjugated goat anti-rabbit globulin (1:50) and third layer of horseradish peroxidase (Dakopatts A/S Denmark) (1:300) were each applied for 30 min at room temperature.

Double Immunostaining Methods. i) Demonstration of two different antigens in the same tissue section. Tramu's antibody elution method (Tramu et al. 1978): Following PAP immunostaining the sections were washed in PBS and immersed in a freshly made solution of 0.05%, 3,3′-diaminobenzidine tetrahydrochloride or 0.025% 4-Cl-1-naphthol in 5 ml of ethanol in PBS containing 0.01% hydrogen peroxide. After washing in distilled water, the sections were mounted in PBS/glycerine. They were then examined under the microscope and the positive areas were photographed. After removal of the coverslips, the sections were immersed in a 1:1 solution of 0.3 M potassium permanganate and 0.02 N H_2SO_4 for 1 minute at room temperature and then bleached with 2.5% sodium thiosulphate (Tramu et al. 1978). We have found that we can remove the reaction product obtained with 4-Cl-1-naphthol by immersion of the sections in graded alcohol and xylene without oxidation and $Na_2S_2O_5$ treatment. After this, the same areas in the wet sections without coverslips were photographed in order to establish whether full removal of the previous PAP immunostaining had occurred. The sections were then incubated with the second antibody (see Table 1) and the PAP technique was followed as before. Photographs of the resulting immunostain were compared with the originals.

ii) Serial (3 μm) sectioning method: In order to compare the cells which were immunoreactive to the different region-specific antisera to motilin and also to compare them with the cells which were immunoreactive for 5-HT, serial 3 micron sections were used. Consecutive sections were immunostained, using the PAP technique, with antiserum to 5-HT (see Table 1). The resultant immunostains were photographed and the corresponding areas compared.

In addition, in order to confirm that some motilin-producing cells were argentaffin EC cells, Masson's silver stain for amine-containing cells was performed on pre-immunostained sections

Table 1. Dilution of antisera used

Immunocytochemistry dilution	*IF*	*PAP*
Antisera		
M1	1:100	1:1000
M4	1:100	1:1000
M25	1:100	1: 400
R1106	1:200	1:2000
R1104	1:400	1:4000
GP71[a]	1:500	1:5000
5-HT	1:400	1:7000

[a] This serum was a gift from Dr. J.C. Brown, British Columbia, Canada

Table 2. Absorption test

Antisera	Motilin	Conjugation		Specificity	PAP dilution	Absorption			
						1–22	1–11	11–22	18–22
M1	Natural	BSA	CDI	N-terminal	1:1000	–	–	+	+
M4	Natural	BSA	CDI	N-terminal	1:1000	–	–	+	+
R1106	Synthetic	PVP	[a]	N-terminal	1:1000	–	–	+	+
GP71[b]	Natural	BSA	CDI	C-terminal	1:2000	–	+	–	+
R1104	Synthetic	PVP	[a]	C-terminal	1:1000	–	+	–	+
M25	Natural	BSA	CDI	C-terminal	1: 500	–	+	–	+

[a] Complete Freund adjuvant, PVP: Polyvinylpyrrolidone
[b] This antiserum was a gift from Dr. J.C. Brown

– = no staining
+ = staining

(Pearse 1972). Only the tissue fixed in Bouin's fluid could be used for this method. The sections were rinsed well in glass-distilled water then incubated in a 5% ammoniacal silver nitrate solution for 1 h at 60° C. They were then rinsed in distilled water and mounted in PBS/glycerine. The positive areas were photographed and compared with the original photographs of the immunostaining.

d) Combined Immunocytochemical and Cytochemical (Masson's and Grimelius') Silver Impregnation

Two different procedures were carried out:

i) Masson's argentaffin reaction was carried out, after immunostaining. Immunostained sections were examined under a Leitz Orthoplan fluorescence microscope and photographed. After photography the slides were soaked in PBS, the coverslips removed and the sections were then rinsed in glass-distilled water for 1–2 h before restaining by Masson's argentaffin silver impregnation technique (Pearse 1972). Sections were incubated in a 5% ammoniacal silver nitrate solution for 1 h at 60° C. They were then rinsed in distilled water and mounted in PBS/glycerine. The positive areas were photographed and compared with the original photographs of the immunostaining.

ii) Grimelius' argyrophilic silver impregnation method was carried out first, prior to immunostaining (Polak et al. 1975a), on tissue fixed in Bouin's fluid. The positive areas of each section were then photographed. The coverslips were removed and the sections were rinsed in PBS for 1 h. Silver deposits were removed by pre-treatment of the sections with potassium cyanide. Immunocytochemistry was carried out on the sections and the positive areas were again photographed and compared with the original photographs showing the results of the Grimelius silver impregnation.

e) Antisera

Antisera to both natural and synthetic motilin as well as to 5-HT (Steinbusch et al. 1978) were used during this investigation. Their specificity and relevant dilutions are shown in Table 1. In order to show that the immunocytochemical reactions were specific, the following tests were performed:

i) prior to immunostaining, the diluted antisera were absorbed with samples of synthetic or purified peptides (Table 2). Included in these samples were synthetic fragments of motilin, in order to show the exact region specificities of the antisera. In addition, preabsorptions were performed using unrelated gut peptides such as secretin, gastrin, somatostatin and glucagon.

ii) normal rabbit serum was used instead of the primary antibody as the first layer.

iii) the FITC second layer was applied alone in the immunofluorescence method.

iv) the PAP complex was applied alone and developed in the unlabelled antibody enzyme technique.

v) formalin fixed sections were observed under U.V. light and photographed prior to immunostaining, in order to rule out the presence of cells showing formaldehyde induced autofluorescence (Kobayashi et al. 1980).

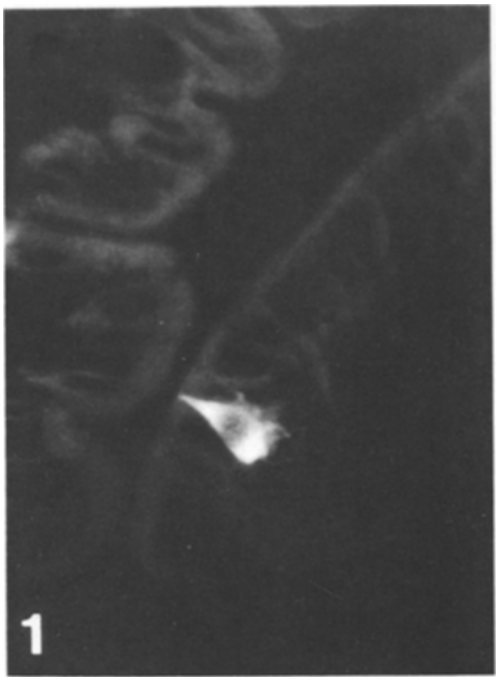

Fig. 1. Motilin cell of the human duodenum immunostained with antibody GP71, which is specific for the C-terminal part of the peptide molecule. (Bouin's fluid fixation ×500)

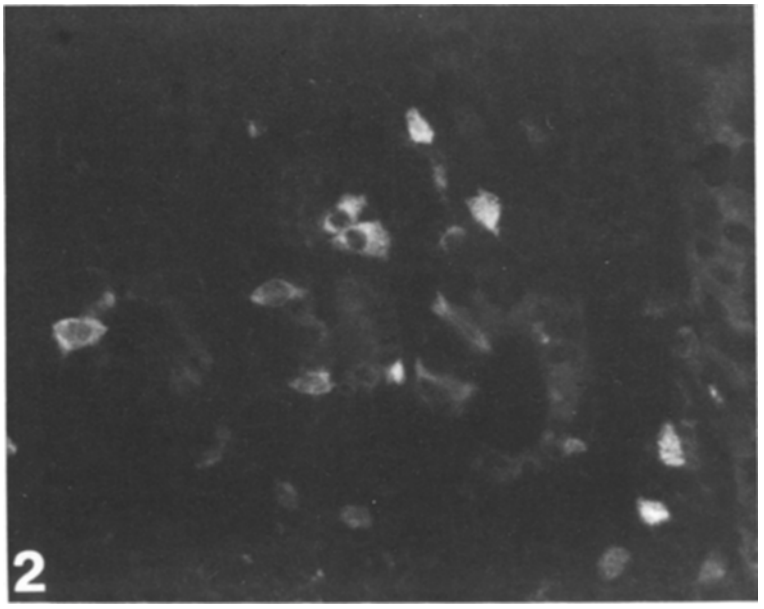

Fig. 2. Enterochromaffin cells of the human intestine immunostained with antibodies to serotonin (Bouin's fluid fixation ×400)

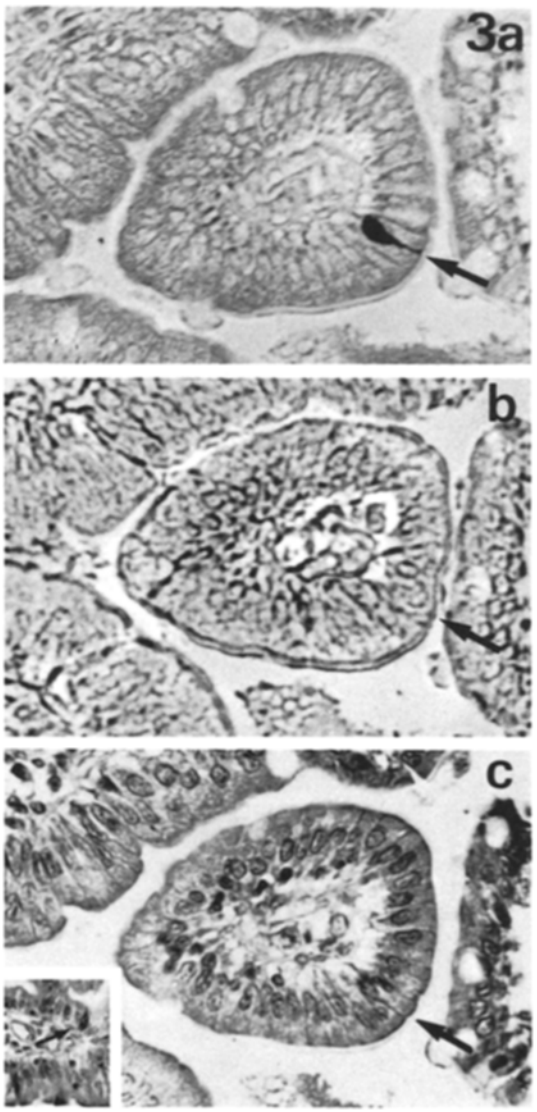

Fig. 3a–c. One 5 μm section of human duodenum consecutively immunostained using Tramu's modification of the PAP technique. **a** Motilin cell immunostained with C-terminal directed antibodies (4-Cl-1-naphthol was used as substrate). **b** The same section after elution of the primary reaction product (seen in (a)) by immersion of the section in graded alcohols and xylene. **c** The same section after re-staining with antibodies to serotonin. The motilin cell seen in (a) (arrow) is unreactive to serotonin antibodies (p-benzoquinone vapour fixation ×500). Insert shows an immunoreactive enterochromaffin cell (×250)

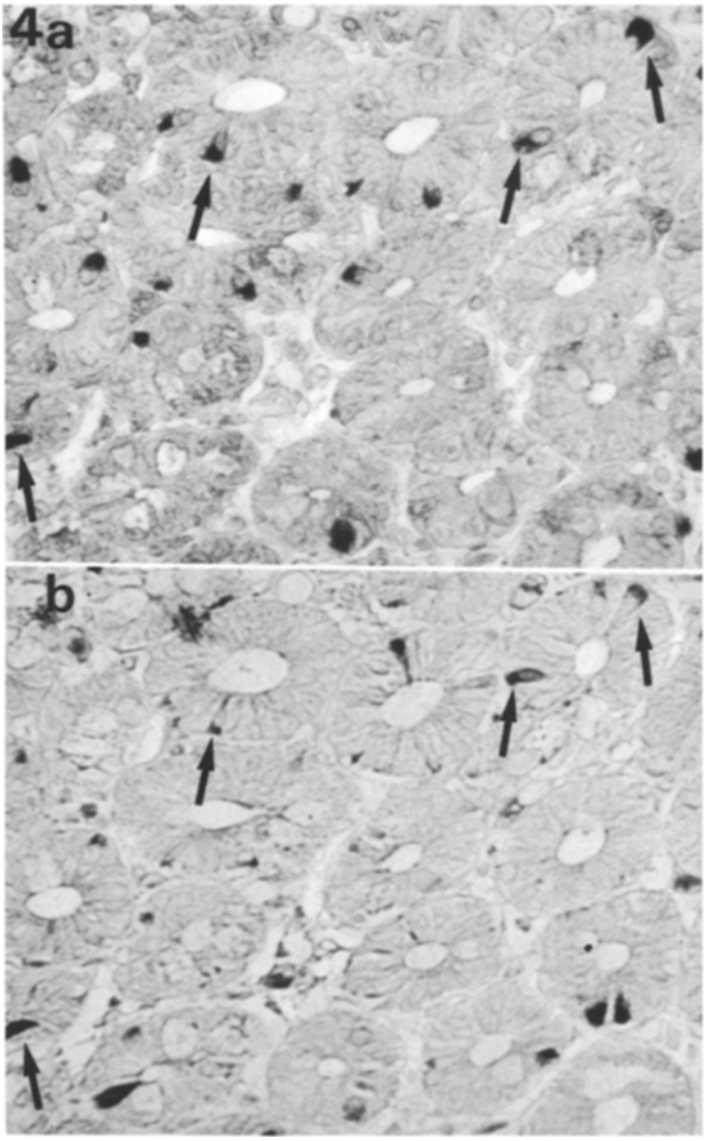

Fig. 4a, b. Canine duodenum stained sequentially by Grimelius' silver impregnation method (**a**) and C-terminal directed antibodies to motilin (**b**) following removal of the silver deposits by pre-treatment of the sections with potassium cyanide (Bouin's fluid fixation ×450)

Results

Immunostaining of the mucosa from human, porcine and canine duodenum with region-specific antisera to motilin as well as with a specific antiserum to 5-HT, revealed the presence of numerous immunoreactive cells (Figs. 1, 2). The majority of these cells were present in the crypts, although some were

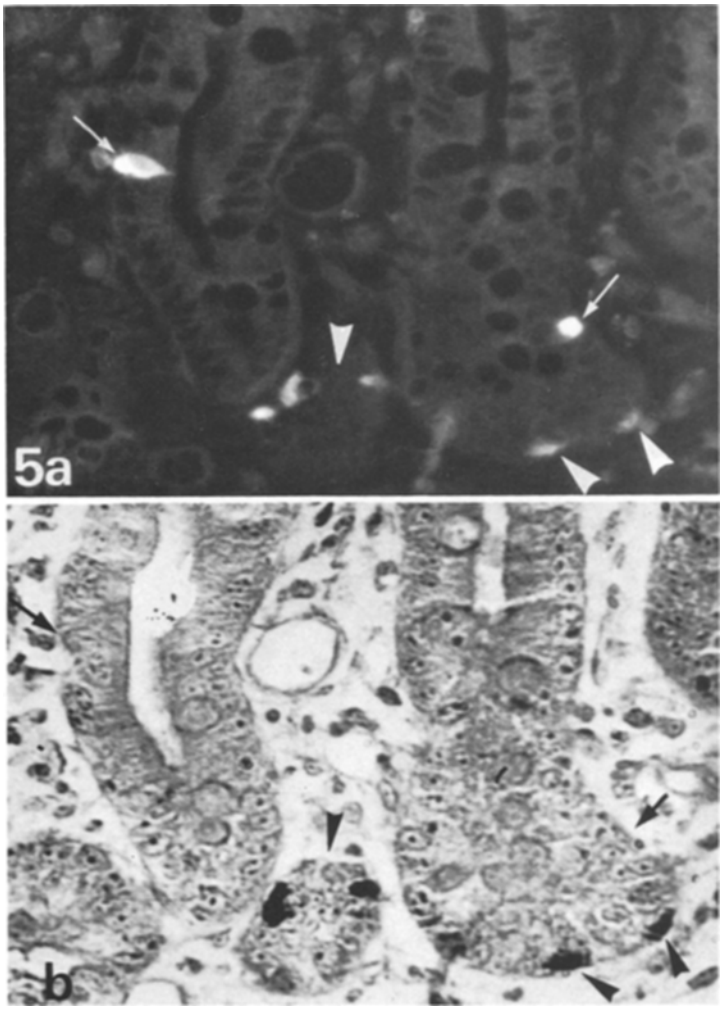

Fig. 5a, b. One 5 μm section of human duodenum sequentially stained by the use of N-terminal directed antibodies (**a**) and by Masson's argentaffin reaction (**b**). Large arrows indicate cells co-storing a motilin-like immunoreactive material and intrinsic amine (serotonin). Fluorescent cells seen in (**a**) correspond to specific immunofluorescence and *not* to fluorescence induced by formaldehyde treatment. This is shown by the fact that 1) control sections did not shown autofluorescence 2) only a proportion of motilin cells contained serotonin, as seen in Fig. 6 (For discussion see Kobayashi et al. 1980) (Bouin's fluid fixation ×400)

found scattered in the villi. The cells showed the classical morphological features of endocrine cells of the APUD series (Pearse et al. 1969).

Antisera to both the C- and N-terminals of the motilin molecule immunostained an identical population of argyrophil, non-argentaffin endocrine cells (Figs. 3a, b, c and 4a, b).

An additional, somewhat smaller endocrine cell type, could be observed only after the use of the N-terminal directed antibodies. These additional cells

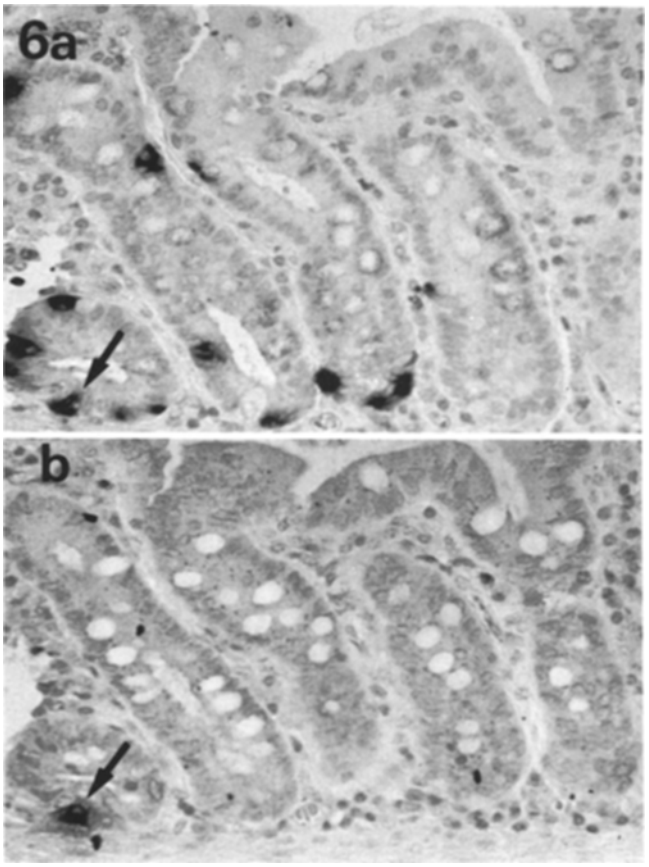

Fig. 6a, b. 3 μm serial sections of canine small intestine demonstrating the presence of a motilin-like immunoreactive material in a subpopulation of enterochromaffin (EC) cells (*arrow*) **a** EC cells immunostained with antiserum to serotonin. **b** Motilin immunoreactive cells immunostained with N-terminal directed antibodies. (p-benzoquinone vapour fixation ×450)

were identified as argentaffin, serotonin-containing (EC) cells by the use of Masson's argentaffin reaction (Fig. 5a, b) or specific antibodies to serotonin (5-HT) (Fig. 6a, b) either on the same section or on serial (3 μm) sections.

The number of motilin/EC cells varied with the species, being more numerous in dog than in the human duodenal mucosa. we were unable to immunostain gut autonomic nerves with any of the antibodies used in the present investigation.

Discussion

A combination of several specialised histological techniques has been used in this study to produce a complete characterisation of the motilin-containing cells in the duodenal mucosa of three mammals including man. The techniques used included immunocytochemistry with antisera specific for different parts of the originally sequenced 22 amino acid peptide motilin, as well as a specific

antiserum to 5-HT. These were applied after two different fixation procedures. In agreement with several previous reports (Forssman et al. 1976; Helmstaedter et al. 1979; Polak and Buchan 1979; Kobayashi et al. 1980) antibodies binding to the C- or N-terminal regions of motilin detect a population of argyrophil, non-argentaffin cells in the gut mucosa. In addition, N-terminal directed antibodies detect a subpopulation of serotonin-containing EC cells.

At a recent international meeting on Gut Hormones (Solcia et al. 1981) the necessity was acknowledged of using a wide variety of region-specific antisera to a single peptide in order to achieve full validation and correct interpretation of immunocytochemical findings. With these criteria in mind, and in view of the present results, it is therefore possible that the motilin-like material detected in the population of argyrophil cells in the duodenal mucosa (by both the C- and N-terminal directed antisera) may be the source of the 22 amino acid peptide originally described (Pearse et al. 1974). It is also possible that the immunoreactive material present in the subpopulation of serotonin-containing EC cells, and detected almost exclusively by the N-terminal directed antisera, may represent another, chemically related peptide, thus supporting the suggestion that there is a family of related motilin-like peptides (Christofides et al. 1981).

However, the variable amounts of serotonin shown to be stored in different APUD cells may affect the antigenic sites of a peptide co-stored in the same cell (Polak and Buchan 1979). This possibility cannot be ruled out at present and is supported by the finding that the proportions of motilin-containing EC and non-EC immunoreactive cells varies when different methods are used. One of the fixatives used contains formaldehyde which is known to react chemically with the indole ring of the serotonin molecule (Falck 1962), thus enhancing the detection of serotonin in tissue fixed by this method. Clearly, biochemical investigations are urgently needed to establish whether there is a "family" of related motilin-like peptides.

Acknowledgement. This work was carried out with the generous support of the Cancer Research Campaign.

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Received January 29, 1981 / Accepted February 3, 1981