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Multiple peptide production and presence of general neuroendocrine markers detected in 12 cases of human phaeochromocytoma and in mammalian adrenal glands

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Summary. In this study, antibodies to a range of markers of neuroendocrine differentiation were evaluated for their use in the histopathological assessment and characterisation of phaeochromocytomas. Routinely processed wax blocks from eleven adrenal phaeochromocytomas (10 benign and 1 malignant) and one benign phaeochromocytoma of the urinary bladder were investigated. In addition to these tumours, normal human, cat and piglet adrenal glands were examined. In the phaeochromocytomas, immunostaining was obtained with 21 of the 25 antisera used. Of the general neuroendocrine markers, neuron-specific enolase was found in all tumours, and chromogranin and protein gene-product 9.5 in most of the cases. A range of regulatory peptide immunoreactivities could be demonstrated, such as enkephalin, neuropeptide tyrosine (NPY), 7B2, galanin and vasoactive intestinal polypeptide (VIP). In addition, two peptides were found which have not been reported previously in these tumours, peptide histidine methionine (PHM) and the cryptic fragment of the precursor encoding VIP. Co-localisation studies revealed that peptides derived from the same precursor or peptide family were found in the same tumour cells (e.g. VIP and PHM, NPY and its C-flanking peptide CPON).

In the normal adrenal medulla, all the peptides previously reported to be present could be demonstrated immunocytochemically. Galanin was present in a subpopulation of cells also immunoreactive for enkephalin. Neuropeptide tyrosine and CPON were demonstrated in another subpopulation. Occasionally, cells were found to contain all four antigen immunoreactivities. Using antisera to enzymes involved in catecholamine synthesis, galanin was found to be present in noradrenalin-containing cells. The study demonstrates the presence of various antigens in chromaffin tissue of the adrenal gland. A range of substances can also be identified immunocytochemically in phaeochromocytoma tissue, using routinely-processed material.

Key words: Histopathology – Immunocytochemistry – Phaeochromocytoma – Adrenal gland – Neuroendocrine markers

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Introduction

Phaeochromocytomas are relatively rare tumours of chromaffin cells. Typically, the tumour cells show a high degree of irregularity in cell size and arrangement, but resemble those of the adrenal medulla and are arranged in cords, trabeculae or, most often, in sheets forming a mosaic-like pattern. However, small cell variants occur, and in some cases there is ganglionic differentiation. About ninety percent of phaeochromocytomas arise in the adrenal gland. However, chromaffin tissue is widespread in the foetus (Coupland 1952), and although most of it is lost by adulthood, some can remain in any site and may give rise to an extra-adrenal tumour. Although the majority are benign, some malignant cases have been reported (for a recent review see Liu et al. 1984). Some phaeochromocytomas are clinically silent, but most produce characteristic symptoms related to the increased production of catecholamines, such as hypertension or hyperglycaemia due to suppressed insulin release.

Recently, regulatory peptides have been found to be produced by both normal adrenal and phaeochromocytoma tissue (Polak and Bloom 1985; Sano et al. 1983; Hassoun et al. 1984). They include peptides of the enkephalin/dynorphin family (Schultzberg et al. 1978; Lundberg et al. 1979; Linniola et al. 1980; Varndell et al. 1981; Wilson et al. 1981a, b; Cox 1982; Livett et al. 1982; Suda 1983; Kobayashi et al. 1983; Yoshimasa et al. 1981, 1983, 1984), neurotensin (Lundberg et al. 1982; Terenghi et al. 1983), somatostatin (Lundberg et al. 1979; Berelowitz et al. 1983; Viale et al. 1985), calcitonin (Hassoun et al. 1984; Viale et al. 1985), vasoactive intestinal polypeptide (Hökfelt et al. 1977; Linnoila et al. 1980; Viale et al. 1985), substance P (Linnoila et al. 1980; Saria et al. 1980; Bucsics et al. 1981; Gamse et al. 1981), 7B2 (Suzuki et al. 1986), adrenocorticotrophic hormone (Berenyi et al. 1977; Hassoun et al. 1984), neuropeptide tyrosine (Adrian et al. 1983; Varndell et al. 1984b; Majane et al. 1985) and its C-flanking peptide CPON (Gulbenkian et al. 1985), and galanin (Bauer et al. 1986). The biological roles of all these substances are not yet fully elucidated. Their functions in this system may include modulation of the release and effects of catecholamines and regulation of adrenal blood flow (e.g. Lundberg et al. 1979; Livett et al. 1982). The clinical symptoms of increased peptide production in phaeochromocytomas appear to be related mainly to oversecretion of catecholamines, and the contribution made by excess circulating peptides has yet to be defined.

A systematic study combining conventional histochemistry and immunocytochemistry has not been made previously. The present investigation was carried out to examine phaeochromocytomas and normal adrenal glands using a wide range of well characterised antibodies to active peptides and other derivatives of their precursors, as well as to general markers of neuroendocrine differentiation.

Materials and methods

Specimens. Tissues from 12 phaeochromocytomas were used in the study (Table 1). Ten of these were benign tumours of the adrenal gland, 1 was a benign phaeochromocytoma of the urinary bladder wall, and 1 was a malignant case of adrenal phaeochromocytoma with metastases in lymph nodes, liver and lung (Pollard and Wing 1984). In addition, normal adrenal glands were sampled from humans ($n=5$), cats ($n=6$) and piglets ($n=5$).

Pieces of tissue were collected freshly from surgery for adrenal tumour resection or after ether anaesthesia (cat, pig) and were either routinely fixed (Bouin's fluid for 4 h, or 10% phosphate-buffered formol saline overnight at 4° C) or snap-frozen in melting Arcton, freeze-dried overnight at −40° C and vapour-fixed with parabenzoquinone at 60° C for 3 h (Pearse and Polak 1975). This was followed by vacuum wax-embedding at 60° C. In addition to these freshly obtained and optimally processed specimens, a range of cases was also selected from routine histopathology files.

Serial sections were cut at 4 µm and mounted on glass slides coated with poly-L-lysine (PLL; Huang et al. 1983) in order to improve section adherence. To study the relationships between cells immunostained for different antigens, sections were mounted consecutively or as mirror images (Bishop et al. 1985). They were then left to air dry at 37° C overnight, dewaxed in xylene and rehydrated in a series of graded alcohols prior to staining.

For semithin resin sections, small samples (1 mm^3) were fixed immediately after removal in 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 h. The samples were then rinsed in buffer, dehydrated through alcohols and embedded in Araldite resin. Semithin sections (0.5 µm thick) were mounted serially on PLL-coated glass slides. Prior to immunostaining the resin was removed from the sections by immersing the slides in sodium ethoxide (saturated NaOH in absolute ethanol) for 3 min, then rinsed well in absolute ethanol and hydrated. Standard light microscopical immunoperoxidase staining was performed on the sections.

Histochemical methods. Conventional haematoxylin and eosin staining was used for routine diagnosis and to assess morphology. Classical silver impregnation methods were employed to aid the demonstration of secretory granules at the light microscopical level. Grimelius' method was applied (Grimelius and Wilander 1984) for the demonstration of argyrophilia, and the method of Masson-Fontana as described by Pearse (1972) to show argentaffinity.

Immunocytochemistry. Immunocytochemistry at the light microscopical level was carried out using the standard peroxidase anti-peroxidase (PAP) technique (Sternberger 1979). In addition, for certain cases, a modified version of the immunogold-silver staining method (IGSS; Holgate et al. 1983) was carried out (Springall et al. 1984; Hacker et al. 1985a and b), in order to improve demonstration of antigens present in low concentration.

Antibodies and specificity controls. Rabbit primary antisera, as well as rat or mouse monoclonal antibodies were used. Their characteristics are shown in Table 2. All antibodies used have been described previously (see Table 2). Specificity controls included preabsorption of the primary antisera with their appropriate antigens (natural purified or synthetic) at concentrations of 0.1 nmol to 10 nmol antigen per ml diluted antiserum, and the use of normal sera or diluent alone in place of the primary antiserum. Secondary or tertiary antisera are summarised in Table 3.

Results

Specificity

Immunostaining was abolished when non-immune rabbit serum or diluent alone was used as first layer, or when immune sera were preabsorbed with appropriate antigens.

Table 1. Summary of cases and light microscopical results of phaeochromocytomas obtained in this study

Case	1	2	3	4	5	6	7	8	9	10	11	12a	12b	12c	12d
Diagn.	PA	PA	PA	PUB	PA	PA	PA	PA	PA	PA	PA	MPA	MPB	MPC	MPD
Sex	F	M	M	M	F	M	F	F	F	F	F	M	M	M	M
Age	49	55	45	53	52	29	52	47	5	62	67	13	13	13	13
Fixative	F	F	F	BQV	F	F	B	B	BQV	F	F	F	F	F	F
Cg	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	−	+	−
NSE	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+	+++
PGP 9.5	nd	nd	+++	+++	nd	+++	+++	+++	+++	+++	+++	+++	+++	+++	−
7B2	−	+	+	+	−	−	−	−	+	+	−	+	+	+	+
NFP	−	−	−	++	++	+	−	+++	+	−	−	−	−	−	−
GFAP	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
S-100	−	−	−	++	+	+++	−	−	−	−	−	++	−	−	+
TH	−	++	+	++	+++	+	−	−	+	−	−	−	−	−	−
DBH	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
PNMT	+	−	+	−	−	++	−	−	−	−	+++	−	−	−	−
LENK	+	−	−	−	+++	−	+	+	+	−	+	+++	+++	+++	++
MENK	+	−	−	−	++	+	−	++	+	−	++	+++	++	+++	+++
ACTH	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
BOM	−	−	−	−	−	++	−	−	−	−	−	nd	nd	nd	nd
CALC	−	−	+	−	−	−	−	−	+	−	−	−	−	−	−
CGRP	+	−	−	−	−	+	−	−	−	−	−	−	−	−	−
SOM	−	−	−	−	++	+	+	+	−	−	−	++	+	+	−
NPY	+	+++	+	++	+++	+	+	++	+	−	−	+++	+	+++	++
CPON	++	+++	++	+	+++	++	++	+++	+	+	++	+++	+++	+++	++
VIP	−	−	−	−	+	−	−	+	−	−	−	−	−	−	−
PHM	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−
CrFrag	+	−	−	+	++	++	−	+	−	−	+	−	−	−	+
NT	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
GAL	+	−	−	−	++	++	++	++	−	−	−	++	+++	+++	−
5HT	−	−	++	−	−	+	−	−	−	+	−	−	−	−	−
Grim	+++	+++	+++	++	+++	++	++	+++	+++	+	+	++	++	+	+
Masson	−	++	−	+++	−	++	−	−	−	−	−	nd	nd	nd	nd

− = negative; + = few scattered immunoreactive cells; ++ = many immunoreactive cells; +++ = very many immunoreactive cells; nd = not done; Grim = Grimelius' argyrophilic silver impregnation; Masson = Masson-Fontana silver impregnation; NSE = Neuron-specific enolase; NFP = Neurofilament protein triplet; GFAP = Glial fibrillary acidic protein; Cg = Chromogranin; 7B2 = Peptide isolated from the anterior pituitary of pig; PGP 9.5 = Protein gene-product 9.5; TH = Tyrosine hydroxylase; DBH = Dopamine beta-hydroxylase; PNMT = Phenylethanolamine-N-methyl-transferase; LENK = Leucine enkephalin; MENK = Methionine enkephalin; ACTH = Adrenocorticotrophic hormone; BOM = Bombesin; CALC = Calcitonin; CGRP = Calcitonin gene-related peptide; SOM = Somatostatin; NPY = Neuropeptide tyrosine; CPON = C-flanking peptide of NPY; VIP = Vasoactive intestinal polypeptide; CrFrag = Cryptic fragment (111–122) of pre-pro-VIP; PHM = Peptide histidine methionine (13–27); NT = Neurotensin; GAL = Galanin; 5HT = Serotonin (5-hydroxy-tryptamine); F = Female; M = Male; PA = Benign phaeochromocytoma of adrenal; PUB = Benign phaeochromocytoma of urinary bladder; MPA = Malignant phaeochromocytoma, primary tumour of adrenal; MPB = Malignant phaeochromocytoma, metastasis of liver; MPC = Malignant phaeochromocytoma, metastasis of lymph node; MPD = Malignant phaeochromocytoma, metastasis of lung; nd = Not done; Grim = Grimelius' argyrophilic silver impregnation; Masson = Masson's argentaffin reaction

Phaeochromocytoma

Using conventional haematoxylin and eosin staining, the 11 benign adrenal and bladder phaeochromocytomas exhibited classical histological features (Fig. 1), showing irregular cells of variable size, structure and arrangement, supported by richly vascular connective tissue. Frequently multinucleated, giant cells were found. In the malignant phaeochromocytoma, clumps of tumour cells showed polymorphism and multinucleation. Conventional histology gave no significant information on malignant potential.

A brief summary of results obtained using silver impregnation methods and immunocytochemistry is given in Table 1. Using Grimelius' silver impregnation method, varying degrees of argyrophilia were demonstrated in all cases. The argentaffin stain of Masson gave positive reactions in 3 cases only.

Using immunocytochemistry, the cytoplasmic neuroendocrine marker neuron-specific enolase (NSE) was found in all cases. Chromogranin, known to be a marker for secretory granules, was positive in all but the malignant case. Antibodies to protein gene-product (PGP) 9.5 (Thompson

Table 2. Primary antisera used in this study. The dilutions shown gave maximal detection of antigen with lowest levels of background

Immunogen	Code	Donor	Type	PAP-dilution	IGSS-dilution
Neuron-specific enolase (human)	839	Rabbit	P	1/200	1/2000
Neurofilament protein triplet (chicken)	1302	Rabbit	P	1/400	1/2000
Glial fibrillary acidic protein (rat)	767	Rabbit	P	1/200	1/2000
S-100 a & b (bovine)	704	Rabbit	P	1/400	1/2000
Chromogranin (human)	1295	Mouse	M	1/200	1/200
7B2 (pig)	1440	Rabbit	P	1/2000	n.d.
Protein gene-product 9.5 (human)	1477	Rabbit	P	1/200	1/2000
Tyrosine hydroxylase (rat)	871	Rabbit	P	1/400	n.d.
Dopamine beta-hydroxylase	1168	Rabbit	P	1/100	n.d.
Leucine enkephalin	855	Rabbit	P	1/400	n.d.
Methionine enkephalin	869	Rabbit	P	1/400	1/4000
Adrenocorticotrophic hormone	133	Rabbit	P	1/2000	n.d.
Bombesin	627	Rabbit	P	1/4000	n.d.
Calcitonin (human)	272	Rabbit	P	1/4000	n.d.
Calcitonin gene-related peptide (rat)	1208	Rabbit	P	1/2000	1/8000
Somatostatin	1082	Rabbit	P	1/2000	n.d.
Neuropeptide tyrosine (porcine)	1086	Rabbit	P	1/400	1/2000
C-flanking peptide of neuropeptide tyrosine	1316	Rabbit	P	1/500	1/1000
Vasoactive intestinal polypeptide (VIP, porcine)	152	Rabbit	P	1/2000	1/2000
Cryptic fragment (111–122) of pre-pro-VIP (human)	YIANG2	Rabbit	P	1/200	n.d.
Peptide histidine methionine (13–27, human)	1108	Rabbit	P	1/400	n.d.
Neurotensin	810	Rabbit	P	1/1600	1/1600
Galanin (porcine)	1152	Rabbit	P	1/1000	1/1000
Serotonin (5-hydroxy-tryptamine)	644	Rabbit	P	1/10000	n.d.
Phenylethanolamine-N-methyl-transferase	1442	Rabbit	P	1/1000	n.d.

PAP = Peroxidase anti-peroxidase method. Incubation of primary antiserum for 24 h at 4° C; IGSS = Immunogold-silver staining method. Incubation of primary antiserum for 90 min at room temperature; P = polyclonal; M = monoclonal; n.d. = not done

Table 3. Secondary and tertiary immune reagents used

Antibody to	Donor	From	Code	Dilution	Method
Rabbit IgG	Goat	Miles	61-003	1/200	PAP
PAP complex	Rabbit	Miles	61-242	1/500	PAP
Mouse IgG	Goat	Miles	61-072-1	1/100	PAP
Clono PAP complex	Mouse	Miles	63-244-1	1/300	PAP
Rabbit IgG[a]	Goat	Janssen	GAR-G5	1/100	IGSS

[a] = adsorbed to 5 nm colloidal gold

IgG = immunoglobulin G; PAP = peroxidase anti-peroxidase; IGSS = immunogold-silver staining

et al. 1983; Rode et al. 1985) gave extremely consistent and clear immunostaining of almost all tumour cells (Fig. 2). Neurofilament protein triplet immunoreactivity was present in 5 cases. Of the glial markers used, only S-100 immunoreactivity was demonstrated (4 cases).

Immunocytochemistry revealed also variable numbers of tumour cells containing regulatory peptide immunoreactivities. In addition to those peptides known to exist in phaeochromocytoma tissue, other peptides could be demonstrated too. These include galanin (Tatemoto et al. 1983) which was found to be present in scattered cells or clusters of tumour cells of 6 cases (Fig. 3). Antisera to newly discovered derivatives of the precursors of neuropeptide tyrosine (NPY) and vasoactive intestinal polypeptide (VIP) appeared to give more consistent staining than those directed either to bioactive NPY or VIP. This was particularly seen with CPON, the C-flanking peptide of NPY, which could be demonstrated in numerous cells of all cases (Fig. 4). To a minor extent, this was also found for peptide histidine methionine (PHM), a second derivative of the VIP precursor. Also, antibodies to the cryptic fragment of the precursor encoding VIP gave dense positive immunostaining. However, these antibodies reacted in only very few isolated cells scattered throughout the tumour (Fig. 5).

Serial wax or semithin resin sections revealed that different peptide immunoreactivities were normally found in different tumour cells. However, in the case of peptides deriving from the same precursor, such as NPY and CPON or leucine- and methionine-enkephalin, sometimes co-localisation was found. 7B2, a peptide originally isolated from the anterior pituitary of pig (Hsi et al. 1982; Seidah

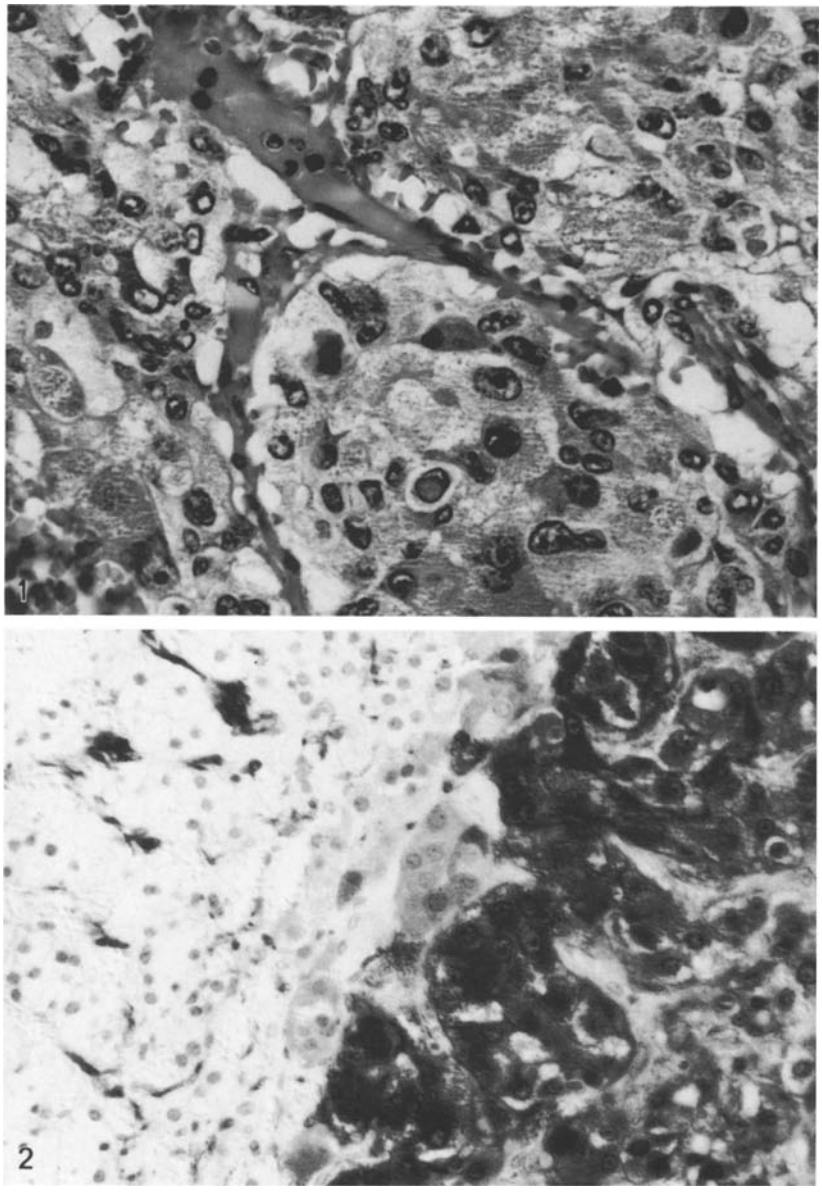

Fig. 1. Benign adrenal phaeochromocytoma, routinely stained with haematoxylin and eosin. Nests of irregular tumour cells are seen, with nuclear and cytoplasmic pleomorphism, supported by connective tissue. Bouin's-fixed, 4 μm wax section. (× 780)

Fig. 2. Adrenal gland with phaeochromocytoma, immunostained with antibodies to protein gene-product (PGP) 9.5. Tumour cells, chromaffin cells and nerve fibres give a positive reaction. Bouin's-fixed, 4 μm wax section. PAP method. (× 780)

et al. 1983), was present in scattered tumour cells in half of the cases. Cell types producing catecholamines were in part visualised with antisera to their synthesising enzymes, dopamine beta-hydroxylase (DBH) and tyrosine hydroxylase (TH), the latter being positive in more cases. Antibodies to phenylethanolamine-N-methyl-transferase (PNMT), the enzyme converting noradrenaline into adrenaline, showed positivity in groups of cells scattered throughout the tissue.

Adrenal gland

In the adrenal gland, peptides and proteins previously reported (see Introduction) could be demonstrated. Adrenal nerve cells as well as a few cortical nerve fibres were detected using antibodies to neurofilament protein triplet. The supporting glial system could be shown using antibodies to S-100. The whole adrenal medulla, and nerve fibres running between the cortical capsule and the medulla, expressed strong immunostaining for PGP 9.5.

Immunoreactivity to the peptide 7B2 was present in human adrenal medulla. Galanin was demonstrated in numerous medullary cells of cat and piglet adrenal, whereas in human adrenals only a few immunoreactive cells were found. In pig and cat, galanin-immunoreactivity was present in well-defined groups of chromaffin cells also immunoreactive to enkephalins, whereas NPY and/or its flanking peptide CPON were found in another sub-

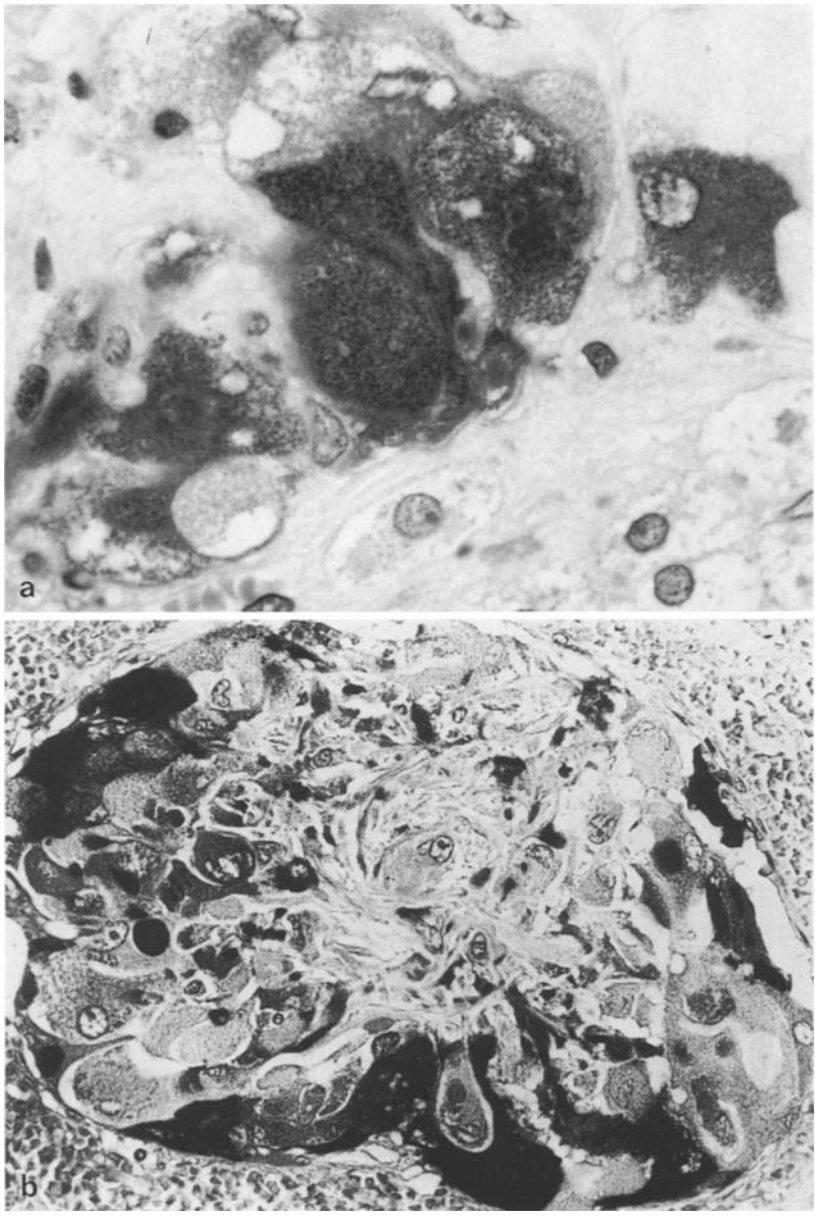

Fig. 3a, b. Clusters of tumour cells immunoreactive to galanin antibodies are seen in benign (**a**) and malignant (**b**) adrenal phaeochromocytoma. Bouin's-fixed (**a**) and formalin-fixed (**b**), 4 μm wax sections, immunostained with the PAP (**a**) and the IGSS (**b**) techniques. (×1100 (**a**) and ×900 (**b**))

population of enkephalin-containing medullary cells. Sometimes, the cells demonstrated all three or four immunoreactivities (Fig. 6). In cat and piglet adrenals, great numbers of galanin-immunoreactive cells were found, whereas in human adrenals, very few cells were immunostained. In cat adrenal cortex, very rarely, galanin-immunoreactive nerve fibres were found when using the immunogold-silver staining method, but not with the PAP technique. Also, some CPON- and NPY-immunoreactive nerves were demonstrated, often closely associated with blood vessels.

Serial sections of cat adrenal immunostained for galanin, CPON, DBH and PNMT showed that galanin immunoreactivity was colocalised with DBH immunoreactivity, but was not present in PNMT-positive cells (Fig. 7). CPON immunoreactivity is present in PNMT-immunoreactive cells and, occasionally, in galanin positive cells.

Grimelius argyrophilic silver impregnation appeared positive in almost all chromaffin cells.

Discussion

The present study was carried out to investigate whether the immunocytochemical use of a range of antibodies to general and specific markers of

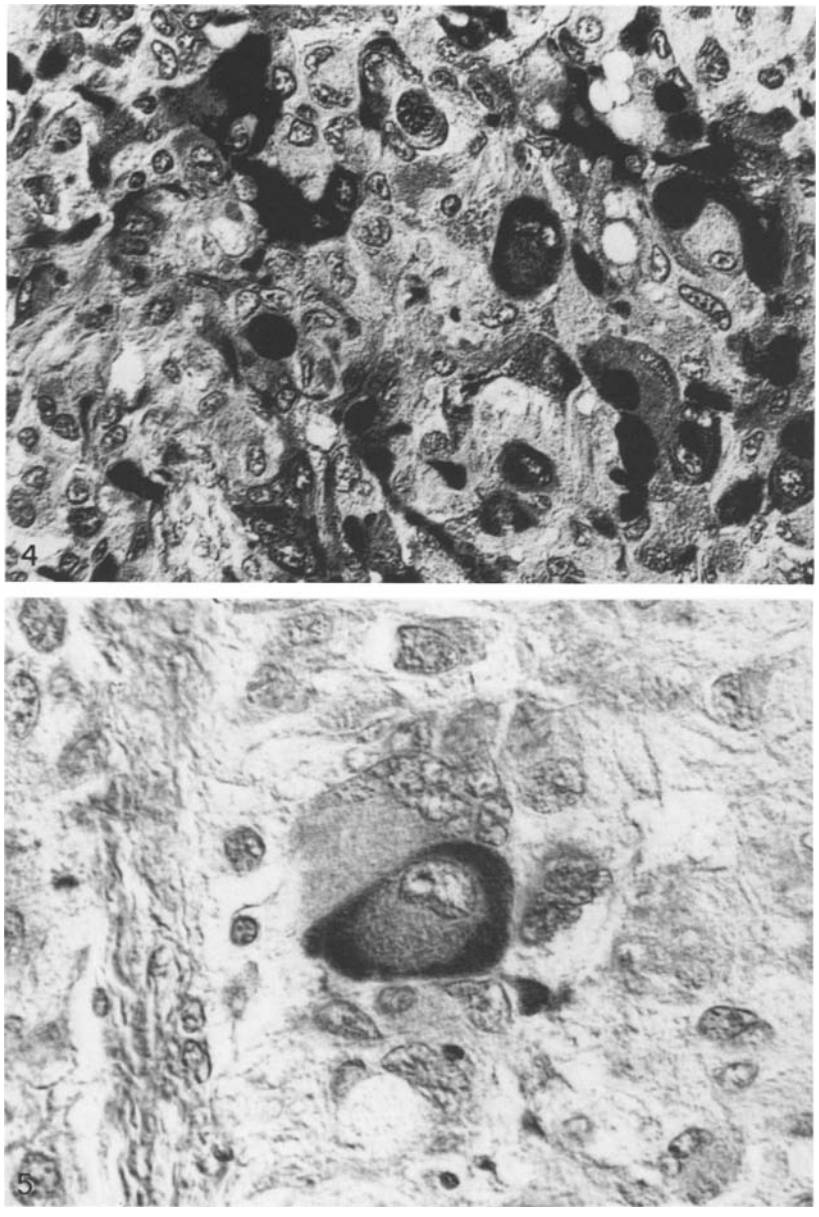

Fig. 4. Tumour cells immunoreactive to antibodies to the C-terminal flanking peptide of neuropeptide tyrosine (CPON), densely scattered throughout a benign adrenal phaeochromocytoma. Bouin's-fixed, 4 μm wax section, PAP method. (×770)

Fig. 5. Single tumour cell of a benign adrenal phaeochromocytoma immunoreactive to antibodies to the cryptic fragment of the precursor encoding vasoactive intestinal polypeptide. Formalin-fixed, 4 μm wax section, PAP method. (×900)

neuroendocrine differentiation would lead to a better characterisation of phaeochromocytomas and to relate the findings to those in the normal adrenal gland. Of the general markers, neuron-specific enolase (Schmechel et al. 1978; Bishop et al. 1982) gave the most consistent immunostaining. The enzyme was found to be present in all tumours and adrenal glands investigated, a finding which confirms the study of Tapia and colleagues (1981). Chromogranin immunoreactivity was detected in all tumours except the malignant one, a finding which may reflect the poor granulation of the tumour. The newly discovered protein PGP 9.5 appears to be a new general marker for neuroendocrine differentiation (Rode et al. 1985) since antibodies to this protein stained the chromaffin system of the normal adrenal gland and large numbers of cells in the tumours. As also demonstrated by Lehto and colleagues (1983), numerous phaeochromocytoma cells were immunoreactive to neurofilament protein triplet antibodies. Immunoreactivity for S-100 protein, reported to be an excellent marker for peripheral glia (Bishop et al. 1985; Hacker et al. 1985c), was demonstrated in 4 cases of phaeochromocytoma, as well as in normal adrenal sustentacular cells in close relationship to chromaffin cells and nerve branches, as was also reported by Lloyd and colleagues (1985).

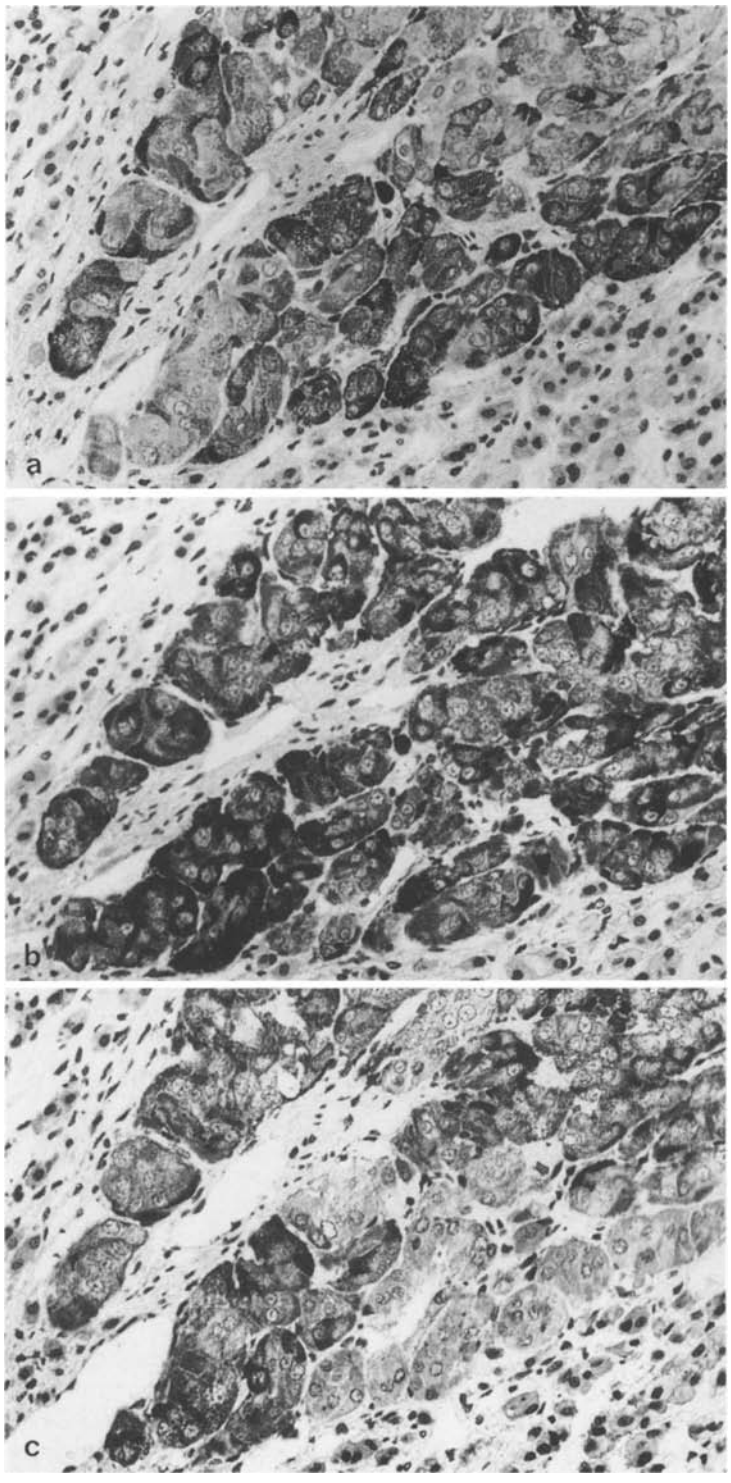

Fig. 6a–c. Consecutive serial sections of cat adrenal gland immunostained for galanin (**a**), met-enkephalin (**b**) and neuropeptide tyrosine (NPY) (**c**). Groups of cells immunostained for galanin also show immunoreactivity for met-enkephalin or for NPY. Very few cells are positive with all three antibodies. Note that the antibody to met-enkephalin revealed a high proportion of medullary cells. Benzoquinone-fixed, 3 μm wax sections, PAP method. (**a**) and (**b**) are mirror image sections, (**c**) is serial. (×400)

In the present examination, a variable number of tumour cells was demonstrated with distinct immunoreactivity to a range of peptide antisera. Similar findings have been reported previously, but using fewer antibodies (Sano et al. 1983; Hassoun et al. 1984; Lloyd et al. 1985). There are several possible explanations for this variability. Firstly, differences in technology and antibody affinities used may lead to different results. Here, masking of antigenic determinants by the fixative and the wax-embedding process used might lead to non-availability to the antibodies of specific epitopes.

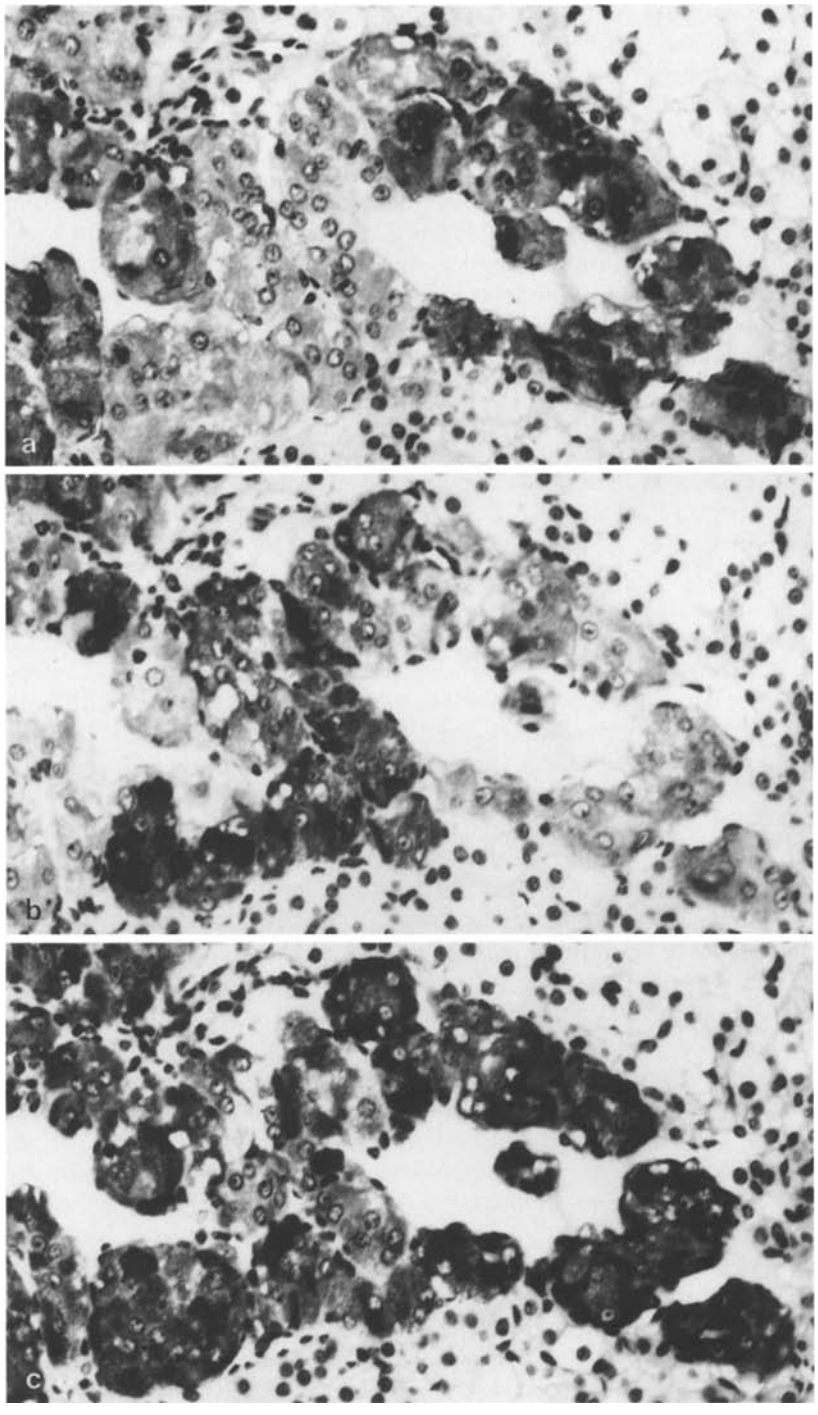

Fig. 7a–c. Consecutive serial sections of cat adrenal gland showing medullary cells immunostained for phenylethanolamine-N-methyl-transferase (PNMT) (**a**), galanin (**b**), and dopamine beta-hydroxylase (DBH) (**c**). The cells immunostained for PNMT do not show any immunoreactivity for galanin, although all medullary cells show a variable degree of DBH-immunoreactivity. Note that the cortical cells do not show immunoreactivity for any of the antisera tested. Benzoquinone vapour fixed, 3 μm wax sections, PAP method. (× 360)

Secondly, these tumours may really store variable amounts of peptides or abnormal molecular forms, as shown for other tumour types (Holst 1983; Hamid et al. 1986). Production of several hormones is the most common form of multidirectional differentiation in neuroendocrine tumours (DeLellis et al. 1984). In many cases of phaeochromocytoma, the substances produced seem identical to those found in the presumed normal cell of origin which is thought to be the chromaffin cell. However, some peptides not commonly found in this cell type may be demonstrated. These include, for instance, VIP and other derivatives of its precursor, known to be present in many types of neurons, but considered to be an ectopic product of mammalian chromaffin cells (Tischler et al. 1984).

However, such substances may also be produced by normal chromaffin cells, but in quantities too small to be detected by standard techniques. In some cases, the new enhancement technique of immunogold-silver staining might be useful (Hacker et al. 1985a) and methods of in situ hybridisation (Gall and Pardue 1971; Hutchinson et al. 1982; Coghlan et al. 1984; Lawrence and Singer 1985; Shivers et al. 1986) might help to demonstrate cells with the potential to produce a certain peptide but expressing low peptide content, by specific visualisation of peptide-encoding mRNA (Varndell et al. 1984).

The nature of chromaffin cells and neurons is not yet fully understood. When chromaffin cells are grown in culture, they extend axon-like processes, indicating their close relationship to neurons (Carmichael and Winkler 1985). It is interesting that immunoreactivity for neurofilament proteins, well known to mark neuronal structures (Bishop et al. 1985; Hacker et al. 1985c), was demonstrated by Lehto et al. (1983) in phaeochromocytoma cells and was confirmed in our study. This might have been caused by alteration of the phenotype of individual tumour cells or even normal chromaffin cells in response to changes in the microenvironment.

Co-localisation studies carried out on serial wax or semithin resin sections of the normal cat adrenal gland revealed the presence of galanin in a subpopulation of met-enkephalin-containing medullary cells, whereas NPY and CPON were found in another subpopulation. Furthermore, co-localisation of all four peptides could sometimes be shown. Recently in the central nervous system the presence of galanin in cholinergic neurons (Melander et al. 1985) and with catecholamines, vasopressin or oxytocin has been shown (Skofitsch et al. 1985). Co-localisation of other peptides with catecholamines appears well established (Viveros et al. 1979 and 1980) and was also found in our study. It seems likely, therefore, that at least a certain number of chromaffin cells have the potential to produce several peptides and catecholamines.

Serial sections of cat adrenals immunostained for CPON, galanin and PNMT show that CPON is present in cells which are positive for PNMT antibodies, whereas galanin-immunoreactive cells were PNMT-negative but positive for DBH. As PNMT catalyses the conversion of noradrenalin to adrenalin, it may be concluded that most of the CPON present in the adrenal medulla occurs in adrenalin-containing cells. Galanin, on the other hand, appears to be in noradrenalin-containing cells, immunoreactive for DBH, the enzyme which converts dopamine into noradrenalin, but negative for PNMT. Some medullary cells were demonstrated to contain immunoreactive CPON and galanin; it appears likely that these are noradrenalin cells, however, only electron microscopical studies could clarify the situation.

Antibodies to regulatory peptides are often judged as not being of any help in phaeochromocytoma diagnosis, mainly because of their variability (Hassoun et al. 1984). However, a single antiserum was found in the present study to give much more consistent and reliable results. The antiserum to CPON revealed the peptide in large numbers of tumour cells in all cases. In addition, a range of other antibodies was found to be particularly useful for revealing general or specific markers in phaeochromocytoma or normal adrenal tissue. To the best of our knowledge, the peptides PHM and the cryptic fragment of the VIP-precursor have been demonstrated for the first time in phaeochromocytoma.

Using antibodies to various derivatives of peptide precursors it appeared that not all tumours produce or store all of their derivatives. However, it seems likely that in phaeochromocytoma multiple molecular forms of peptides, both normal and abnormal, are produced, as suggested for other endocrine tumours (Holst 1983; Hamid et al. 1986). Using only one antibody to a defined "normal" peptide might result in misleading negative results. Using a panel antisera, it is often possible to demonstrate at least one of the various derivatives of peptide precursors. Some of these, especially macromolecular forms, may not have biological activity.

Clinical features of phaeochromocytomas are varied but have been considered previously to derive from increased catecholamine production by the tumour (Ch'ng et al. 1985). Little is known about the clinical manifestation of the peptides demonstrated in phaeochromocytomas. The peptides produced may act as mediators of catecholamine production, release or function or they may well cause symptoms by themselves, depending on the concentrations of biologically active molecular forms secreted. Often, the quantities of peptides released into the bloodstream can be measured by using immunoassay techniques, but sometimes tumour products cannot be detected in serum, as they act in a paracrine or local manner. In these cases, immunocytochemistry is likely to be the preferable method for detection of the product and thus tumour characterisation. The new method of immunogold-silver staining (Holgate et al. 1983; Springall et al. 1984; Hacker et al. 1985a and b)

sometimes appeared to be helpful in situations where only little antigen is available to its specific antibodies, because of masking of epitopes or the production of the substances of interest in very small amounts. In the present study, the number of cells demonstrated by each of the two immunocytochemical techniques was comparable. However, primary antibodies could often be used in much higher dilutions for the IGSS than for the PAP method. The IGSS method also appeared to be more suitable for the demonstration of nerve fibres, as shown here in nerves of the adrenal cortex.

Using silver impregnation techniques, phaeochromocytomas and normal adrenal medulla displayed very similar reactions. Sometimes, there appeared to be a high correlation between the results of Grimelius' silver staining and immunostaining for chromogranin, both being granular markers (Grimelius and Wilander 1985; Facer et al. 1985).

In conclusion, the regulatory peptide content of phaeochromocytomas appears to be highly diverse and unpredictable. Until now, this variability has ruled out the use of any peptide antiserum as a reliable immunocytochemical marker from chromaffin tumour cells. However, in the present study, some antibodies including antibodies to the peptide CPON were found to give consistent results in all cases tested. In addition, similarly consistent results were obtained with antibodies to the general neuroendocrine markers neuron-specific enolase, protein gene-product 9.5 and, in benign cases, chromogranin. Our results emphasise the importance of using antibodies to all known derivatives of peptide precursors in order to characterise tumours fully. This was found to be necessary as not all of these derivatives or molecular forms are always produced or expressed in parallel.

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