Upsala Journal of Medical Sciences

ISSN: 0300-9734 (Print) 2000-1967 (Online) Journal homepage: https://www.tandfonline.com/loi/iups20

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To cite this article: Lars Grimelius (2008) Methods in Neuroendocrine Histopathology, A Methodological Overview, Upsala Journal of Medical Sciences, 113:3, 243-260, DOI: 10.3109/2000-1967-238

To link to this article: https://doi.org/10.3109/2000-1967-238

Published online: 12 Jul 2009.

Upsala J Med Sci 113 (3): 243–260, 2008

Methods in Neuroendocrine Histopathology
A Methodological Overview

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Abstract

Light microscopy is still the main tool in diagnostic histopathology, though it does not always lead to a definitive diagnosis. It has therefore been a constant ambition to develop methods which can add further information to the diagnosis.

In endocrine pathology, a major problem has been to distinguish between neuroendocrine and non-neuroendocrine tumours. The silver stains, such as the Bodian, Grimelius and Sevier-Munger methods, were the first useful "general neuroendocrine" markers. Electron microscopy can also be useful for identifying neuroendocrine tumours.

A further step forward was the introduction of histochemical fluorescence methods, as these could identify biogenic amines. With the introduction of immunohistochemical techniques, tumours could be characterized in a more specific way regarding peptide hormones and biogenic amines content, proliferation factors, hormone receptors, etc. Another method, DNA cytometry, has been used mainly in predicting the prognosis. *In situ* hybridization can be a useful complement to the histopathological diagnosis when other methods have failed to demonstrate the neuroendocrine nature of the tumour. Some endocrine tumours, especially the well-differentiated ones, still cause diagnostic problems in predicting tumour behaviour, why further complementary methods would be of great value.

Introduction

Histopathological tumour diagnosis is still based mainly on light-microscopical structure patterns. During the second half of the 19th century, up to the late 1960s several histotechnical stains were developed. In histology and histopathology they became some help in identifying particular cell types, cell structures, and different types of neoplasms. Substances such as lipids, different types of carbohydrates (*e.g.* glycogen, mucus) could be demonstrated as well as pigment, heavy metals, calcium deposits and amyloid structures, etc. Some of them were introduced in histopathological diagnostic laboratories all over the world. Most of them were developed empirically, based on simple tinctorial reactions, while their precise chemical background remained mainly unknown (1).

The purpose of the present overview is to describe some of the tinctorial and histochemical staining methods used in neuroendocrine pathology during the 20th century and to evaluate their usefulness when compared with that of modern diagnostic procedures.

Received 17 June 2008
Accepted 27 June 2008

Silver stains

Silver stain methods have gained a wide field of applications in histology and histopathology (1). Several tissues and cell types can be identified with silver stains, including most types of neuroendocrine cells. Up to the late 1960s, the silver stains mostly used for endocrine cells and tumours were modifications of the Masson, Gros-Schultze and Bodian methods.

In 1914 Masson described a silver stain, initially developed for demonstrating neuronal structures. However, it was soon found that this stain could also visualize Kulchitsky cells in the crypts of Lieberkühn and carcinoid tumours in the small intestinal tract, a tumour type first described by Oberndorfer in 1907 (2–5). These silver-positive intestinal cells are called enterochromaffin (EC) cells. The recognition of carcinoids as neuroendocrine neoplasms was outlined by Gosset and Masson in 1914 (3).

The Masson method, empirically developed, was later modified (6–8), which improved its reproducibility (Fig. 1). This staining method, based on an ammoniacal silver solution, is a "one-step" procedure, *i.e.* the cells contain chemical substance(s) that can retain silver ions and reduce them to metallic silver without assistance from an external reducer; the cells visualized by this technique were termed "argentaffin". Baker and Pearse suggested that these argentaffin cells contain serotonin (5-hydroxytryptamine) and that the reaction product of this monoamine with formaldehyde, beta-carboline, causes the argentaffin reaction (9, 10). By using a histochemical model, the dot blot technique, Lundqvist et al. showed in 1990 that also other biogenic amines, such as Dopa, dopamine, norepinephrine, epinephrine and 5-hydroxytryptophan, can give rise to the argentaffin reaction (11).

Another principally different type of silver staining technique was also developed on an empirical basis with an initial silver impregnation of the sections fol-

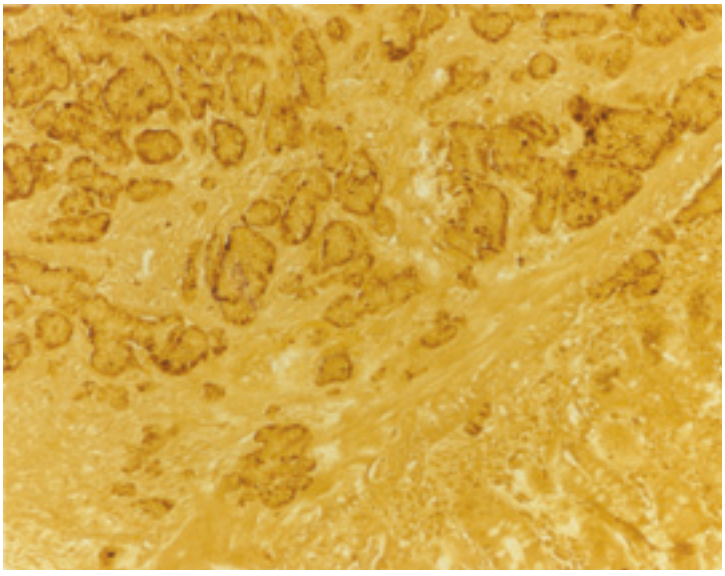

Figure 1. Midgut carcinoid tumour showing argentaffin reaction (Masson method).

lowed by a reduction process by an external reducer. The neuroendocrine cells visualized by this silver technique were called ”argyrophil”. By using various silver salts, solvent solutions, and reducing substances, different cell types and tissue components could be visualized. Most of these silver staining techniques were initially developed with focus on neural tissue but some were found to be useful also for demonstrating neuroendocrine cells and their neoplasms. One of these stains, the Gros-Schultze technique, later modified by Hamperl, demonstrated argyrophil cells in the mucosa of the gastrointestinal (GI) tract and in the islets of Langerhans (12). However, this method presented a number of technical difficulties and did not give reproducible staining results (13).

Another silver stain, the Bodian method, used a silver proteinate (“protargol”) solution for the initial impregnation of the sections, followed by reduction by an external reducer of hydroquinone and sodium sulphite (14). This stain was also initially developed for nerve structures but subsequently modified to demonstrate neuroendocrine cells in mucosa of the GI tract and endocrine pancreas (15, 16). The technique is simple, giving reproducible results, but it was found that only a few of the manufactured batches of the silver proteinate were useful.

Already in 1929/30, Davenport described an alcoholic silver nitrate stain for neuronal structures (17, 18). In Uppsala, Hellerström and Hellman, who modified this staining method in 1960, were able to demonstrate a minor fraction of non-insulin cells in rat pancreatic islets and this cell fraction was termed A1 (19). These A1 cells corresponded to an earlier described cell type in man and other mammals, called D cells (Fig. 2). Polak et al. showed in 1975, partly by a restaining technique, that these A1 cells were actually somatostatin cells, a type also occurring in the mucosa of the GI tract (20).

In 1968 an argyrophil staining method was developed in our laboratory in Uppsala, based on a low silver nitrate concentration at slightly acid pH (21). The reducer was the same as that used in the Bodian stain. The stain was modified slightly in 1980 (22). By using “dot blot” technique, Rindi et al. (23) showed that chrom-

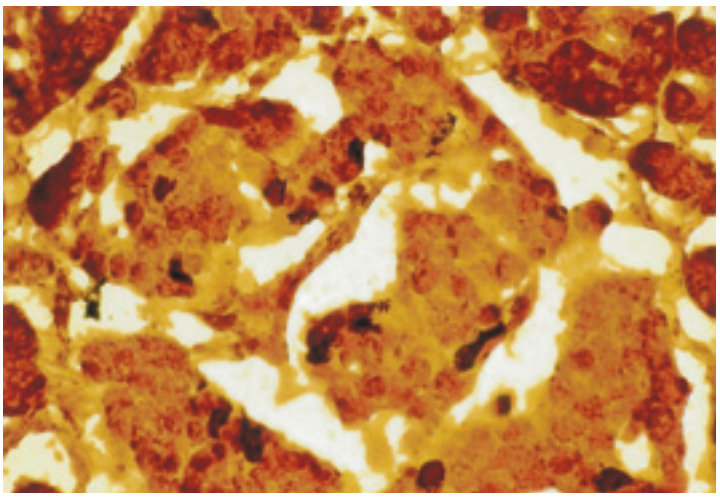

Figure 2. Human pancreatic islet stained with the Hellerstöm-Hellman modification of the Davenport technique. The black cells represent A1 (D) cells.

ogranin A gives rise to the argyrophil reaction and Lundqvist et al. (11) showed that dopamine, norepinephrine and 5-hydroxytryptamine also elicited the argyrophil reaction. The argyrophil cells visualized by the Grimelius method were found to constitute a separate cell type in the islets of Langerhans, different from the A1 and B cells in man (21).

With the introduction of immunohistochemical (IHC) methods, the Grimelius silver-positive cells could be related to specific peptide hormones and biogenic amines. They were found to correspond to the glucagon and pancreatic polypeptide (PP) cells of endocrine pancreas, but also to most cell types in the mucosa of the GI tract (Fig. 3); exceptions were insulin, somatostatin, CCK and polypeptide YY cells (22, 24). An argyrophil reaction was observed in the chromaffin cells of adrenal medulla, but not in the cortex (25). Parenchymal cells of the anterior lobe of the pituitary gland (26), and C cells of the thyroid gland were also argyrophilic, but follicular cells were not (25, 27). Argyrophil cells also appeared in parathyroid gland as well as in other organs (28–30). The neoplastic cells of neuroendocrine tumours derived from these argyrophil cells also demonstrated the silver reaction (Fig. 4). With few exceptions the Grimelius method stained almost all neuroendocrine tumours and was therefore used as a general neuroendocrine marker. A few neuroendocrine tumours (some insulinomas, some hindgut carcinoids and somatostatinomas) lacked the argyrophil reaction (22, 31).

In 1965 Sevier and Munger (32) developed another silver stain for identifying nerve structures, but this stain also visualized endocrine cells in the gastric mucosa (22, 25, 33–36) in the adrenal medulla and C cells of the thyroid gland (37).

In 1979, Churukian and Schenk modified another silver stain, originally developed by Pascual; the modified method is similar to the Grimelius stain; the impregnation solution contains a higher concentration of silver nitrate and its pH is

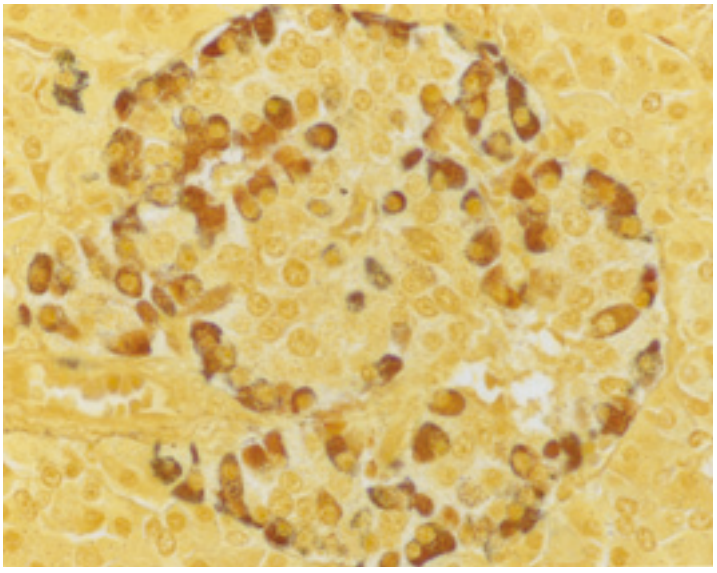

Figure 3. Human pancreatic islet stained with the Grimelius method. The silver–positive cells represent glucagon and PP cells.

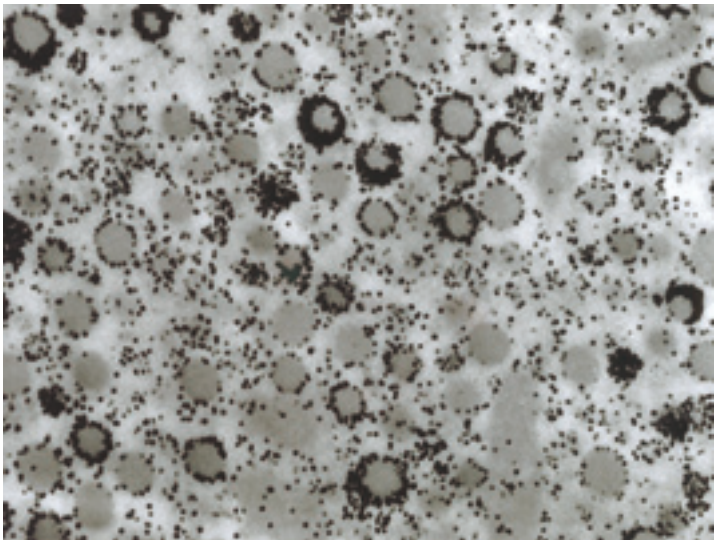

Figure 4. Electron micrograph of human pancreatic glucagon (A) cell stained with the Grimelius technique. The silver grains are concentrated over the peripheral less electron-dense mantle of the secretory granules.

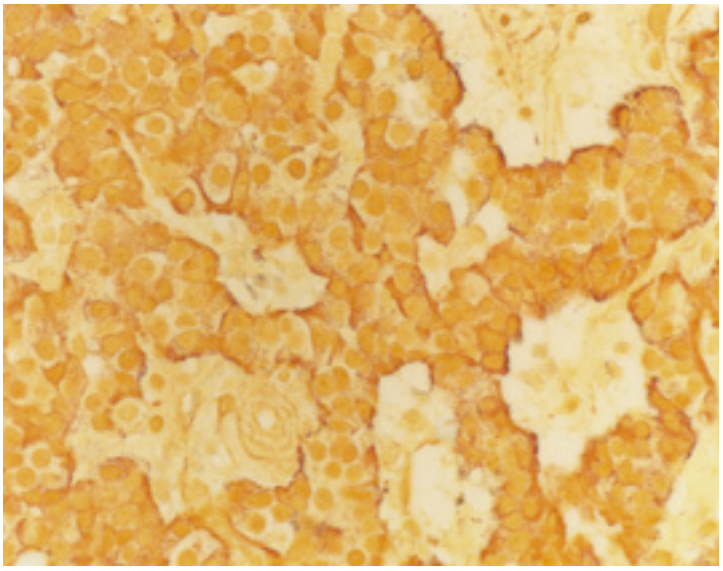

Figure 5. Medullary thyroid cancer stained with the Grimelius technique. A stronger silver reaction is seen in the neoplastic cells facing the fibrovascular stroma. The staining intensity reflects the frequency of secretory granules.

lower. The method visualizes endocrine cells in the pancreatic islets and in various neuroendocrine tumours (38).

Electron microscopical analyses of silver-stained sections showed that the metallic silver grains were located in the secretory granules in the Masson, Davenport and Grimelius stains (36, 39, 40). The frequency of silver grains varied within these granules and their localization differed to some extent in the different endocrine cell types (36, 41) (Fig. 5).

The above-mentioned silver stains used in neuroendocrine histopathology diagnosis do not give identical results. The Grimelius, Bodian, Sevier-Munger and Churukian & Schenk methods seem to visualize more or less the same endocrine

cell populations (22) whereas the Masson and Davenport methods visualize specific cell types. Therefore, when argentaffin and/or argyrophil neuroendocrine cells are described, it is important to clarify the staining method used.

A silver stain has also been used to identify so-called nucleolar organizer regions (NORs) which are believed to consist of proteins associated with ribosomal RNA activity (42), protein synthesis (43), and cell proliferation (44). The stain has been widely applied in a variety of cell kinetic studies, using the mean number of argyrophil "(Ag) NORs" of the tumour cells mainly as a marker for cell proliferation. The staining method, initially developed by Goodpasture & Bloom (42), modified by Howell & Hsu (45) and Li et al. (46), has provided additional information to predict malignancy in some endocrine tumours (47–49).

Non-silver stains

In the pre-IHC era, some neuroendocrine cell types could be visualized by stains other than silver-based. Insulin cells could be demonstrated with Gomori aldehyde fuchsin and chrome haematoxylin stains (50, 51) and also by the pseudo-isocyanin stain after permanganate oxidation (52). Basic dyes, such as toluidine blue and Azure A, were also used to identify endocrine cell types in endocrine pancreas and in the mucosa of the GI tract, in pituitary gland (anterior lobe), and C-cells of the thyroid and adrenal medulla. In addition, diazonium salts and xanthydrol demonstrated neuroendocrine cells. The bromine-alcian blue-OFG method was often used to identify acidophilic, basophilic and chromophobic cells of the pituitary gland and its tumours (53). The lead-haematoxylin method developed by MacConaill (54) and modified by Solcia et al. (25) was also used to also demonstrate neuroendocrine cells in various organs.

Fluorescence histochemical methods

Falck and Hillarp and their co-workers introduced in 1961 a gas phase fluorescence histochemical procedure to demonstrate biogenic amines in neuroendocrine cells (55, 56). This procedure was more sensitive than those previously used. The main reason was that the conventional techniques used at that time involved a water phase, in which inadvertant extraction of biogenic amines in the tissue could not be entirely avoided. The new technique was based on a condensation reaction between gaseous formaldehyde and the biogenic amines in freeze-dried tissues; some specific residues of peptides could also be demonstrated. By exposing the sections to different light excitation spectra, biogenic amines as well as some peptides could be identified (57).

When catecholamines and 5-hydroxytryptamine react with gaseous formaldehyde, the reaction products give rise to a green fluorescence with the aforementioned amines, yellow with the latter (55–57) (Fig. 6). Thus, the adrenal medullary

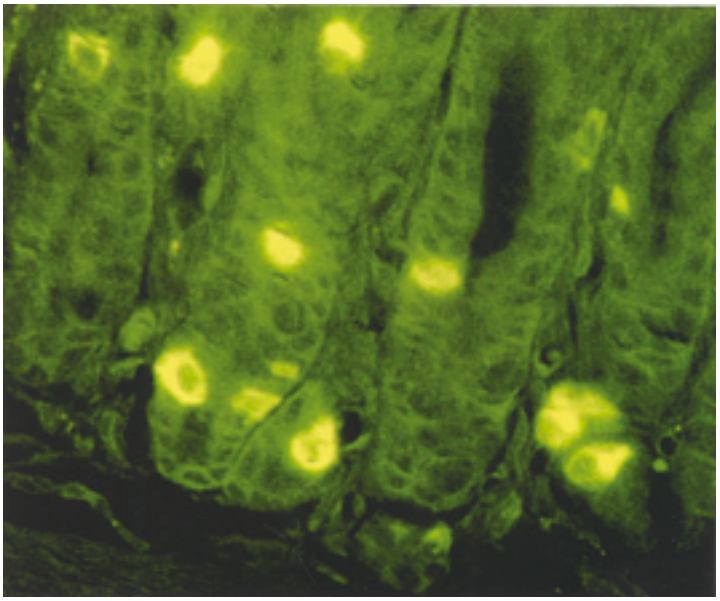

Figure 6. Unstained antral mucosa section fixed with paraformaldehyde showing enterochromaffin cells. The reaction product between paraformaldehyde and 5-hydroxytryptamine, beta-carboline, gives rise to the yellow fluorescence.

cells and the EC cells in the mucosa of the GI tract could be reliably visualized by this method. Some cells of the pituitary anterior lobe were also found to display this specific fluorescence (58).

The paraformaldehyde-ozone method is similar to the above-mentioned technique but the condensation reaction takes place in the presence of ozone which acts as an oxidizing catalyst. In histochemical models, tryptamine and peptides with the NH2-terminal tryptophan give rise to a yellow fluorescence (59, 60). With this method, ACTH, gastrin and glucagon cells as well as C-cells of the thyroid display fluorescence (61–64).

If the paraformaldehyde condensation process takes place in an acid environment (the formaldehyde-HCl method), intense fluorescence is seen in a variety of cells, not only in the neuroendocrine. Tryptophan residues, irrespective of their position in the peptide molecule, were found to give rise to fluorescence. Gastrin and other cells in the mucosa of the GI tract, as well as the pancreatic glucagon cells, were demonstrated by this method (65, 66).

Histochemical models have shown that the reaction products between o-phthaldialdehyde and histamine, as well as NH2-terminal histidine, give rise to fluorescence (67). This aldehyde probably reacts with other proteins too. Mast cells and EC-like cells are visualized by this method, as glucagon cells. All three cell types are equipped with peptide hormones or biogenic amines, containing histidine in the NH2-terminal position (67–72).

The fluorescamine method is another technique, that in formaldehyde-fixed tissue specimens can be used to demonstrate certain peptide hormone-secreting cells such as pituitary GH cells, gastrin and insulin cells, as well as the C-cells of the

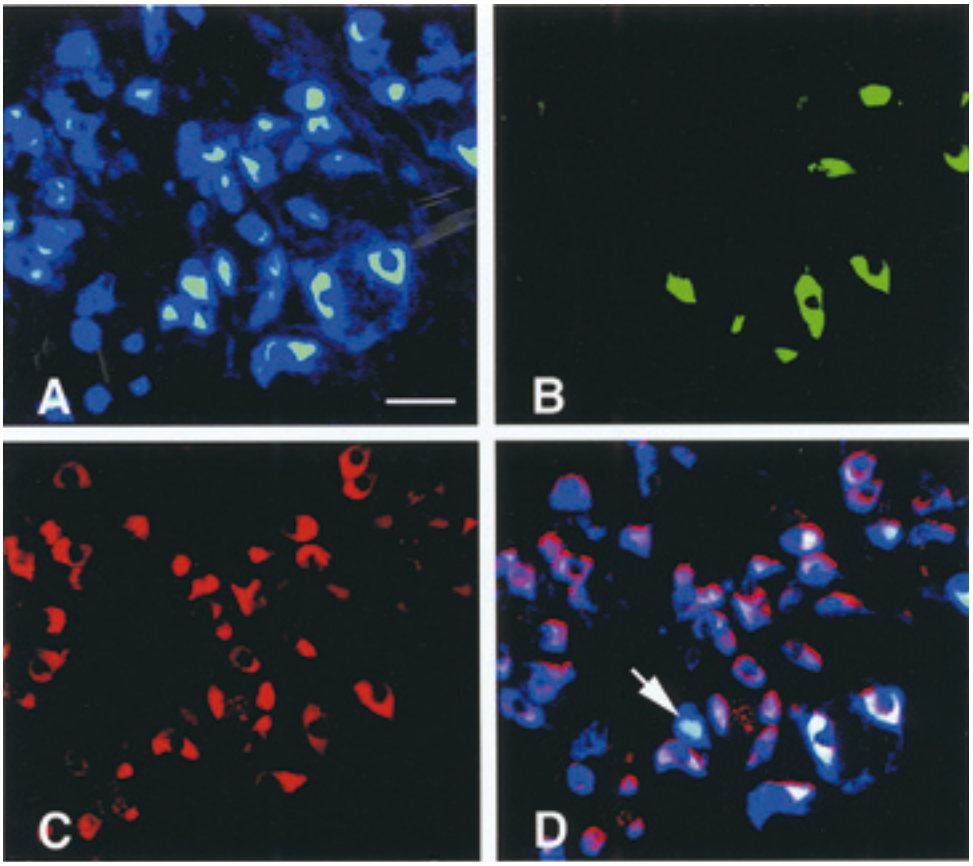

Figure 7. Human antral mucosa triple immunostained for (A) gastrin (AMCA), (B) chromogranin A (FITC) and (C) chromogranin B (Texas red). (D) Double exposure through the double-band filter for FITC and Texas red and through the AMCA filter is shown. Most gastrin cells (blue) (A) show stronger immunoreactivity to chromogranin B (red) (C) than to chromogranin A (green) (B), evident as varying intensities of magenta colour (D). A few gastrin cells are more strongly immunoreactive to chromogranin A, visualized as cyan colour (D) (arrow). The white cells in D reflect the co-localization of the three fluorochromes together.
(Reproduced, with permission, from Portela-Gomes, GM, Stridsberg, M, Johansson, H, and Grimelius, L, Journal of Histochemistry & Cytochemistry , 45 (6), 815–822, 1997.)

thyroid gland. However, it was found that this fluorescence was not related to the presence of the respective hormones but rather to some granule component(s) distinct from the hormones (73–76).

Immunohistochemistry

The immunofluorescence technique was introduced in 1941 by Coons et al. (77). Initially, a direct staining procedure was used, *i.e.* the primary antibodies were la-

belled with a fluorophore. During the 1950s, with the introduction of the unlabelled antibody (sandwich) method, the staining procedure became simpler, more sensitive and useful for the characterization of neuroendocrine cells and tumours (78). The primary antibody was unlabelled, but the second, which was raised against the immunoglobulins of the species from which the primary antibody was raised, was labelled. Initially the antibodies were polyclonal, *i.e.* raised against several epitopes. By raising antibodies to a limited amino acid sequence, their specificity was increased and the introduction of monoclonal antibodies also improved their specificity. The staining sensitivity was further increased by the introduction of the PAP (peroxidase-antiperoxidase) (79, 80), ABC (avidin-biotin complex) (81), and biotin-streptavidin methods (82, 83). Further improvements in sensitivity were achieved with tyramide signal amplification techniques and polymer-based detection systems (84, 85).

Double- and even triple-immunostaining can be performed in order to identify co-localization of two and three proteins, respectively, in the same cells in the same section (86) (Fig. 7 A–D). With this staining technique, fluorophores are preferred to chromogens, as it is possible to alternate between single and double/triple stainings by changing the filter sets. In single immunostaining, a variety of chromogens can be used, of which diaminobenzidine and amino-ethyl carbazole are the most common.

The secondary antibody can be labelled with gold particles of nano size and the protein in question can then be identified in the electron microscope (Fig. 8). An ultrastructural co-localization of different proteins can be performed by labelling the various antibodies with gold particles of different size (87).

Holgate et al. (88) introduced the immunogold-silver stain for light microscopy. The method was modified by Hacker et al. (89, 90) (Fig. 9). The secondary antibodies were labelled with gold particles of nanometer size, but these particles are too small to be identifiable under the light microscope. By applying a silver acetate autometallography method, the gold particles catalysed the reduction of silver ions to

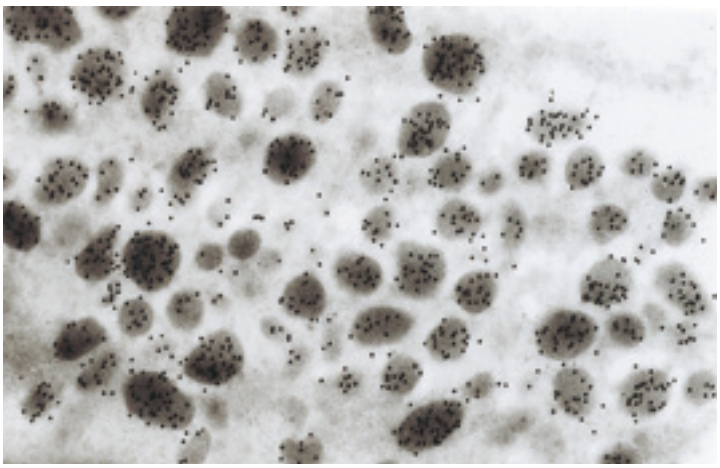

Figure 8. Electron microscopic picture of an enterochromaffin cell from rectum, immunostained with serotonin antibodies labelled with colloidal gold particles (15 nm). The gold particles are concentrated to the secretory granules.

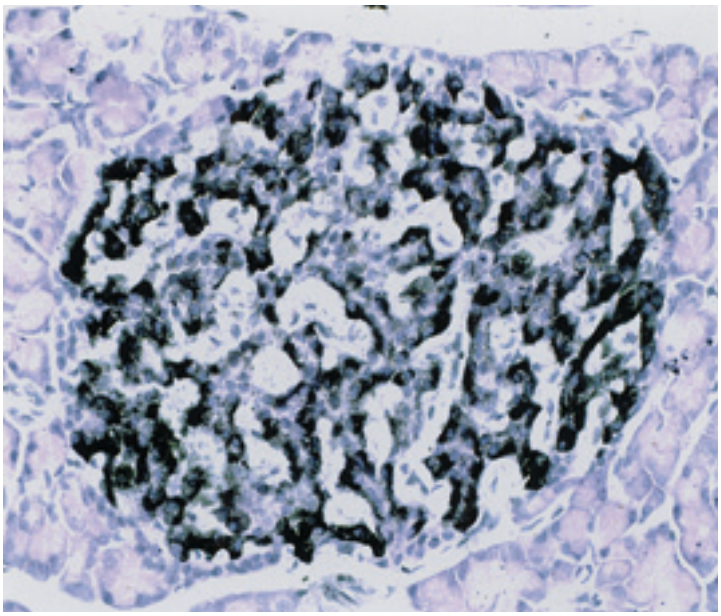

Figure 9. Human pancreatic islet immunostained with antibodies to islet amyloid polypeptide (IAPP), using the immunogold-silver technique. The peptide is produced by the insulin (B) cells.

metallic silver on the surface of the gold particles. These gold-silver particles grow in size until they become visible even under the light microscope (91).

In diagnostic histopathology, formalin is the main fixative. The fixation process induces various reactions between the fixative and tissue proteins, often with the formation of "cross-links" between protein end-groups, which may mask epitopes. However, these cross-links can be disrupted by using antigen retrieval techniques (exposure to high temperature, enzymatic, or strong alkaline treatment) which unmask the epitopes and increase the usefulness of IHC for routine histopathological diagnosis (92).

IHC techniques have become an integral part of histopathological diagnosis, particularly for tumour characterization. The IHC staining process is now largely performed, to a great extent, by using automated devices.

DNA cytometry

Cytometrical assessment of the nuclear DNA content of tumour cell nuclei was introduced as an additional tool in attempts to conduct histopathological grading of the malignancy of the neoplasms and in efforts to evaluate their prognosis, supplementary to the findings of conventional clinical and histopathological investigations.

In 1924 Feulgen and Rossenbeck (93) described a fluorescence staining method for DNA, and showed that the intensity of the fluorescence could be assessed by a microspectophotometric technique. Caspersson and colleagues developed a high-resolution scanning technique which allowed measurement of DNA by the fluores-

cence intensity of each individual cell (94, 95). These innovations formed the basis for the development of image DNA cytometry. Nowadays, this kind of analyses can be performed not only on imprints or fine-needle aspirates but also on thin, deparaffinized histopathological sections, again using a cytophotometric method based on light-transmission measurements of ”Feulgen-stained” nuclei. With the introduction of flow DNA cytometry, a greater number of nuclei could be analysed (96) but the results were invalidated by the fact that in flow DNA cytometry one cannot be certain that the nuclei analysed are neoplastic cells. However, the cytometric DNA assessment is of mainly prognostic value in neuroendocrine pathology (97–100).

Electron microscopy

When a pathologist is in doubt whether or not a neoplasm is of neuroendocrine nature, the ultimate recourse is electron microscopy, the reason being that neuroendocrine cells, by definition, contain secretory granules. Their size, density and configuration can differ somewhat between the different cell types. These differences are, however, not retained in neuroendocrine tumour cells, with some exceptions, *i.e.* the crystalline configuration of the granules seen in normal insulin cells can often be recognized in insulinomas. Ultrastructural examinations are thus used chiefly to distinguish between neuroendocrine tumours and other tumour types (101).

In situ hybridization

Neuroendocrine tumours can be functioning or non-functioning. The former tumour type gives rise to symptoms of hormonal overproduction, the latter type not. Sometimes it can be difficult to ascertain the hormone in question by immunohistochemical means, either because (i) the epitopes have become masked, or (ii) the amount of stored protein is below the detection limit, or (iii) the hormone has been released, or (iv) is not synthesized. In such a situation *in situ* hybridization can help. This technique can allow one to demonstrate the presence of mRNA sequences specific for the peptide hormone or the biogenic amine in question. Probes labelled with isotopes, chromophores (”CISH”), or fluorophores (”FISH”) are used in histopathological diagnosis (102–106). The sensitivity can be improved as in conventional IHC methods, *e.g.* by tyramide amplification (84, 107).

Another sensitive non-fluorescence method in *in situ* hybridization is the use of probes labelled with colloidal gold of nanometer size. By using the same amplification process as for the IHC immunogold-silver technique, the gold-silver particles grow in size until visible in the light microscope (108–110).

Density gradient technique

A common issue in daily routine histopathological diagnostic service is to assess the structural basis for the symptoms of a patient suffering from hyperparathyroidism. Here the parathyroid histopathological diagnosis is based on glandular weight, cellular appearance and arrangement and also clinical information. Normal parathyroid glands in adults consist of parenchyma and interstitial fat cells. The quantity of fat cells varies to some extent with age and body constitution. Thus obese persons usually have a greater fat cell content than lean persons. Even pathological glands can contain fat cells, especially hyperplastic glands. The distribution pattern of fat cells can thus vary to some extent in both normal and abnormal glands. In the histopathological diagnosis, the parenchymal cell mass plays an important role as it reflects the gland's functional state. Parenchymal cell weight can be calculated easily by using a percoll density gradient, a rapid and simple method, also useful in intraoperative diagnosis (111–112).

General conclusions

With the introduction of the Grimelius silver stain it was possible in an easy way to distinguish between neuroendocrine and non-neuroendocrine tumours. With the introduction of IHC techniques, neuroendocrine tumours can be carefully analysed regarding their peptide hormone and biogenic amine content, growth factors, hormone receptors, etc. The IHC tumour cell proliferation index is a useful complement to the conventional assessment of mitotic activity in trials to establish a prognosis.

Electron microscopy, *in situ* hybridization, and density gradient technique can also serve as complementary methods for the characterization of neuroendocrine tumours.

The traditional "old" signs of histopathological assessment of malignancy, such as the occurrence of metastases, infiltrative growth into the peritumoral tissue, and the invasion of blood and lymphatic vessels, are still the most useful signs for confirming histopathological malignancy. However, the well-differentiated and the more or less well demarcated neuroendocrine tumours still often cause diagnostic problems. Tumour size and tumour cell proliferation can be of importance to predict tumour behaviour, but the appearance of necroses and atypical mitoses can also be of important diagnostic help. For these well-differentiated tumour groups, there is a need for further diagnostic tumour markers.

Acknowledgements

I am grateful for valuable comments on this article from the present and previous collaborators: Professors Sture Falkmer, Guida M Portela-Gomes, Henry Johans-

son and Gerhard W Hacker. I also thank Professor Guida Portela-Gomes and Agneta Lukinius, PhD for contributing with Figs. 6 and 8, respectively and Apostolos Tsolakis MD, PhD for arranging the figures.

This work has been supported by the Swedish Medical Research Council and the Lions Foundation.

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