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Calcitonin–like immunoreactivity of amyloid fibrils in medullary thyroid carcinomas

An immunoelectron microscope study

Gérard Berger [1], Nicole Berger [2], Marie-Hélène Guillaud [2], Jacqueline Trouillas [3], and Jean-Louis Vauzelle [1]

[1] Laboratoire d'Anatomie Pathologique, Hôpital Edouard Herriot, place d'Arsonval, F-69374 Lyon Cédex 03, France
[2] Laboratoire d'Anatomie Pathologique, Hôtel-Dieu, place de l'Hôpital, 69002 Lyon, France
[3] Laboratoire d'Histologie (Pr Christian Girod), Faculté Alexis Carrel, rue Guillaume Paradin, 69008 Lyon, France

Summary. Using 3 polyclonal antisera directed against synthetic human calcitonin, we investigated at the electron microscope level the intra-or-extracellular fibrillar/filamentous aggregates found in 4 amyloid-rich medullary thyroid carcinomas (MTC) and in a number of other endocrine polypeptide tumours with or without demonstrable amyloid deposition. The antisera were applied by the immunogold procedure on ultrathin sections of glutaraldehyde-fixed, usually osmium-postfixed, tissues. In MTC cases, a strong labelling was present over two types of aggregates: one composed of rigid, criss-crossing fibrils 7–10 nm in diameter, suggestive of amyloid, and the other consisting of loosely arranged fibrils, 4–7 nm in width, often wavy or poorly defined. In both cases, the labelling was closely associated with that part of the sectioned fibril exposed to the antiserum. Amorphous material was sometimes present adjacent to the latter aggregates, but did not bind the calcitonin antibodies. In contrast, no labelling occurred over the amyloid deposits found in two non-calcitonin-producing endocrine tumours of the pancreas, nor over the cytoskeletal filaments stored in various endocrine polypeptide tumours. The specific value of the labelling for calcitonin-like immunoreactivity was assessed by control tests, such as absorption of the antiserum by excess calcitonin and comparative use of normal serum and antisera directed against human IgG and P component. No immunoreactivity of the MTC amyloid fibrils was found using antibodies directed against katacalcin and human prealbumin. We conclude that in tumour tissues conventionally processed for electron microscopy, MTC amyloid fibrils of varying morphology can be selectively and specifically labelled for calcitonin-like immunoreactivity.

Offprint requests to: G. Berger

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Introduction

In a previous light microscope investigation of medullary thyroid carcinomas (MTC), we found an uneven staining of the amyloid stroma by the calcitonin antiserum (Berger et al. 1983). This finding was in accordance with observations reported by Arnal-Monreal et al. (1977), Mendelsohn et al. (1978) and Goltzman et al. (1979), although contradictory results have also been presented (Charpin et al. 1982).

The current study was carried out in order to localize ultrastructurally the sites involved in this staining, a goal that could be attained on ultrathin sections of 4 double-fixed MTC using the immunogold procedure. Since amyloid has been claimed to behave like a sponge and to adsorb immunoglobulins and various plasma proteins (Van de Kaa et al. 1986), including P component (Pepys et al. 1977; Skinner et al. 1980), control tests were carefully performed and control tissues were comparatively investigated. This allowed us to answer two questions: 1) Is the immunoelectron microscope labelling helpful in the selective detection of MTC-related amyloid among other fibrillar/filamentous aggregates intra-or-extracellularly present in endocrine polypeptide tumours? 2) Does the labelling reflect a specific calcitonin-like immunoreactivity of the MTC amyloid fibrils? Further, in an attempt to better define the significance of the staining, additional labellings were carried out on the same material and on unosmicated material, using a) an antiserum directed against katacalcin, the C-terminal fragment of the calcitonin precursor

and b) human prealbumin antibodies, known to cross-react with a number of polypeptide hormones and pro-hormones (Jörnwall et al. 1981) and with the major protein found in senile cardiac (Cornwell et al. 1981) and cerebral (Shirahama et al. 1982) amyloid.

Materials and methods

Four cases of sporadic primary MTC were included in this study. Amyloid, characterized by affinity for Congo red, was present in the form of intracellular inclusions and large interstitial clumps. By the peroxidase/antiperoxidase procedure on deparaffinized sections, most of the tumour cells stained for calcitonin; only a few stained for somatostatin in one case and for ACTH in another case. The amyloid clumps variably stained for calcitonin only.

Other amyloid-rich tumour tissues consisted of one pancreatic insulinoma and one GRF (Growth Hormone-Releasing Factor) – producing carcinoid tumour of the pancreas (Berger et al. 1984b). At the light microscope level, no calcitonin-like immunoreactivity was found in these tumours.

A number of endocrine tumours that did not contain histochemically detectable amyloid was also investigated because of the presence within tumour cells of abundant cytoskeletal filaments. This group included a bronchial carcinoid whose cells contained filamentous inclusions related to cytokeratins (Berger et al. 1984a), two duodenal carcinoids whose cells stained for somatostatin or gastrin and two pituitary adenomas secreting growth hormone or prolactin.

Three polyclonal antisera directed to calcitonin were obtained from Dakopatts a/s (Glostrup, Denmark) (code n° A 576), Milab (Malmö, Sweden) (code n° B 10) and Calbiochem-Behring (La Jolla, USA) (code n° 869046). They were raised in rabbits using synthetic human calcitonin, either unconjugated (n° A 576) or conjugated to hemocyanin (n° B 10) or bovine thyroglobulin (n° 869046). When used at the electron microscope level, they were optimally diluted 1:12000, 1:5000 and 1:3000 respectively. The katacalcin antiserum, obtained from Cambridge Research Biochemicals Ltd (Cambridge, England), was raised in rabbit using katacalcin (PDN-21) bound to ovalbumin. By radioimmunoassay, no cross-reactivity in amounts up to 100-fold more than the katacalcin concentration was found with human calcitonin. The working dilution was 1:2000. IgG fractions of rabbit antisera directed to human prealbumin (Dakopatts a/s, code n° A 002), human P component (Dakopatts a/s code n° A 302) and human IgG (Nordic Immunological Lab., Tilburg, The Netherlands) were used at a dilution of 1:1000. Affinity purified goat antibodies to rabbit IgG coating gold particules of 10 nm or 5 nm mean diameter were obtained from Janssen (Beerse, Belgium).

For electron microscopy, tissues were fixed at 4° C for 1 h in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.30, post-fixed for 1 h in 1% buffered osmium tetroxide and embedded in Epon 812. In addition, unosmicated tumour material was available from one patient (case 4). Ultrathin sections were serially cut and mounted on uncoated 300-mesh nickel grids for immunoelectron microscopy or directly contrasted.

For immunogold staining the procedure was that reported by Varndell et al. (1982), except for the following modifications: a) Each section of osmicated tissue was slightly etched (H_2O_2 10% for 2 min) b) Normal goat serum used as blocking reagent was heat-decomplemented c) Primary antisera were applied for 24 h at 4° C.

The specificity of the labelling for calcitonin-like immunoreactivity was assessed as follows:

1) The primary antiserum was replaced by normal rabbit serum, complement-deleted or not, diluted 1:12000 and 1:3000, or by buffer.

2) Antibodies directed to human P component or human IgG and antisera directed to unrelated peptides (gastrin and insulin) were applied instead of calcitonin antiserum.

3) To 1 ml of calcitonin antiserum diluted 1:12000 (antiserum A 576) or 1:5000 (antiserum B 10), 20 µg of pure human calcitonin, from two different sources (Calbiochem-Behring; Cambridge Research Biochemicals) were added for 24 h at 4° C in presence of 0.1% bovine serum albumin. Then, the inactivated antiserum was applied over the sections for 24 h at 4° C and the results were compared with those obtained using the non-absorbed but similarly diluted antiserum kept at 4° C for 24 h.

In addition, cross-absorption of the katacalcin antiserum was similarly investigated adding 20 µg of calcitonin to 1 ml of the diluted antiserum.

Results

Whatever calcitonin antiserum was used at the optimal dilution, a strong labelling was present over both the secretory granules and intra-or-extracellular aggregates of fibrils in MTC. Most of these fibrils consisted of rigid nonbranching structures, approximately 7–10 nm thick, haphazardly arranged in rather dense clumps highly suggestive of amyloid. In tumours 1 and 2, the intracellular fibrils formed round inclusions together with non-reactive, fragmented or degenerative granules and various cellular debris (Fig. 1A). Some fibrils were stored within tumour cell vacuoles bounded by a non-coated membrane, or were present between cell organelles (Fig. 1B). The extracellular fibrils made up large interstitial deposits, adjacent to non-labelled collagen fibers and small aggregates closely associated with deep invaginations of the tumour cell membrane. A strong labelling also occurred over fibrils of another type, present in all the cases but predominant in tumours 3 and 4. These fibrils were thinner (about 4–7 nm in width) and loosely arranged, often wavy or poorly defined. They composed intracellular bundles (Fig. 2A) and large extracellular clumps merging into areas of floccular appearance (Fig. 2B). Fibrils of both types were sometimes found together in the same aggregates. On enlarged micrographs, the labelling over the various aggregates consisted mainly of a single 10 nm gold particle (or two or three 5 nm particles) in contact with one extremity of each fibril segment, whereas the interfibrillar spaces were not labelled (Fig. 1B and 2A). In addition, some areas composed of homogeneous or finely granular material were present between the tumour cells in case 4. They were usually adjacent to amyloid fibrillar aggregates, but, in contrast to

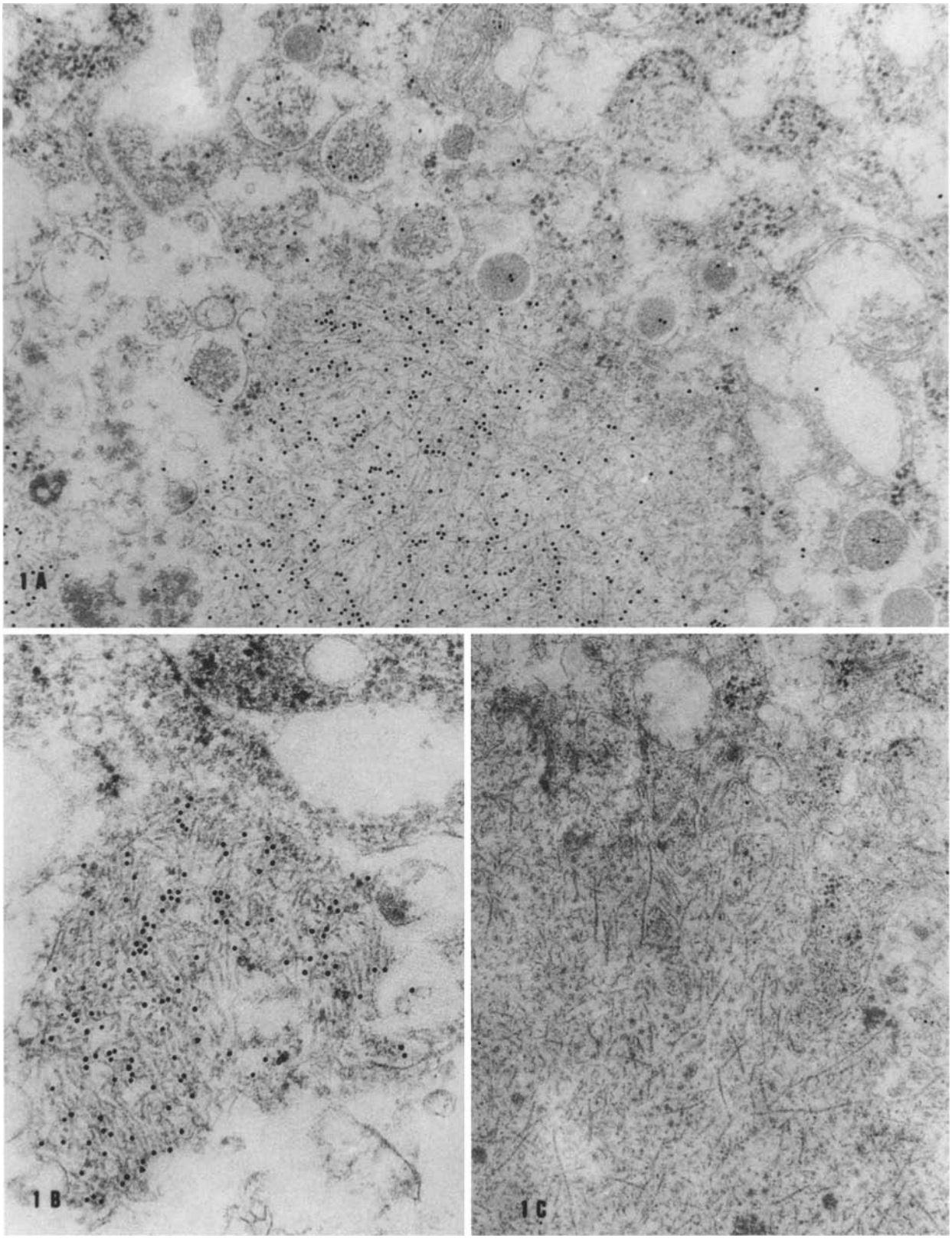

Fig. 1A–C. Immunolabelling of case 1 MTC using calcitonin antiserum code n° A 576 and 10 nm gold. **A** The gold particles are present over an amyloid inclusion composed of criss-crossing rigid fibrils and over secretory granules. ×49000. **B** Intracellular aggregate of typical amyloid fibrils approx. 7–10 nm in width. The gold particles are mainly associated with one extremity of each fibril segment. ×70000. **C** Absorption test. The fibrils do not bind the inactivated antiserum. ×42000

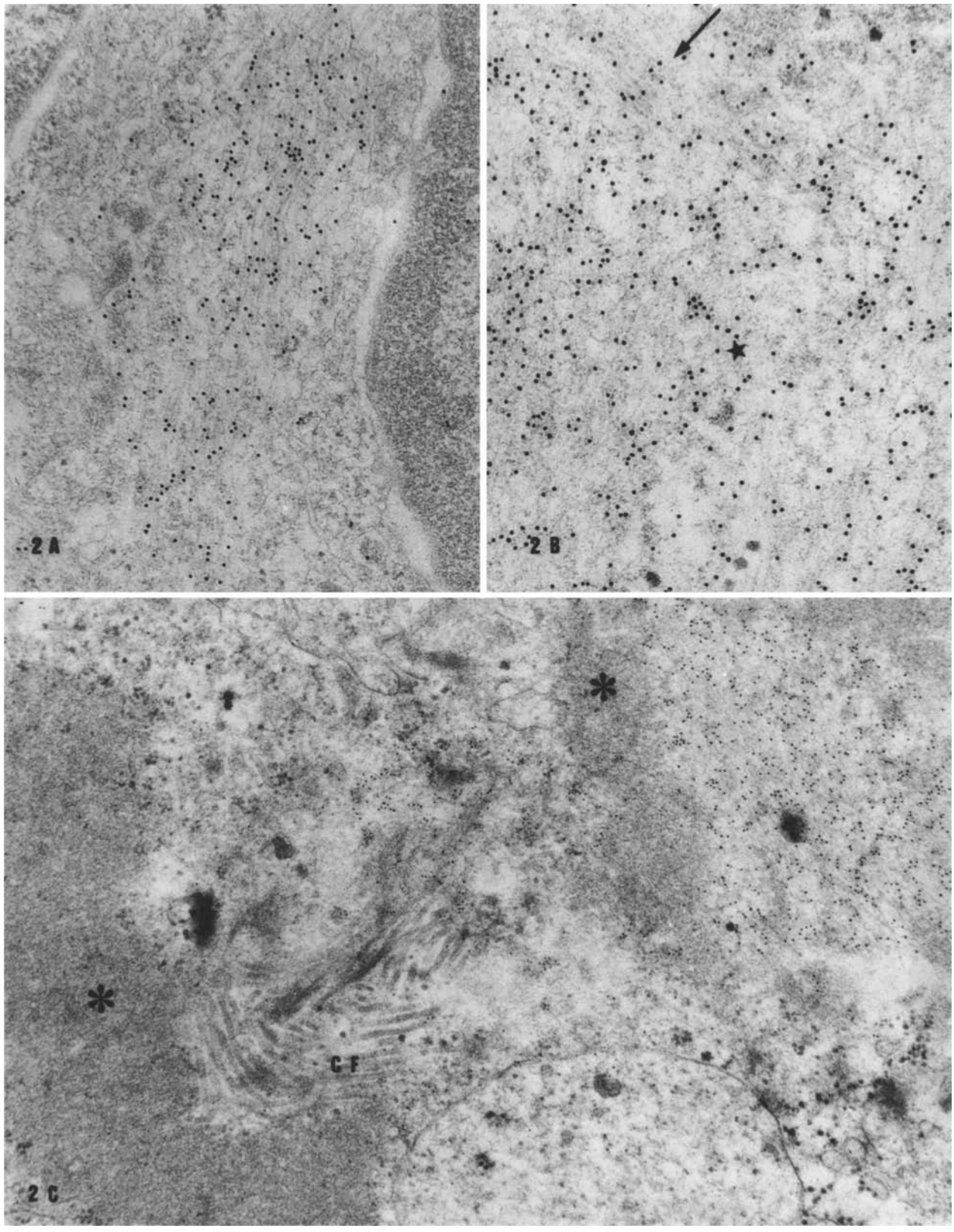

Fig. 2A–C. Immunolabelling of case 3 MTC using calcitonin antiserum code n° B 10 and 10 nm gold. **A** The labelling is located over thin amyloid fibrils of wavy appearance, forming intracellular bundles. ×42000. **B** Extracellular amyloid clump. Both the fibrillar (*arrow*) and the floccular (*star*) areas are immunoreactive. ×55000. **C** A non-immunoreactive material of homogeneous appearance (*asterisks*) is present in the vicinity of strongly labelled amyloid fibrils; microfilaments stored in a cell process (*bottom*) are not labelled. *CF:* collagen fibrils. ×35000

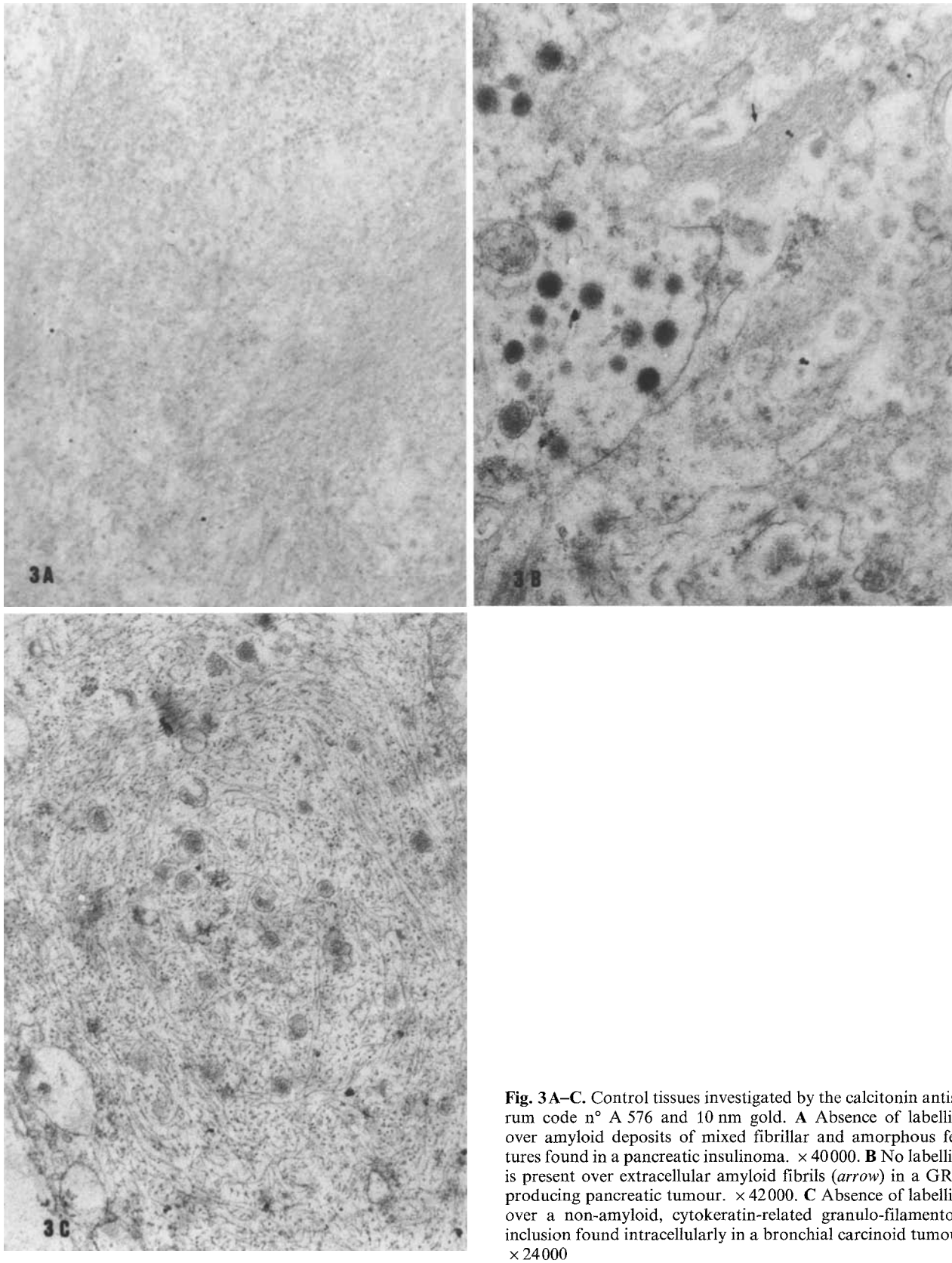

Fig. 3A–C. Control tissues investigated by the calcitonin antiserum code n° A 576 and 10 nm gold. **A** Absence of labelling over amyloid deposits of mixed fibrillar and amorphous features found in a pancreatic insulinoma. ×40000. **B** No labelling is present over extracellular amyloid fibrils (*arrow*) in a GRF-producing pancreatic tumour. ×42000. **C** Absence of labelling over a non-amyloid, cytokeratin-related granulo-filamentous inclusion found intracellularly in a bronchial carcinoid tumour. ×24000

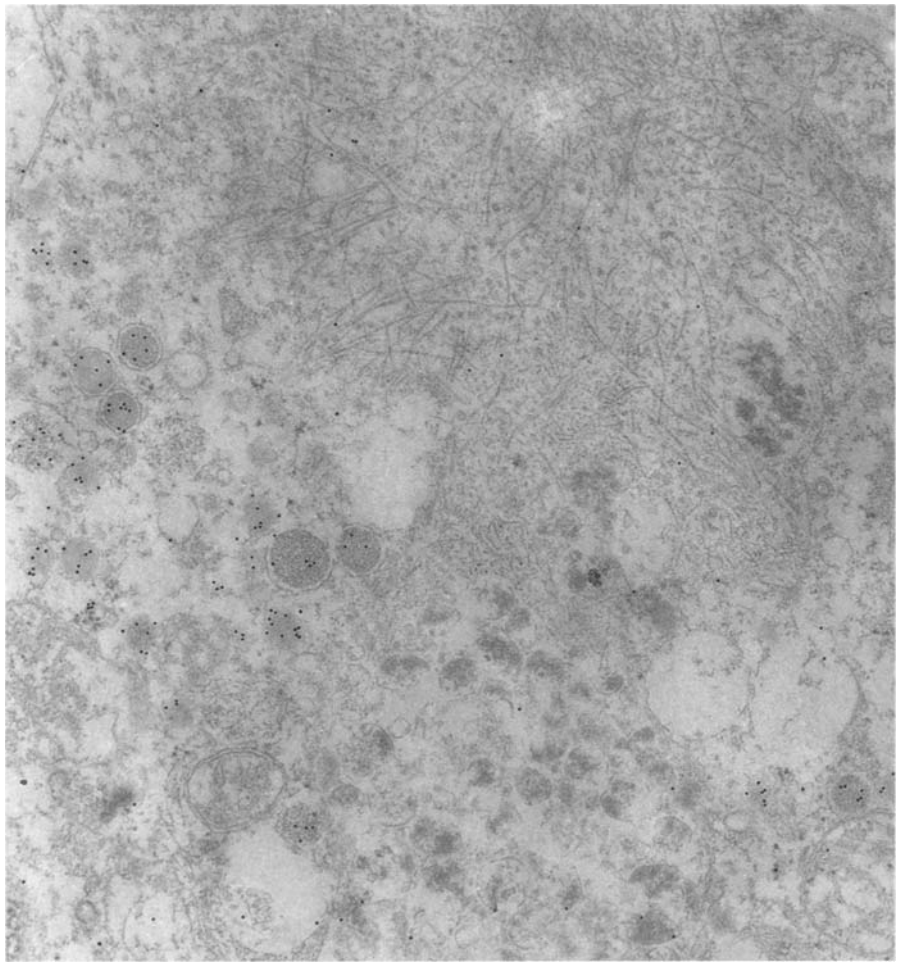

Fig. 4. Immunolabelling of case 2 MTC using the katacalcin antiserum and 10 nm gold. The tumour secretory granules are labelled, whereas the amyloid fibrils are not. Double fixation. ×38000

the latter, did not show any labelling (Fig. 2C). Lastly, non-labelled cytofilaments could be found within the tumour cells but were not a prominent feature. They consisted of a) microfilaments scattered diffusely within the cytoplasmic area or associated with microtubules b) focal deposits composed of microfilaments and polysomes and c) localized filaments (tonofilaments?) attached to junctional structures.

A large amount of extracellular amyloid was present in the insulinoma and showed a finely fibrillar or a rather homogeneous appearance. In the GRF-producing pancreatic tumour, amyloid was found in the center of acinar structures and intracellularly and consisted of thin dispersed fibrils. In neither tumour was labelling by the calcitonin antisera observed (Fig. 3A, B).

In the cells of non amyloid producing endocrine tumours, no labelling by the calcitonin antisera could be found over the intermediate filaments, arranged in whorls or bundles or making up granulofilamentous inclusion bodies (Fig. 3C), nor over the finely dispersed microfilaments.

When the calcitonin antiserum was replaced by normal rabbit serum, rabbit anti-human IgG antibodies, or antisera directed to unrelated peptide hormones, the MTC amyloid fibrils did not reveal any binding. Using P component antibodies, the result was also negative, even on unosmicated material. When the calcitonin antiserum was inactivated by excess calcitonin, no significant labelling was obtained over the MTC sections, when compared with the control sections (Fig. 1C).

In both osmicated and unosmicated materials, a strong staining by the katacalcin antiserum was obtained over the MTC secretory granules. The staining intensity was not reduced by cross-absorption of the antiserum with calcitonin. In contrast, the amyloid fibrils were unreactive (Fig. 4).

Using prealbumin antibodies, no labelling occurred over the fibrils nor over the secretory granules.

Discussion

The strong labelling that we found over MTC amyloid fibrils in presence of calcitonin antisera was quite unexpected, since no similar immunoreactivity has been mentioned in previous investigations of such tumours by post-embedding immunoelectron microscopy. However, reagents and procedures adopted in most of these studies differed from the current ones, including the use of peroxidase (Charpin et al. 1983) or 20 nm gold (Dämmrich et al. 1984) and/or brief incubations of poorly diluted antisera (Charpin et al. 1983; Dämmrich et al. 1984; Suzuki et al. 1985). In our experience, the use of 10 nm or 5 nm gold (a particulate label not so prone to steric hindrance problems as the large-sized particles) is essential to obtain a clear-cut staining of the fibrils. In preliminary investigations, carried out by the peroxidase antiperoxidase method or by the 20 nm or 40 nm immunogold procedure, we too were unable to recognize the staining of MTC amyloid fibrils, considering it to be a DAB diffusion artefact or an excess of background labelling. In fact, the present procedure is very similar to that used by Sikri et al. (1985) to investigate two MTC, but these authors did not mention the presence of amyloid in the sections. However, our results can be compared with those obtained by Huang et al. (1978), who investigated one MTC by the indirect ferritin pre-embedding method; indeed, particles of ferritin were demonstrated at the peripheral zone of cytoplasmic amyloid inclusions, but no labelling was present over the extracellular amyloid. This may be due to inadequate penetration of the label. Further, although our results were similar using any of the three calcitonin antisera, we cannot exclude that some antisera directed against this peptide may fail to stain MTC amyloid, because of the region specificity or other biological characteristics.

In contrast, we did not find any labelling over control fibrillar/filamentous structures, that is, the amyloid in non-calcitonin-secreting endocrine tumours and cytoskeletal filaments present in endocrine polypeptide tumours (including MTC). Since the procedure allows conventional fixation and processing of tissues and results in fairly good ultrastructural morphology, it appears to be useful as a diagnostic tool to detect and localize MTC-related amyloid fibrils. Moreover, the immunological nature of the labelling, indicating a specific calcitonin-like immunoreactivity, was strongly suggested by a number of control tests, such as absorption of the primary antisera with synthetic human calcitonin or comparative use of non-immune rabbit serum and rabbit anti-human IgG antibodies. These tests, together with the use of highly diluted calcitonin antisera, exclude a confusion with a non-specific trapping by the fibrils of either an unrelated plasma component or one of the staining reagents. Our failure to detect P component, a serum glycoprotein present in saline extracts of all amyloids including that of MTC (Sletten et al. 1976) in this material, is probably related to the post-embedding methodology since Breathnach et al. (1983) were able to detect it in localized cutaneous amyloidosis, using the pre-embedding method. However, our results are consistent with what is known regarding the chemical composition of MTC amyloid. As demonstrated by Sletten et al. (1976), the fibrils consist of a major protein including an amino-acid sequence of 11 residues corresponding to residues 9 to 19 of human calcitonin. This unidentified protein, designated AEt, is considered as a calcitonin precursor or fragment of precursor, because it is much larger than the human hormone. The common amino-acid sequence may account for the property of MTC amyloid to cross-react with a number of calcitonin antisera in both immunochemical (Takahashi et al. 1977) and immunocytochemical investigations.

In contrast, even in unosmicated tissue, the amyloid fibrils of MTC did not bind the antibodies directed to katacalcin or human prealbumin. Since, in agreement with Sikri et al. (1985), we found a strong staining of the secretory granules for katacalcin, the C-terminal sequence of the human calcitonin precursor is probably not represented (or is significantly modified or masked) in the AEt protein. Our failure to stain either the secretory granules or the amyloid deposits of MTC for human prealbumin is consistent with the light microscope immunocytochemical findings of Bussolati et al. (1984). These negative results do not favour the presence of any significant sequence homology between this serum protein and the calcitonin-related peptides (including AEt) stored in MTC.

Finally, our results indicate a morphological heterogeneity of amyloid fibrils in MTC. In addition to typical deposits, characterized by criss-crossing rigid fibrils 7–10 nm in width, we found aggregates of rather wavy fibrils, measuring 4–7 nm in width, making up intracellular bundles, or loosely arranged in the extracellular spaces, including areas of floccular appearance. Since the latter structures were also present in conventional sections, they could not represent etching artefacts. Variations in fibril morphology have been noted in systemic or localized amyloidosis (Glenner et al. 1974; Glenner 1980). The differences in fibril width

seem to depend on the state of lateral aggregation of protofibrils, as suggested by high resolution microscopy of extracted amyloid (Cohen and Shirahama 1973). Wavy fibrils of small diameter, thought to be protofibrils, have been extracted from amyloid of human pancreatic islets (Westermark et al. 1977) and extractions from MTC amyloid resulted in somewhat similar material (Westermark 1975). Considering that both types of fibril were equally immunoreactive, our results suggest a strong similarity, if not identity, of the respective major proteins, and, thus, are consistent with the protofibril-fibril concept. However, the reasons that govern the predominance of one type of fibril in each tumour and the various patterns of fibrillar organization are unclear. The amorphous or finely granular material closely associated with some aggregates of thin fibrils raises another problem, since it did not bind the calcitonin antibodies. A morphologically similar material has been previously noted within MTC cells (Charpin et al. 1982) and may also represent a large component of the extracellular amyloid produced by human islets of Langerhans (Westermark 1977) and human insulinomas (see one of our control cases). Although Westermark suggested that in islet amyloid this material may consist of fibrils too thin to be visualized by conventional electron microscopy, the present data indicate that in MTC it may differ in chemical composition (or nature) from the amyloid fibrils.

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