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REVIEW

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Applications of immunogold and lectin-gold labeling in tumor research and diagnosis

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Abstract Immunohistochemistry and carbohydrate histochemistry have had an enormous impact on both tumor research and diagnosis. In particular, immunogold labeling has provided significant advantages over classical fluorescence and enzyme-based techniques. In light microscopy, the silver-intensified gold labeling has proven highly sensitive and precise in localization. In electron microscopy, the gold particle marker was a prerequisite for successful and unequivocal antigen detection in electron-dense cellular structures such as secretory granules. In this review we demonstrate the usefulness of light and electron microscopical gold labeling techniques as applied in tumor research and diagnosis. The examples include expression of β-1,6 branches and specific sialoglycoconjugates in colon carcinoma, b-12 carbohydrate epitope in breast carcinoma, polysialic acid in neuroendocrine tumors of lung, adrenal and thyroid, as well as studies on proinsulin to insulin conversion in insulinomas. In addition, practical hints for prevention of background staining, tissue fixation, and silver intensification of gold labeling are given.

Introduction

Histochemical techniques have always been important tools in tumor research and diagnosis and modern approaches such as immunocytochemistry and in situ hybridization have initiated a revolution. The immunoperoxidase and immunogold techniques provided a degree of sensitivity which made them suitable for applications on conventionally fixed and paraffin-embedded tissues (Hsu and Raine 1984; Nakane and Pierce 1966; Roth and Warhol 1992; Roth et al. 1992b, c; Springall et al. 1984; Sternberger et al. 1970). Furthermore, the introduction of sensitive, non-radioactive detection systems additionally advanced the methods of in situ hybridization and in situ PCR (Embretson et al. 1993; Humbel et al. 1995; Komminoth 1992; Komminoth and Long 1993; Komminoth et al. 1992; Lamarcq et al. 1995; Long et al. 1993; Nuovo et al. 1992; Roth et al.1992b; Thiry 1995; Zehbe et al. 1992). Antigen retrieval techniques such as microwave pretreatment of sections from paraffin-embedded tissues (Boon and Kok 1988; Werner et al. 1996) and signal intensification techniques (Adams 1981; Danscher and Rytter Nörgaard 1983; Holgate et al. 1983; Werner et al. 1996) provided the basis for immunocytochemistry and in situ hybridization to be successfully performed on samples which were fixed under various conditions. Very recently, it has also been demonstrated that pretreatment of tissue sections with sodium dodecyl sulfate permits the use of antibodies raised against denatured antigens which otherwise failed to work (Brown et al. 1996). Further useful techniques, especially for studies on cellular kinetics in tumors, are represented by the technique of silver staining of nucleolar organizer regions (AgNOR) (Li et al. 1995; Öfner and Schmid 1996; Ploton et al. 1986) and techniques to detect internucleosomal DNA fragmentation occurring in apoptosis (Ansari et al. 1993; Gavrieli et al. 1992; Wijysman et al. 1993).

A common phenomenon occurring in malignant tumors is changes in the composition and structure of cell surface glycoconjugates (Brockhausen 1993; Dennis 1992; Feizi 1985; Hakomori 1989) which can be detected by the use of lectins and monoclonal anti-carbohydrate antibodies. Furthermore, in the diagnosis and classification of neuroendocrine tumors, immunocytochemistry has become indispensable (Roth et al. 1996). In particular, immunogold labeling has had a major impact on electron microscopic analysis of neuroendocrine cells and tumors derived from them (Roth 1983c, Roth et al. 1981).

In this paper, examples of studies on tumor-associated glycoconjugates and on neuroendocrine tumors using lectin-gold and immunogold labeling are reviewed. This is preceded by notes on prevention of background stain-

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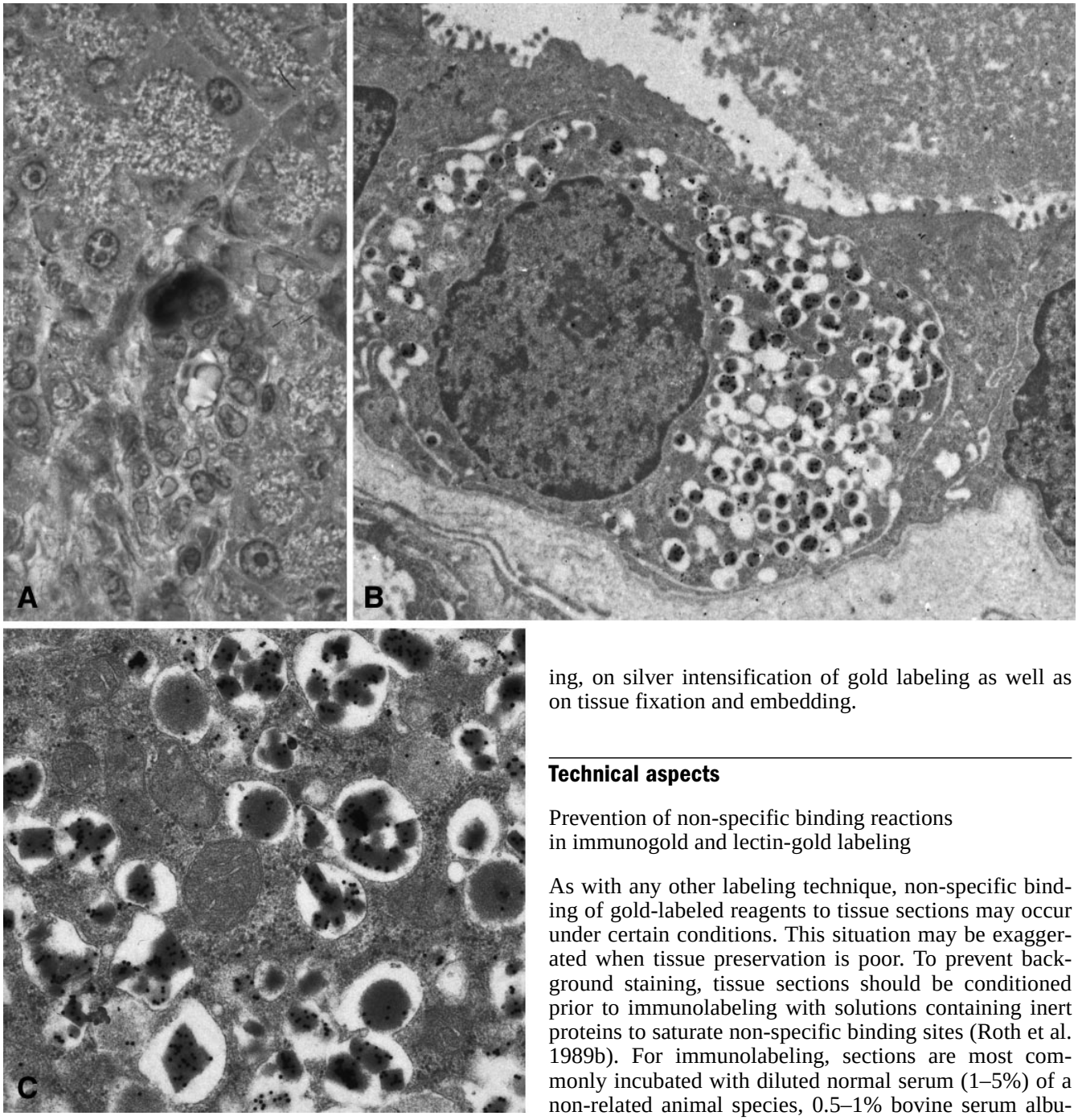


Fig. 1A–C Detection of insulin immunoreactivity with a polyclonal antiserum applying the protein A-gold technique. **A** Rat pancreas, Bouin's fixative, paraffin embedding. A single insulin immunoreactive cell is present in a secretory duct. **B** Rat pancreas, 1% glutaraldehyde, Epon embedding. A single cell exhibiting gold particle labeling for insulin over the secretory granules is located between secretory duct epithelial cells. **C** Human pancreas, 1% glutaraldehyde followed by 1% osmium tetroxide, Epon embedding. Pretreatment of the thin sections with periodic acid permits the detection of insulin immunoreactivity. (**A** ×250; **B** ×3500; **C** ×14000)

ing, on silver intensification of gold labeling as well as on tissue fixation and embedding.

Technical aspects

Prevention of non-specific binding reactions in immunogold and lectin-gold labeling

As with any other labeling technique, non-specific binding of gold-labeled reagents to tissue sections may occur under certain conditions. This situation may be exaggerated when tissue preservation is poor. To prevent background staining, tissue sections should be conditioned prior to immunolabeling with solutions containing inert proteins to saturate non-specific binding sites (Roth et al. 1989b). For immunolabeling, sections are most commonly incubated with diluted normal serum (1–5%) of a non-related animal species, 0.5–1% bovine serum albumin or egg albumin for 5–10 min at room temperature. Conditioning solutions containing albumin may be further supplemented with Triton X-100 (0.01–0.1%) and Tween 20 (0.01–0.1%). Most effective for paraffin sections from surgical or autopsy specimens is defatted milk powder dissolved in phosphate-buffered isotonic saline. The concentration of the defatted milk powder may vary between 1% and 4%. For lectin-gold labeling, conditioning with isotonic buffered salt solutions containing 0.05% Tween 20 has proven to be highly effective.

Another factor of paramount importance is the proper dilution of the gold-labeled reagents, since they will produce non-specific binding if applied in too high a con-

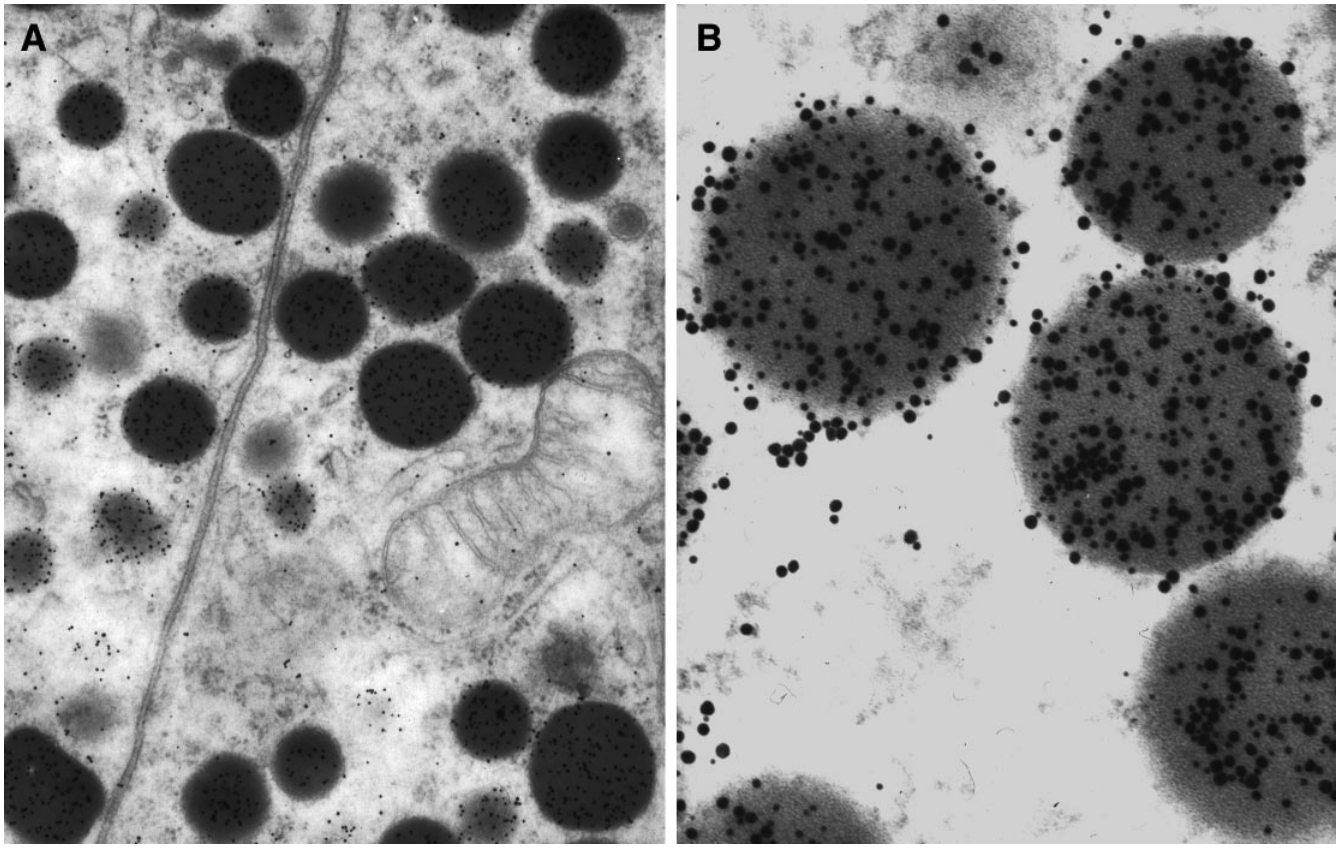


Fig. 2A, B Human pituitary, glutaraldehyde/osmium tetroxide fixation, Epon embedding, protein A-gold technique. Pretreatment of thin sections with periodic acid allows the detection of growth hormone (**A**), and the co-existence of growth hormone (large gold particles) and prolactin (small gold particles) in secretory granules by double protein A-gold labeling. (**A** ×23000; **B** ×95000)

centration (Roth 1989; Roth et al. 1989b). An objective measure for the dilution of gold-labeled reagents is the absorbance at 525 nm as determined with a standard spectrophotometer. From our experience we recommend the use of the following dilutions of protein A-gold and IgG-gold complexes. For protein A-gold made with 15- or 20-nm gold particles, A_{525nm}=0.4; for protein A-gold made with 6- to 10-nm gold particles, A_{525nm}=0.06; for secondary IgG-gold made with 15- or 20-nm gold particles, A_{525nm}=0.1–0.2 and for secondary IgG-gold made with 6- to 10-nm gold particles, A_{525nm}=0.05–0.1. These are standard dilutions which are used, independent of the dilution of the primary antibody and the tissue or cell type under investigation. These values also apply for gold-labeled anti-digoxigenin antibodies used in conjunction with digoxigenin-conjugated lectins (Sata et al. 1990) and probes for in situ hybridization (Komminoth et al. 1992, 1995).

Tissue fixation and embedding

Mono- and oligosaccharidic structures of sugar side chains of proteins are less affected by aldehyde fixation than proteins (Roth 1983a, b, 1989). Thus, section pretreatment by microwave or other antigen retrieval techniques (Werner et al. 1996) are not required, even after uncontrolled aldehyde fixation and standard paraffin embedding. For electron microscopic analysis, Lowicryl K4M embedding and ultrathin cryosections are most suitable. Furthermore, many of the peptide hormones synthesized and stored by neuroendocrine cells and their tumors can be detected even after prolonged glutaraldehyde fixation followed by embedding in resins such as Epon (Fig. 1) (Roth et al. 1978, 1981). Retrospective studies on aldehyde/osmium-fixed and Epon-embedded tumors may be possible by section pretreatment with sodium metaperiodate (Bendayan and Zollinger 1983) or periodic acid (Figs. 1, 2) (Roth et al. 1985).

Silver intensification of gold labeling

The silver intensification of immunogold or lectin-gold labeling in tissue sections (for review, see Lackie 1996) provides several advantages such as: (1) primary antibodies or lectins and secondary gold-labeled reagents can be used in dilutions as applied for electron microscopy; (2) greatly improved detection limit (Holgate et al. 1983); and (3) high contrast due to the black tint (Holgate et al. 1983; Taatjes et al. 1987). The introduction of silver acetate as the silver ion source was of great practical importance since its relative insensitivity towards light allows the entire procedure to be per-

formed under normal daylight (Hacker et al. 1988; Skutelsky et al. 1987). The silver-intensified gold particle labeling results in a non-diffusible, permanent staining. Sections can be counterstained with nuclear fast red, with hematoxylin alone or in combination with eosin and then dehydrated and mounted in xylene-based mountants.

Commercial kits for silver intensification are avilable. In our laboratories we perform the following procedure, which has proven to be reliable and highly reproducible over the last 10 years. For the visualization of the immunogold or lectin-gold labeling, slides with the sections are placed vertically in solution B (0.5% w/v hydroquinone in 0.05 M citrate buffer, pH 3.8) to which an equal volume of distilled water has been added for 2–5 min at ambient temperature. Then they are transferred to the developer consisting of equal volumes of solution B and A [0.2% w/v silver acetate (Fluka) in double-distilled water] and incubated for 18–25 min at 20–22° C. This is followed by brief rinses with double-distilled water and immersion in a photographic fixative (e.g., Superfix, Agfa, 1:9 dilution) for 2–3 min. After a thorough rinse with tap water, the sections are counterstained as required and mounted. Solutions A and B can be kept in the refrigerator for about 1 week.

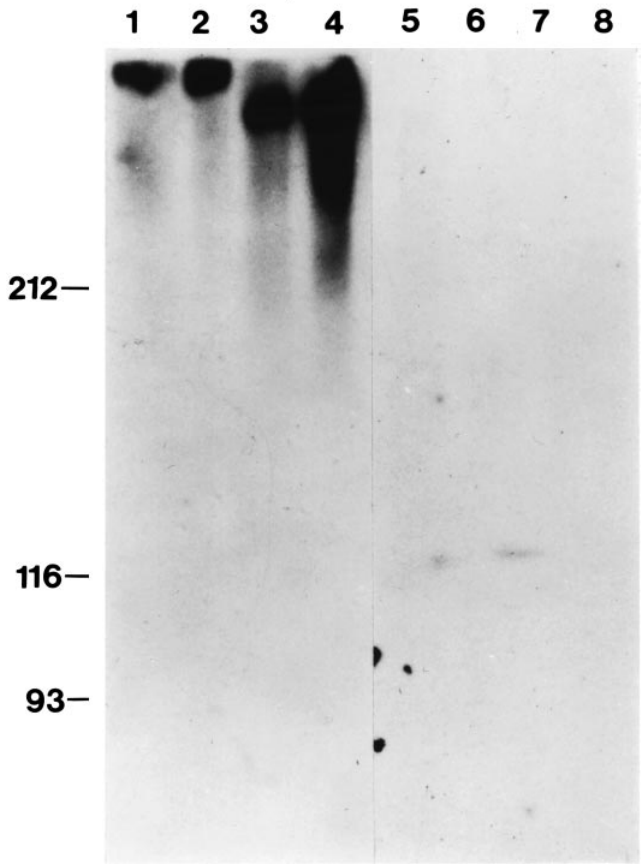


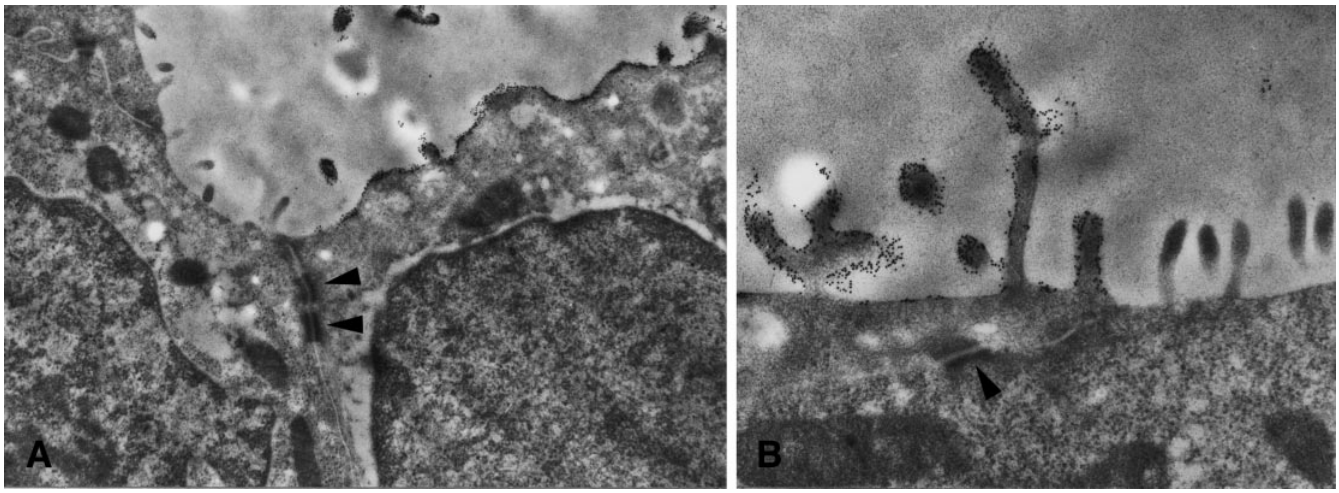
Fig. 3 Western blot analysis of invasive ductular breast carcinomas by mAb b-12. Broad immunoreactive bands of the high molecular mass (>350 kDa) are visible (*lanes 1–4*). These bands become undetectable following β-elimination to remove *O*-glycosidically linked oligosaccharides of protein aliquots of the same carcinomas (*lanes 5–8*)

Applications in tumor research and diagnosis

Studies on β-1,6 branches of asparagine-linked oligosaccharides in colon carcinoma

In malignant tumors, a most commonly observed change is the formation of larger complex-type asparagine-linked oligosaccharide side chains (Dennis 1992; Yamashita et al. 1984) due to an increased number of the *N*-acetylglucosamine β-1,6 mannose α-1,6 mannose β1 structure of the mannosyl core, the so-called β-1,6 branches. The increase in β-1,6 branches has been shown

Fig. 4A, B Normal human breast duct epithelium, Lowicryl K4M thin sections, protein A-gold technique. The apical parts of duct epithelia are shown with monoclonal antibody (mAb) b-12 positive and negative labeling along the apical plasma membrane. Note the absence of gold particle labeling along the lateral plasma membrane. (*Arrowheads* Desmosomes). (**A** ×14000; **B** ×23000)



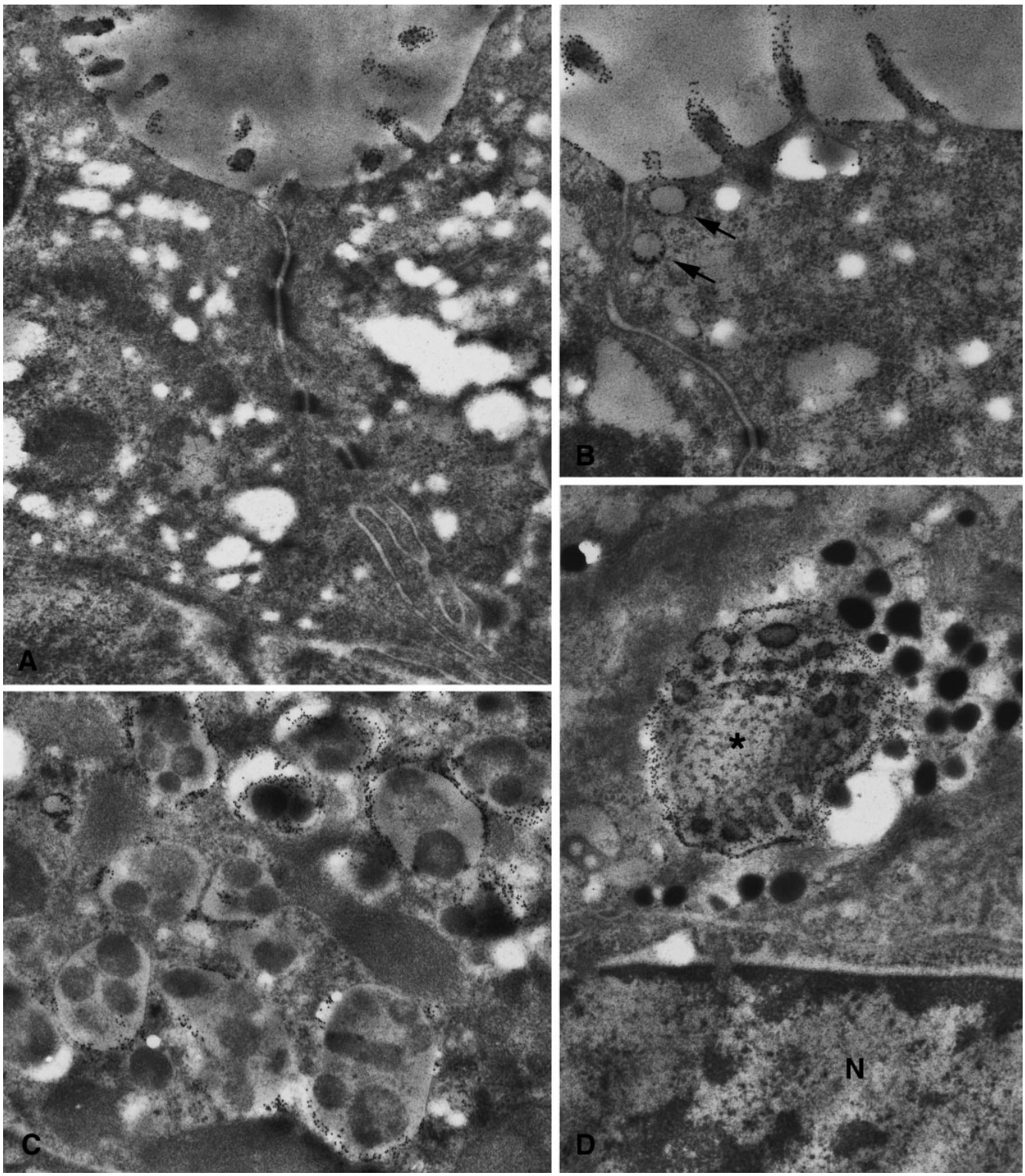


Fig. 5A–D Same tissue and methods as in Fig. 4. **A, B** Immunolabeling with mAb b-12 is restricted to the apical plasma membrane and also detectable at the limiting membrane of cytoplasmic vesicles (*arrows* in **B**). **C** The limiting membrane of secretory granules exhibits immunolabeling. **D** The membrane of an intracytoplasmic lumen (*asterisk*) is labeled whereas secretory granules with highly electron dense cores are unlabeled. (*N* Nucleus). (**A, C, D** ×25000; **B** ×46000)

to be correlated with the potential of tumor cell lines to form metastases (Dennis 1992; Dennis et al. 1987). Based on these data, we carried out a study as to whether such a correlation also existed for variants of metastasizing human colon carcinoma HCT116 cells. The *Phaseolus vulgaris* leukoagglutinating lectin (PHA-L) which binds to β-1,6 branched oligosaccharides (Wang and Cummings 1988) has been shown to be an excellent tool

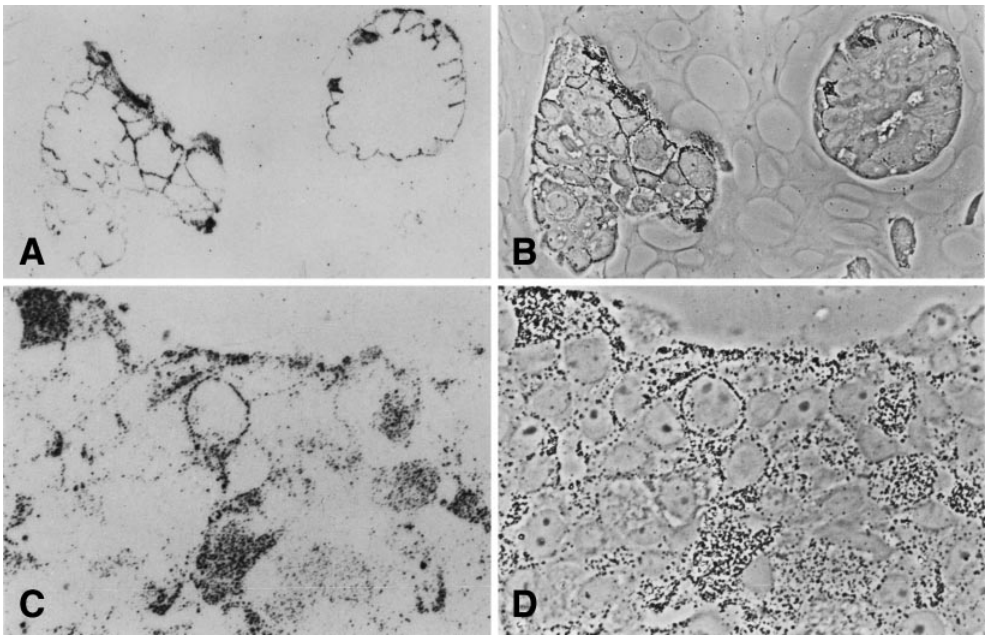


Fig. 6A–D Semithin sections of Lowicryl K4M-embedded invasive ductular breast carcinoma, silver-intensified protein A-gold labeling. **A, B** Immunostaining by mAb b-12 is restricted to the basolateral surface of the tumor cells (**A** bright field; **B** phase contrast). **C, D** Another carcinoma exhibits both intracellular and cell surface immunostaining albeit of varying intensity (**C** bright field; **D** phase contrast). (**A, B** ×550; **C, D** ×670)

for histochemical studies at the light and electron microscope levels (Li et al. 1993). A quantitative analysis by electron microscopy of the plasma membrane labeling by PHA-L-gold revealed a significantly higher labeling intensity in the more aggressive HCT116a cells compared to that in the less aggressive HCT116b cells (Li et al. 1994). The difference in plasma membrane labeling intensity could also be observed in paraffin sections of the cell lines following silver intensification of the lectin-gold labeling.

Studies on sialoglycoconjugates in colon carcinoma

The β-1,6 branches of oligosaccharides may be extended by polylactosamine structures carrying sialic acid moieties at the non-reducing terminus. In human colon carcinomas, as in other carcinomas, tumor-associated changes in the expression of cell surface sialic acid have been observed (Itzkowitz 1992; Kokoglu et al. 1992). Higher amounts of sialic acid were detected in colon carcinoma than in normal colonic mucosa, with a further increase in metastases as well as in colon carcinoma cell lines differing in their metastatic potential (Fogel et al. 1983; Hoff et al. 1989). The importance of increased amounts of sialic acids as one among many other factors in determining the metastatic potential of colon carcinoma cells has been demonstrated experimentally by various investigators (Harvey and Thomas 1993; Harvey et al. 1992; Kijima-Suda et al. 1986, 1988; Saitoh et al. 1992; Wagner et al. 1990). Furthermore, a sialic acid-dependent adhesion to collagen IV and laminin and its correlation with in vivo tumorigenicity of sublines of the colon carcinoma cell line HCT116 could be demonstrated (Morgenthaler et al. 1990). Previously, we demonstrated the colon carcinoma-associated de novo synthesis of α-2,6-linked sialic acid (Sata et al. 1991) as detected with the *Sambucus nigra* agglutinin I (Shibuya et al. 1987). In contrast, oligosaccharides terminated by α-2,3-linked sialic acid, as recognized by the *Maackia amurensis* leukoagglutinin and the *Amaranthus caudatus* agglutinin, were detectable in both normal mucosa and carcinoma (Sata et al. 1991, 1992). From these and other studies it can be concluded that the differences in the amount of sialic acid between normal colonic mucosa and colon carcinoma may be due to both an overall increased sialylation of glycoproteins and the de novo occurrence of specific sialylated sequences. It also should be emphasized that such differences in staining with the *S. nigra* agglutinin I were not observed in tumors derived from other organs such as mammary gland and kidney, since their epithelia normally express binding sites for this lectin.

Studies on the b-12 epitope in breast carcinoma

A number of monoclonal antibodies have been produced for diagnostic purposes that show selective reactivity against cells of human breast tumors. Such monoclonal antibodies may recognize glycoproteins expressed by normal and malignant epithelia of the breast. The monoclonal antibody (mAb) b-12 (Stähli et al. 1985) belongs to this group. In human breast carcinomas, strong immunostaining was observed in all histological types independent of their stage (Zenklusen et al. 1988). In addition, the mAb b-12 showed reactivity with a limited number of cell types in normal human tissues, among them the epithelial cells of the ducts and ductules of the non-lactating breast and material present in the duct lumen (Stähli et al. 1985; Zenklusen et al. 1988). In a first step, we attempted the further characterization of the epi-

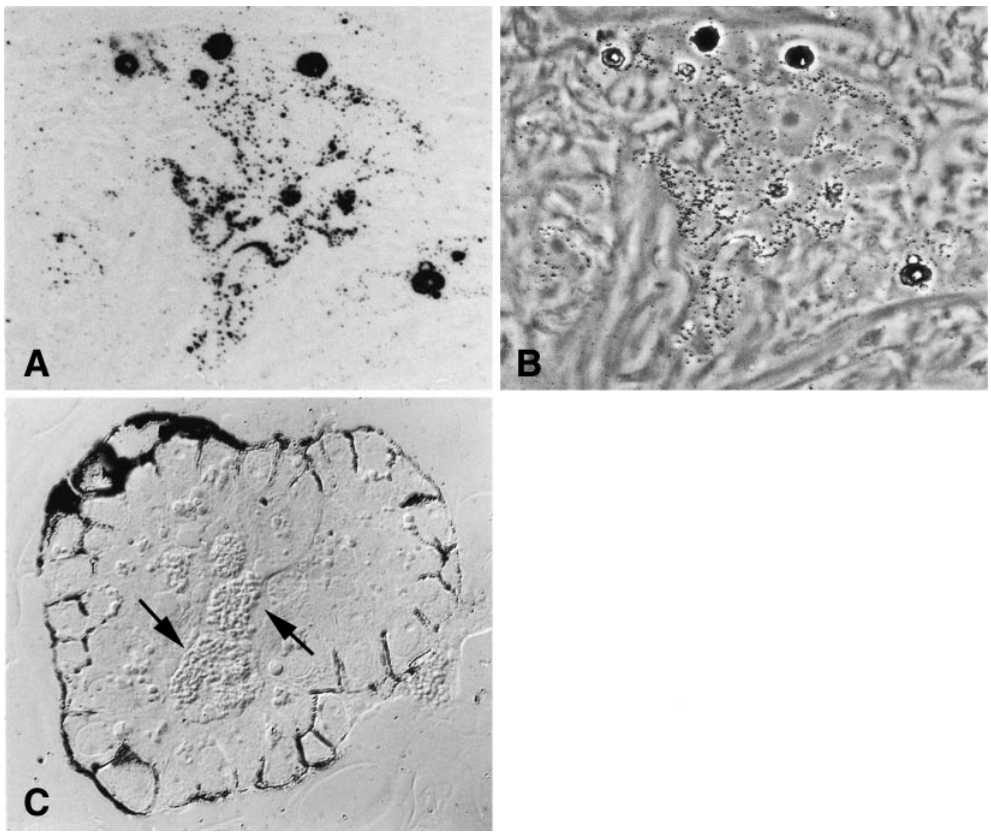


Fig. 7A–C Same tissues and methods as in Fig. 6. **A, B** The carcinoma cells show cell surface-associated and cytoplasmic labeling by mAb b-12. The positive, large cytoplasmic structures may correspond to intracytoplasmic lumina (see Fig. 8) (**A** bright field; **B** phase contrast). **C** Part of a carcinoma with intense cell surface staining. Secretory granules (*arrows*) are unstained. Normaski interference contrast. (**A, B** ×860; **C** ×1150)

tope recognized by mAb b-12 using Western blotting. When electrophoretically resolved and nitrocellulose-transferred glycoproteins from the homogenates of five breast carcinomas were probed with the monoclonal antibody, a band of apparent molecular mass of >350 kDa was observed (Fig. 3, lanes 1–4), in accordance with earlier studies (Stähli et al. 1985). Pretreatment of glycoproteins after transfer to PVDF membranes by the β-elimination reaction abolished the immunoreactive band (Fig. 3, lanes 5–8), whereas pretreatment of isolated glycoproteins with *N*-glycanase had no effect. Therefore, the epitope recognized by mAb b-12 is a carbohydrate structure which is part of an *O*-glycosidically linked oligosaccharide side chain of a glycoprotein. Our subsequent studies were aimed at the subcellular localization of this epitope in Lowicryl K4M sections of normal human breast and breast carcinoma applying the protein A-gold technique. In semithin and ultrathin Lowicryl K4M sections of normal breast ducts and ductules, the immunostaining was restricted to the apical plasma membrane of the epithelia (Figs. 4, 5). However, by light and electron microscopic immunolabeling, positive and negative cells were clearly seen (Fig. 4). The positive cells corresponded to the clear epithelial cells (Nesland et al.1983; Ozello 1971). Intracellular labeling was confined to the inner surface of the limiting membrane of small lucent vesicles (Fig. 5B) and secretory granules exhibiting dense cores surrounded by a lucent halo (Fig. 5C), and to so-called intracytoplasmic lumina (Fig. 5D). Secretory granules with a tightly fitting electron dense core were unlabeled (Fig. 5D). In breast carcinoma, varying labeling patterns were observed by light microscopy in semithin Lowicryl K4M sections. As a rule, most of the tumor cells in the carcinomas studied were positive although the extent of cytoplasmic labeling varied greatly (Figs. 6A, B, 7C). In particular, secretory granules existing in such tumor cells were unreactive (Fig. 7C). In other carcinomas, the cytoplasm was filled with fine punctate immunolabeling in addition to cell surface staining (Figs. 6C, D, 7A, B). In addition, large globular cytoplasmic structures displayed positive reaction in some tumor cells (Fig. 7A, B). By electron microscopy, immunolabeling was observed all along the apical plasma membrane of the tumor cells and over the luminal content (Fig. 8A, B). Large, electron-lucent and smaller electron-dense secretory granules were unlabeled (Fig. 7A, C). However, as already observed in normal duct epithelia, the inner surfaces of small, lucent vesicles (Figs. 5B, 8B) and intracyctoplasmic lumina were labeled (Fig. 8C). The strong labeling of the latter may account for the large positive cytoplasmic elements seen in Fig. 7A and B. Immunolabeling was also present along the basolateral plasma membrane domains, as illustrated in Fig. 9A, C, and D. In addition, the labeling pattern by the gold particles was suggestive of membrane-associated and non-membrane-associated labeling. In Fig. 9C and D, the immunolabeling is seen along the basal plasma membrane and free in the extracellular space. From these data the following conclusions can be drawn. The mAb b-12 reacts with a carbohydrate epitope present in *O*-glycosidically linked oligosaccharide side

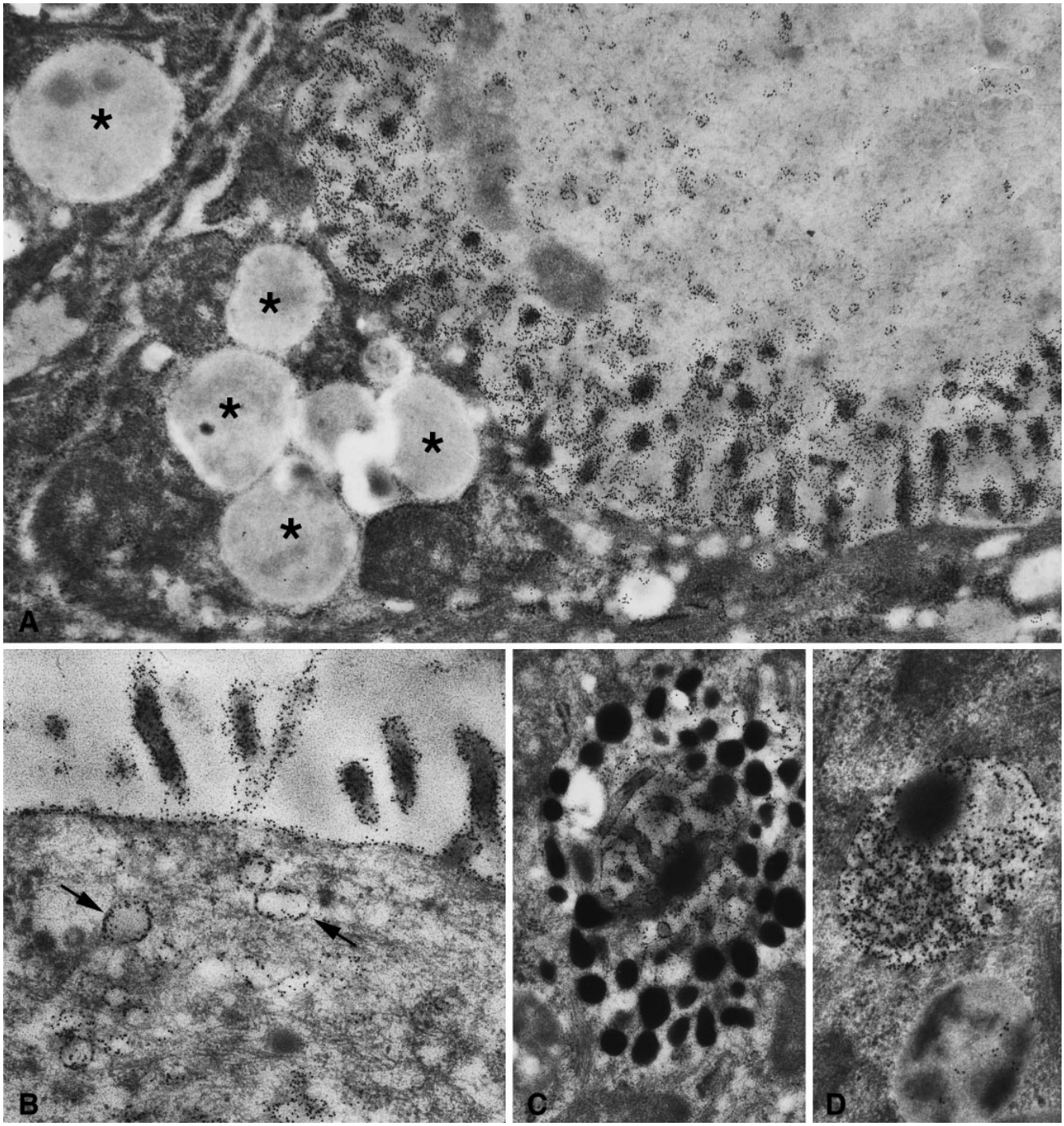
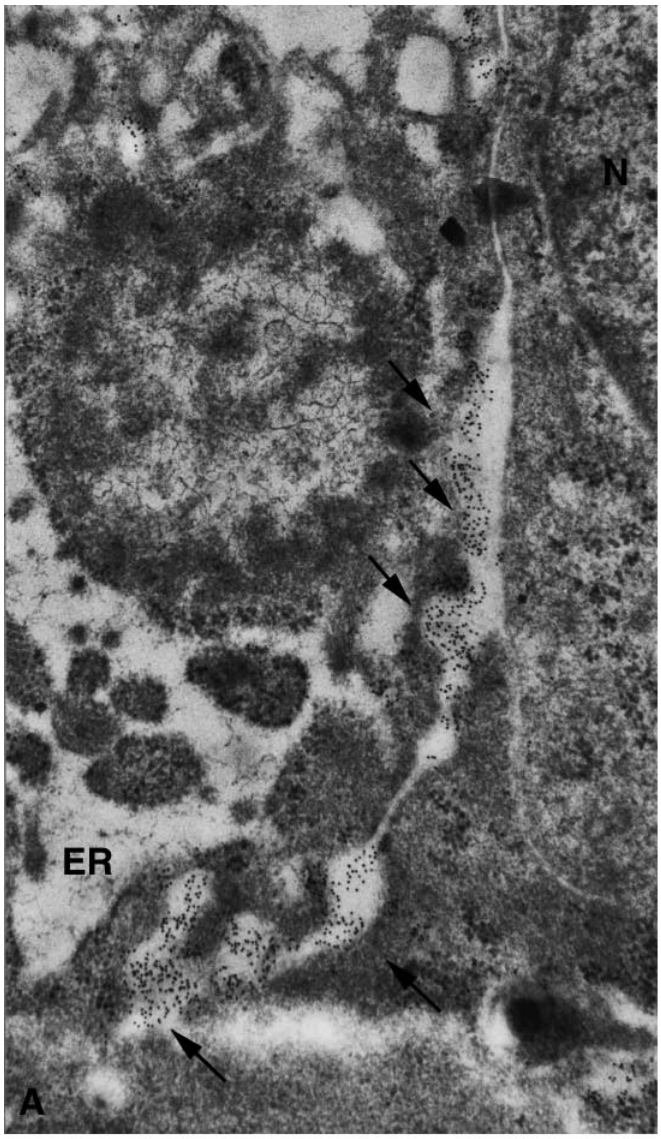
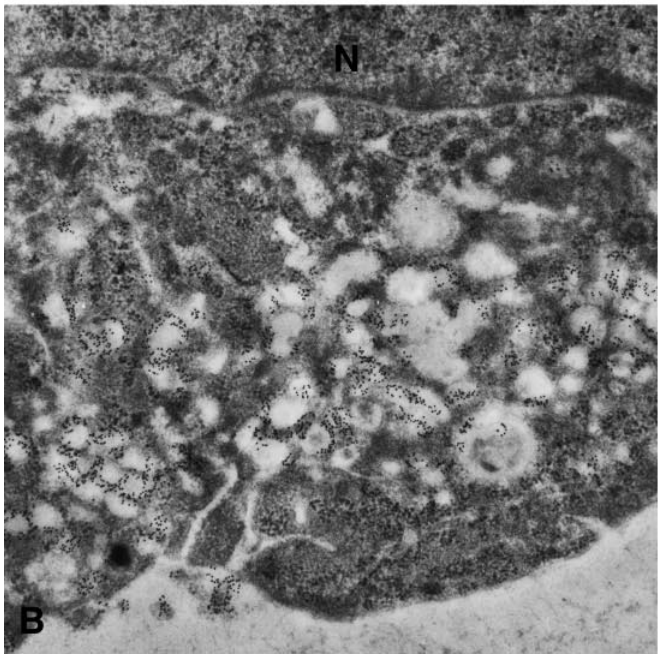
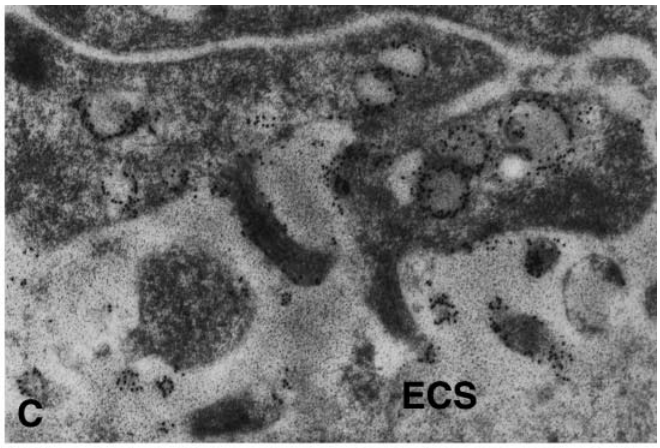


Fig. 8A–D Thin sections of Lowicryl K4M-embedded invasive ductular breast carcinoma, protein A-gold technique. **A, B** The mAb b-12 results in intense gold particle labeling along the plasma membrane and over the lumen. Secretory granules (*asterisks* in **A**) are unlabeled whereas the limiting membrane of cytoplasmic vesicles shows intense labeling (*arrows* in **B**). **C** The membrane of an intracytoplasmic lumen exhibits gold particles labeling but the adjacent secretory granules are unlabeled. **D** An unidentified cytoplasmic structure with intense immunolabeling. (**A** ×25000; **B** ×37000; **C** ×23000; **D** ×35000)

chains of high molecular weight membrane glycoproteins of normal breast epithelia and breast carcinoma cells. Although this epitope could be detected in secretory granules of normal breast duct epithelia, it was undetectable in those of the breast carcinomas studied. In both normal and tumor cells, the epitope was present in lucent, presumably exocytotic, vesicles and intracytoplasmic lumina. The strictly polarized apical plasma membrane expression in normal duct epithelia was lost in carcinoma cells. In the carcinomas, the epitope seemed to undergo shedding from the basolateral (and







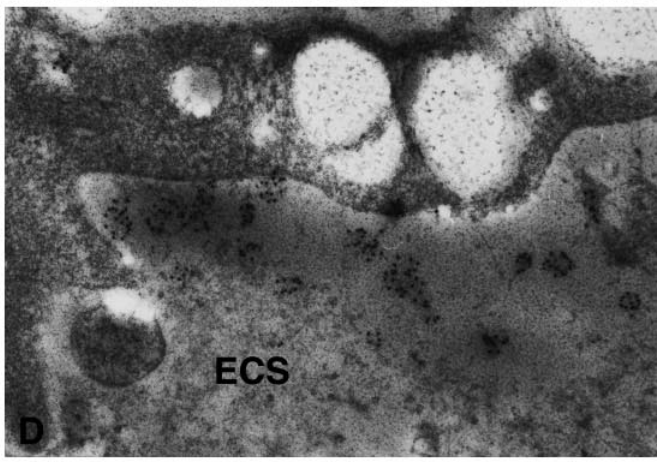


Fig. 9A–D Basal parts of tumor cells in breast carcinoma, mAb b-12. **A** Immunolabeling is confined to the intercellular space (*arrows*). Part of a nucleus (*N*) and the rough endoplasmic reticulum (*ER*) are unlabeled. **B** Intensely labeled vesicles are present in basal cytoplasm of a tumor cell (*N* nucleus). **C, D** Labeling by mAb b-12 is found in cytoplasmic vesicles and the plasma membrane as well as over the extracellular space (*ECS*). (**A** ×33000; **B** ×30000; **C** ×36000; **D** ×39000)

apical) plasma membrane and was found free in the extracellular space from where it may be transported to the lymphatics. This phenomenon may account in part for the observed elevated serum levels in breast carcinoma patients.

Studies on poly(α-2,8)*N*-acetylneuraminic acid in neuroendocrine tumors

Neuroendocrine cells in various organs and tumors derived thereof can be detected and characterized by using antibodies directed against typical secretory products or specific membrane glycoproteins (Heitz et al. 1991; Roth et al. 1996).

Despite its designation, neither the neural cell adhesion molecule (NCAM) nor its polysialic acid represent a specific marker for neuroendocrine cells. Immunocytochemical studies applying the highly specific and powerful mAb 735 directed against polysialic acid consisting

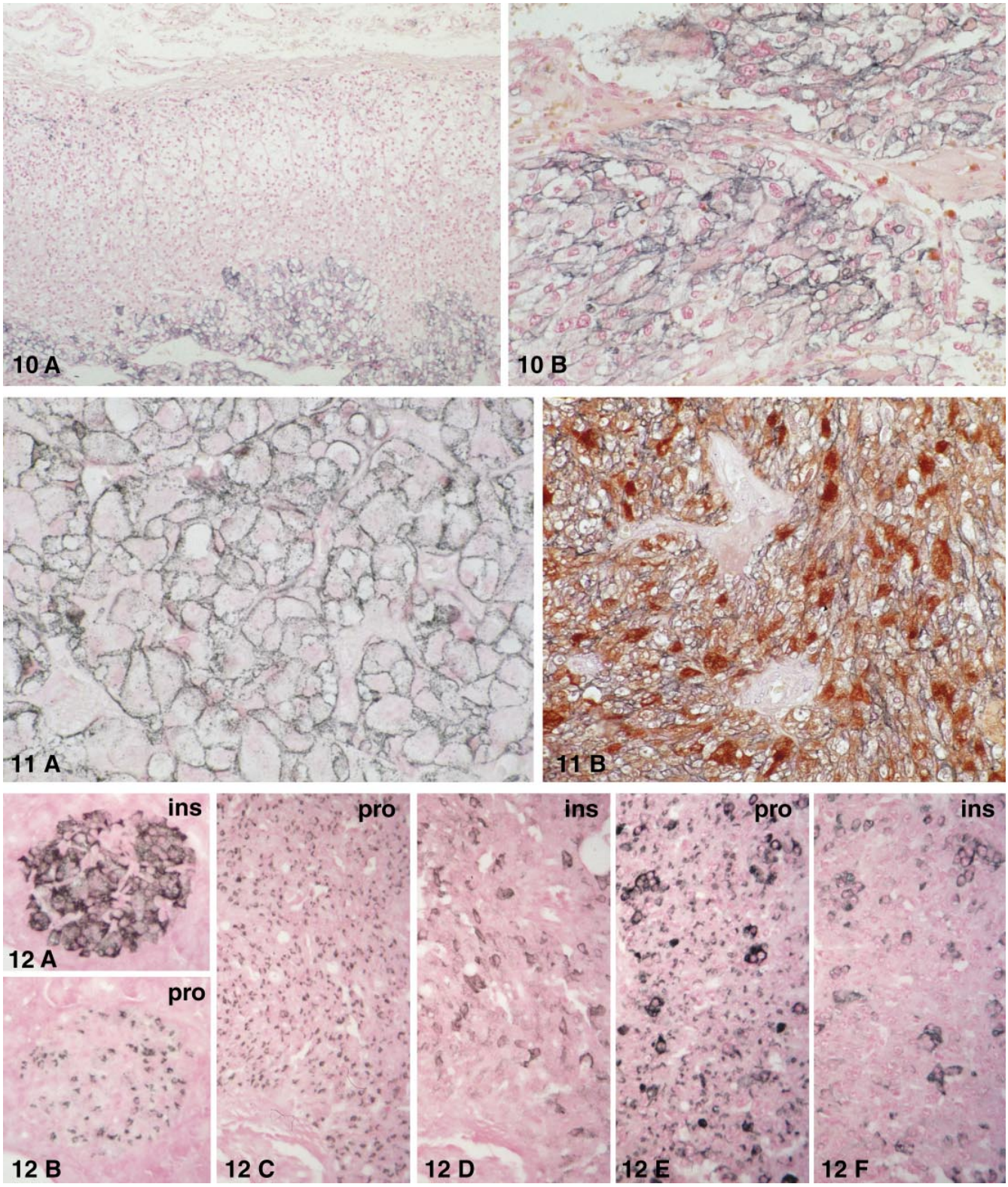


Fig. 10A, B Human adrenal, paraffin sections, directly gold-labeled mAb 735, silver intensification, nuclear fast counterstaining. **A** In the normal adrenal gland immunostaining for polysialic acid is present in the medulla and cortical nerve fibers. The endocrine cells of the cortex are unstained. **B** A pheochromocytoma with cell surface staining for polysialic acid. (**A** ×70; **B** ×500)

Fig. 11A, B Medullary thyroid carcinoma, paraffin sections. **A** The tumor cells show intense cell surface immunostaining for polysialic acid. **B** Immunogold-silver staining for polysialic acid (*black*) and immunoperoxidase staining for calcitonin (*brown*) reveals that many tumor cells are positive for both antigens (**A** ×500; **B** ×300)

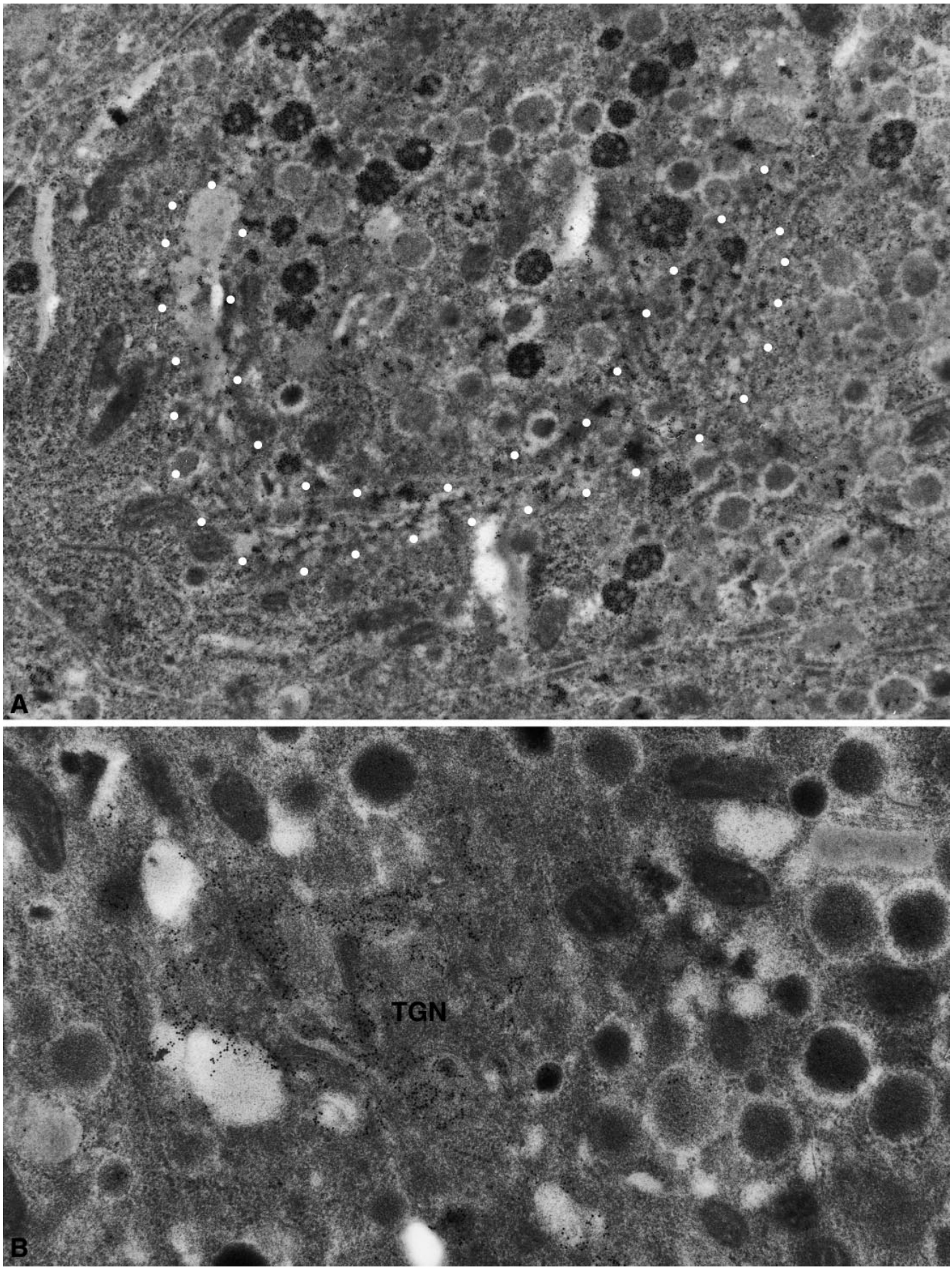
of homopolymers of α-2,8-linked *N*-acetylneuraminic acid residues (Frosch et al. 1985) have demonstrated this very clearly. Thus, polysialic acid on the NCAM was detected in a developmentally regulated manner in the mesodermally derived rat and human kidney (Lackie et al. 1990; Roth et al. 1987) and in many other tissues of non-neuroectodermal origin (Lackie et al. 1994). Studies on the highly malignant Wilms tumor of the kidney demonstrated for the first time that polysialic acid of NCAM represented an oncodevelopmental antigen (Roth et al. 1988a, b). This was particularly interesting in view of the documented function of polysialic acid of the NCAM in cell-cell interactions (Roth et al. 1993; Rutishauser et al. 1988; Yang et al. 1994). In a study of clonal lines of human small cell lung carcinoma, the importance of polysialic acid for invasive and metastatic growth could be demonstrated experimentally (Scheidegger et al. 1994). Several immunohistochemical studies on the presence of polysialic acid have demonstrated its usefulness in the differential diagnosis of certain neuroendocrine tumors. This is also underlined by the fact that mAb 735 works extremely well on routinely formalin-fixed and paraffin-embedded tissues without need for antigen retrieval.

Polysialic acid was found to be temporarily expressed during lung development but was undetectable in adult lung (Lackie et al. 1994), and in squamous cell carcinomas as well as adenocarcinomas of the lung. In contrast, small cell lung carcinomas were positive for polysialic acid (Komminoth et al. 1991, Scheidegger et al. 1994). The small cell lung carcinomas, composed of the typical fusiform cells with high nuclear cytoplasmic ratio showing nuclear molding and high mitotic rate, of small round uniform cells, or containing regions with larger fusiform or polygonal cells (the so-called intermediate type of small cell carcinomas or the mixed small cell-large cell variant), behaved similarly with respect to the presence of polysialic acid. In strong contrast, the well and moderately well-differentiated bronchial (as well as gastrointestinal) neuroendocrine tumors (so-called carcinoids) showed no immunostaining for polysialic acid albeit they were immunoreactive for NCAM protein. These immunohistochemical data demonstrated that all neuroendocrine lung tumors contained NCAM polypeptide but that they differed in the extent of polysialylation of NCAM. This difference may be very helpful in the immunohistochemical distinction of these tumors in daily routine.

Fig. 12A–F Human pancreas, pairs of consecutive serial paraffin sections, immunogold-silver staining for proinsulin (*pro*) and insulin (*ins*). **A, B** Diffuse cytoplasmic immunolabeling for insulin and perinuclear, sharp, crescent-shaped immunolabeling for proinsulin in an islet of Langerhans. **C, D** Solid insulinoma exhibiting perinuclear crescent-shaped immunostaining for proinsulin in all tumor cells and diffuse immunostaining for insulin in only a few tumor cells. **E**, **F** Another solid insulinoma presenting crescent-shaped and diffuse immunostaining for proinsulin and diffuse immunostaining for insulin in a percentage of tumor cells. (**A, B** ×280; **C–F** ×240)

Several investigators have demonstrated NCAM polypeptide and messenger RNA expression both in the normal adrenal cortex and medulla as well as their tumors (Garin-Chesa et al. 1991; Jin et al. 1991; Lahr et al. 1993). However, the reported cellular distribution of polysialic acid in the adrenal is somewhat contradictory. Some investigators reported immunostaining for polysialic acid to be restricted to the medulla in normal and neoplastic human adrenal cells (Heitz et al. 1990; Tome et al. 1993), while others described additional immunoreactivity in the adrenal cortex of the rat (Bensch et al. 1968; Lahr et al. 1993) by using the same monoclonal antibody, mAb 735. Our additional studies in normal human adrenal gland (Komminoth et al. 1995) gave the following results. We observed a strong cell surface-associated immunostaining for polysialic acid in medullary cells of all 13 adrenal glands studied (Fig. 10A). The staining was abolished by pretreatment of the tissue sections with endo N, a sialidase which specifically hydrolyzes α-2,8-linked polysialic acid, therefore unequivocally demonstrating the specificity of the immunostaining. The adrenal cortex lacked detectable polysialic acid immunoreactivity with the exception of cortical small nerve fibers and larger nerves present in the adrenal capsule (Fig. 10A). Thus, the differences in polysialic acid immunostaining between human and rat adrenal may be due to species differences since, in the rat adrenal gland, chromaffin cells are frequently found within the cortex and may even be present in the subcapsular part of the zona glomerulosa (Bornstein et al. 1991). Another interesting conclusion derived from these studies was that, while NCAM polypeptides have been demonstrated both in the medulla and the cortex of the adrenal gland (Garin-Chesa et al. 1991; Tome et al. 1993), the polysialylated form of NCAM appears to be restricted to medullary cells in the human adrenal gland.

Two types of neoplasms of the adrenal gland can be found: the adrenocortical tumors and the pheochromocytomas, the latter being derived from the medulla. Their distinction may be sometimes difficult because of similarities in histological appearance (Ramsay et al. 1987). Since they behave clinically differently, their differential diagnosis is important (Lack 1990). All 28 pheochromocytomas we examined exhibited a strong cell surface-associated immunostaining for polysialic acid (Fig. 10B), irrespective of the histological type (Komminoth et al. 1995). A similar distribution and intensity of immunostaining was observed in the six tumors of patients with multiple endocrine neoplasia type 2A, the four clinically malignant, and the remaining sporadically occurring pheochromocytomas. In contrast, the majority of the 27 adrenocortical carcinomas examined was found to be negative for polysialic acid (Komminoth et al. 1995). Only in 8 of the 27 adrenocortical tumors was immunoreactivity detectable and it should be emphasized that the staining was strictly focal. The results of these studies indicate that several markers such as polysialic acid, vimentin, neuron-specific enolase, and synaptophysin may show overlapping patterns in both types of adrenal tu-


A
TGN
B

mors (Komminoth et al. 1995). Furthermore, they provided evidence of neuroendocrine differentiation in adrenocortical carcinoma.

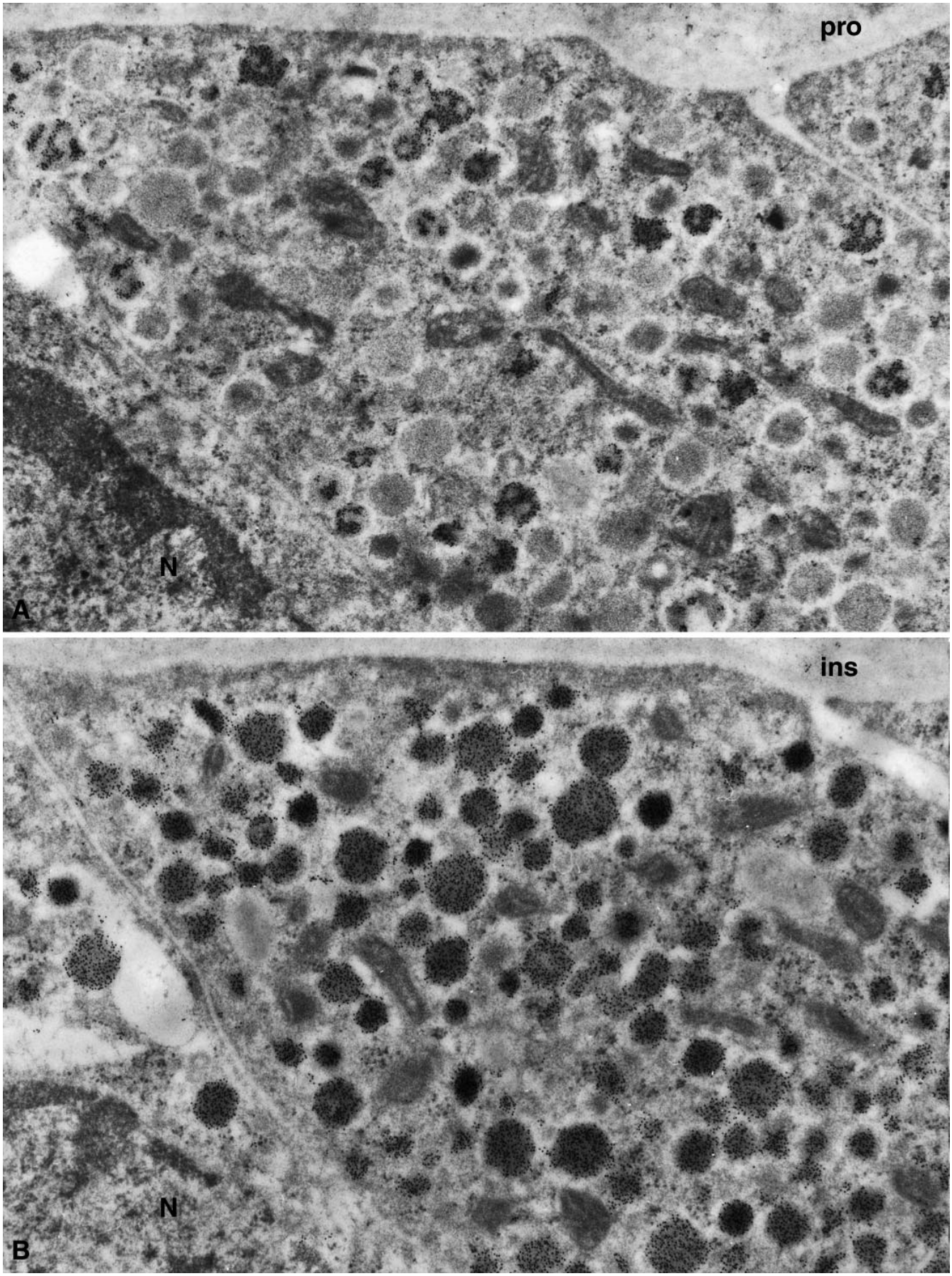
Among the various histological types of thyroid carcinomas, medullary thyroid carcinomas comprise 5–10%. The latter occur in sporadic and familiar forms (Murray 1991). Medullary thyroid carcinomas probably originate from neural crest-derived C-cells (Le Douarin 1982) and the familial forms are preceded by bilateral, multicentric C-cell hyperplasia (Wolfe et al. 1973). A residual C-cell hyperplasia adjacent to the carcinoma is considered a hallmark of familial forms (Wolfe et al. 1980). However, reactive or secondary C-cell hyperplasia has been observed adjacent to tumors of follicular cell origin and in other conditions (Albores-Saavedra et al. 1988). The morphological appearance of medullary thyroid carcinomas can be variable and may be mistaken for other types of thyroid carcinomas (Sambade et al. 1988). However, precise diagnosis of thyroid carcinomas is of importance because of differences in clinical course. Useful immunohistochemical markers in the diagnosis of medullary thyroid carcinoma are calcitonin, carcinoembryonic antigen, and calcitonin-related gene product. In a recent study (Komminoth et al. 1994) we evaluated the usefulness of immunostaining for polysialic acid to distinguish medullary thyroid carcinoma from other types of thyroid carcinomas and primary from secondary C-cell hyperplasia. Furthermore, we were interested if changes in polysialic acid immunostaining were detectable during progression from C-cell hyperplasia to medullary thyroid carcinoma. All 33 medullary thyroid carcinomas showed polysialic acid immunoreactivity along the tumor cell surface (Fig. 11A) and in the majority of the carcinomas virtually all tumor cells were positive. Double immunolabeling revealed co-expression of polysialic acid and calcitonin in the tumor cells (Fig. 11B). In contrast to medullary thyroid carcinomas, the anaplastic, follicular, and papillary thyroid carcinomas studied were unreactive for polysialic acid. Occasionally, small polysialic acid-positive foci were observed in some anaplastic (2/16), follicular (1/13), and papillary (2/11) carcinomas. These foci were negative for calcitonin but positive for thyroglobin. No difference in pattern for polysialic acid immunostaining could be observed between sporadic and familial forms of medullary thyroid carcinoma. C-cell hyperplasia associated with medullary thyroid carcinoma exhibited immunostaining for polysialic acid whereas secondary C-cell hyperplasia and apparently normal C-cells were unreactive. Interestingly, the intensity of immunostaining for polysialic acid was highest in primary C-cell hyperplasia associated with medullary thyroid carcinoma. Thus, the immunostaining for polysialic acid in normal, hyperplastic, and neoplastic C-cells appears to be correlated with their neoplastic progression since hyperplastic C-cells in patients with family history of medullary thyroid carcinoma (MEN II) and neoplastic C-cells were positive. In summary, polysialic acid appears to represent a valuable marker to distinguish medullary carcinomas from other types of thyroid carcinomas and seems to allow the discrimination of primary from secondary C-cell hyperplasia.

Fig. 13A, B Human insulinoma, Lowicryl K4M thin sections, proinsulin immunolabeling. **A** Intense gold particle labeling is present over the Golgi apparatus (outlined by *white spots*) and a percentage of secretory granules. **B** A grazing section through the intensely labeled *trans* Golgi network (*TGN*). Most of the secretory granules are not immunoreactive for proinsulin. (**A** ×16000; **B** ×30000)

Studies on proinsulin to insulin conversion in functioning insulinomas

Insulin is synthesized in the pancreatic B-cells of the islets of Langerhans (Bailyes et al. 1992) as the prohormone proinsulin, which becomes converted by limited endoproteolysis to the mature active hormone insulin (Hutton 1994; Steiner et al. 1992). In normal pancreatic B-cells, the subcellular site of proinsulin to insulin conversion has been determined by immunoelectron microscopy with the use of non-crossreacting monoclonal antibodies to proinsulin (Madsen et al. 1983, 1984) and insulin (Storch et al. 1985) and shown to take place in acidic immature secretory granules of the Golgi apparatus (Orci et al. 1985, 1986, 1987, 1994; Steiner et al. 1987). In light microscopical sections, these monoclonal antibodies produced a conspicuous labeling pattern. The proinsulin-specific monoclonal antibody created a crescent-shaped perinuclear staining (Fig. 12B) whereas the insulin-specific monoclonal antibody consistently gave a diffuse cytoplasmic staining (Fig. 12A) of islet cells.

Insulinomas are pancreatic neuroendocrine tumors which can cause hyperinsulinemic hypoglycemia and can exhibit many structural and immunohistochemical features in common with normal pancreatic B-cells (Roth et al. 1996). However, even within a given insulinoma, insulin immunostaining and ultrastructural details of secretory granule morphology may vary greatly (Creutzfeldt 1985; Klöppel and Heitz 1988; Roth et al. 1996). Based on various morpho-clinical findings, Creutzfeldt and coworkers (1985) concluded that a major defect in insulinomas was uncontrolled insulin release due to impaired cellular storage capacity. Later, Berger and coworkers (Berger et al. 1983) proposed a classification of insulinomas into two groups based on morphological studies, hormone analysis, and suppressibility of serum insulin by somatostatin and diazoxide of 12 insulinoms. Group A was characterized by well-granulated, insulin-positive trabecular tumors with moderate proinsulin elevation and complete suppressibility of serum insulin by somatostatin and diazoxide. In contrast, group B consisted of solid tumors containing few insulin-positive cells, elevated proinsulin serum levels, and marked resistance to somatostatin and diaxozide treatment. Our immunohistochemical studies on a large series of 76 human insulinomas did not support the concept that trabecular and solid insulinomas represent two entities since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substantial variations in the number of insulin-immunoreactive cells were found in both histological tumor types (Roth et al. 1989a, 1992a). Three categories of immunostaining pattern were observed. A "near-normal" staining pattern, characterized by perinuclear proinsulin, and diffuse or polarized insulin immunostaining. An "intermediate" staining pattern, consisting of a combination of intense perinuclear and weaker diffuse proinsulin staining, and diffuse or polarized insulin immunostaining. An "abnormal" staining pattern with diffuse immunostaining for both proinsulin and insulin. Examples of the near-normal and the abnormal staining patterns are shown in Fig. 12C–F. Based on these findings, we proposed that the abnormal pattern of proinsulin immunostaining observed in 60% of the trabecular and solid insulinomas was an expression of topographical abnormalities of hormone conversion. This hypothesis was proved in subsequent immunoelectron microscopic studies (Roth et al. 1995). In insulinomas, immunoreactivity for both proinsulin and insulin was confined to the Golgi apparatus and secretory granules and was not detectable over the cytosol. Intense proinsulin immunolabeling was consistently observed throughout the cisternal stack of the Golgi apparatus and to a variable degree in the *trans* Golgi network (Fig. 13A), similar to the situation described for normal pancreatic B-cells (Orci et al. 1985, 1986). In contrast to normal pancreatic B-cells, immunolabeling for insulin could also be detected over *trans* Golgi cisternae and the *trans* Golgi network. Further differences were observed with regard to proinsulin immunolabeling of secretory granules. Although secretory granules at the *trans* side of the Golgi apparatus were labeled for proinsulin, the labeling intensity and the number of positive secretory granules showed substantial variation, irrespective of their morphology even in a single insulinoma. In Fig. 13B, the extreme of such a situation is illustrated with intense proinsulin labeling over the *trans* Golgi network and virtually none detectable in secretory granules. On the other hand, intense immunolabeling for insulin was consistently observed over all secretory granules with a tightly fitting core and present in the Golgi area. Secretory granules containing proinsulin immunoreactivity were not only detected in the Golgi apparatus area but also in the periphery of the tumor cells, as shown in Fig. 14A. Figure 14B is an adjacent thin section showing the same cells and labeling for insulin over all secretory granules.

Collectively, our light and electron microscopic studies demonstrated that there is no correlation between histological tumor type and both proinsulin and insulin immunostaining patterns. This may be due to the substantial subcellular variability in distribution of proinsulin and insulin immunoreactivity among individual tumor cells shown in our studies. This variability at the level of single tumor cells in a given insulinoma and between the studied insulinomas was observed, irrespective of the tumor size, its histological type, and dignity. Further, by immunoelectron microscopy, we obtained direct evidence for abnormalities in the topography of proinsulin to insulin conversion occurring at different levels in functioning human insulinomas. Thus, the proinsulin to insulin conversion already started in the *trans* Golgi apparatus suggests that proinsulin conversion is not linked to secretory granule formation and can take place prior to sorting into secretory granules has occurred. Further, proinsulin immunoreactivity was not only detectable in the Golgi apparatus and to a varying degree in immature secretory granules of the Golgi area but also in secretory granules of different morphology in the periphery of the tumor cells and in proximity to the plasma membrane. This abnormal distribution is in situ evidence for incomplete proinsulin to insulin conversion in insulinoma. Finally, the immunoelectron microscopic studies showed that, in insulinoma, both proinsulin and insulin are sorted and stored in typical elements of the regulated secretory pathway, the secretory granules.

Fig. 14A, B Adjacent Lowicryl K4M thin sections of human insulinoma labeled for proinsulin (*pro*) and insulin (*ins*). **A** Proinsulin-positive secretory granules are present in the periphery of the tumor cells and adjacent to the plasma membrane. **B** All secretory granules are positive for insulin irrespective of their shape and size (*N* nucleus). (**A, B** ×24000)

Conclusions

The high sensitivity, resolution, and contrast of immunogold labeling techniques have provided invaluable new insights in many fields of tumor research and diagnosis. In general, the labeling reagents can be applied to conventionally processed tissues in light and electron microscoppy. Such studies have revealed a colon carcinoma-associated expression of highly β-1,6 branched oligosaccharides and of sialic acid in α-2,6 linkage. They have demonstrated that the b-12 epitope in breast carcinoma is not a secretory glycoprotein of the regulated pathway but is shed from the plasma membrane into the lumen and extracellular space. Furthermore, the polysialic acid of the neural cell adhesion molecule has proven to represent a useful marker in the differential diagnosis of neuroendocrine tumors. Finally, studies in human insulinomas provided evidence for topographic abnormalities in proinsulin to insulin conversion commencing already in the Golgi apparatus but remaining incomplete and resulting in the formation of secretory granules containing both proinsulin and insulin.

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