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Polysialic Acid of the Neural Cell Adhesion Molecule Distinguishes Small Cell Lung Carcinoma from Carcinoids

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The neural cell adhesion molecule (NCAM) exists in various types of neuroendocrine cells and their tumors. A typical feature of NCAM is polysialic acid, of which the chain length is developmentally regulated. The authors have performed a comparative immunohistochemical study on small cell lung carcinomas and bronchial as well as gastrointestinal carcinoids with the monoclonal antibody (MAb) 735 reactive with the long-chain form of polysialic acid. The small cell lung carcinomas, irrespective of their histological type, were positive for polysialic acid. Metastatic tumor cell complexes also exhibited immunostaining. The tumor cell-surface–associated immunostaining for polysialic acid was sensitive to endoneuraminidase. The mature and atypical bronchial and gastrointestinal carcinoids were not immunoreactive for polysialic acid. Cytoplasmic staining in groups of cells of carcinoids (2 of 28 cases) was due to nonspecific antibody binding, which could be prevented by increased ion strength. These data indicate that neuroendocrine tumors of the lung can be distinguished by their content of highly sialylated NCAM. (Am J Pathol 1991, 139:297–304)

Bronchopulmonary neuroendocrine neoplasms are assumed to derive from normal neuroendocrine cells of the lung.[1–4] In the lung these are represented by solitary Kultchitsky cells and compact clusters of neuroendocrine cells forming the neuroepithelial bodies.[1,2,5–8] Bensch et al[9] pointed out the ultrastructural similarities of the oat (small) cell lung carcinoma and bronchial carcinoids with the neuroendocrine cells. In numerous subsequent studies, the neuroendocrine characteristics of small cell lung carcinomas (SCLC) and bronchial carcinoids have been firmly established.[10–22] Currently it is not clear if there is a common (neuroendocrine) cell of origin for the spectrum of neuroendocrine neoplasms of the lung.[2,9,23–25] It has been speculated, however, that the endocrine characteristics of tumors do not necessarily demonstrate their origin from an endocrine cell type and could equally well reflect a particular mode of differentiation.[26] Such a standpoint would be at variance with view that each type of lung neoplasm develops from a distinct respiratory epithelial cell type.[27]

Neuroendocrine cells such as chromaffin cells of the adrenal medulla and tumors derived from them,[28,29] cells of the anterior pituitary and pituitary adenomas,[30,31] and endocrine pancreatic cells and insulinomas[32,33] have been shown to contain immunoreactivity for the neural cell adhesion molecule (NCAM). Neural cell adhesion molecule undergoes a series of post-translational modifications.[34,35] The most characteristic is the acquisition of α 2,8-linked polysialic acid,[35,36] the length of which is developmentally regulated and is involved in the modulation of the adhesive properties of NCAM.[30,31,37] Interestingly some neuroendocrine tumors such as pheochromocytoma,[38] medullary thyroid carcinoma,[32] and pituitary adenoma[32] have been shown to express the highly sialylated form of NCAM, whereas human insulinomas and a transplantable rat insulinoma contain the less sialylated form of NCAM.[32]

In the present study we have used a monoclonal antibody (MAb) that recognizes the long-chain form of polysialic acid characteristically found on NCAM in embryonic brain[39,40] to study its expression in SCLCs and bronchial carcinoids as well as carcinoids of the gastrointestinal tract. This MAb can be applied to paraffin-embedded tissues as shown in our previous studies

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on brain, kidney, and Wilms's tumor.[41–43] We found all cases of SCLC immunohistochemically positive for polysialic acid, whereas the bronchial as well as gastrointestinal carcinoids were unreactive. Therefore the MAb against polysialic acid can be used to distinguish these two neuroendocrine lung tumors.

Materials and Methods

Reagents

A mouse monoclonal IgG2a antibody, MAb 753, raised against live group B meningococci, was used to detect polysialic acid.[39,40] The MAb requires for binding to polysialic acid the presence of at least eight α 2,8-linked *N*-acetylneuraminic acid residues[41,44] and has no cross-reactivity with polynucleotides and denatured DNA.[40] The protein A-purified MAb 735 was directly complexed to particles of colloidal gold (8 nm in diameter) as described elsewhere.[45] The crude gamma G immunoglobulin (IgG)-gold complexes were centrifuged for 45 minutes at 90,000*g*. The supernatant was discarded and the sedimented IgG-gold complexes resuspended in 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (10 mmol/l [millimolar] phosphate buffer, pH 7.2, 0.15 mol/l [molar] NaCl). This was followed by centrifugation in a continuous 10% to 30% glycerol/PBS density gradient at 45,000*g* for 45 minutes. The upper red band was collected from the gradient and used for immunolabeling.

A rabbit antiserum raised against mouse brain NCAM exhibiting cross-reactivity with human NCAM has been described in detail previously[46] and was a gift from Dr. C. Goridis (INSERM, Marseille).

Protein A-gold was prepared using 8-nm gold particles according to Roth et al.[47,48] Centrifugation of the crude protein A-gold complexes was performed as described above for the MAb 735-gold complexes. Protein A was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Two bacteriophage-encoded endosialidases specifically hydrolyzing α 2,8-linked *N*-acetylneuraminic acid were used: 1) bacteriophage PK1A-bound endosialidase[49] and 2) soluble, purified bacteriophage E-encoded endosialidase.[50] Bacteriophage PK1A-bound endosialidase has a reported strict substrate specificity: it requires for cleavage the presence of at least eight α 2,8-linked *N*-acetylneuraminic acid residues. Bacteriophage E-bound endosialidase was a gift from Dr. C. Weisgerber (Medical School, Hannover, FRG). All other reagents were of the highest purity available.

Tissue Fixation and Embedding

Twenty-five SCLCs, seven bronchial carcinoids, three gastric carcinoids, six duodenal carcinoids, seven ileal carcinoids, and five colon carcinoids were investigated (classified according to the World Health Organization nomenclature[51]). Tissue pieces were routinely fixed in 10% phosphate-buffered formaldehyde and embedded in paraffin according to standard procedures.

Immunohistochemistry

Immunoreactive sites for polysialic acid were detected in a one-step labeling protocol applying the MAb 735-gold complex. Dewaxed and rehydrated tissue sections were preblocked with PBS containing 2% wt/vol fat-free dried milk powder for 5 to 10 minutes. Sections were then incubated for 1 hour with gold-labeled MAb 735 diluted in PBS containing 1% BSA and 0.05% Triton X-100 and Tween 20 to optical density $(OD)_{525\ nm} = 0.04$. Antibody–gold complexes were also diluted in PBS containing 0.2 mol/l, 0.3 mol/l, or 0.5 mol/l NaCl and used to incubate sections from small cell lung cancer and carcinoids. The sections then were washed (2 × 5 minutes in PBS). Afterwards the sections were postfixed in 1% glutaraldehyde for 20 minutes and washed well in distilled water before air drying. Gold particle label intensification using silver acetate developer was carried out following the method detailed in Roth.[48] Sections were counterstained with nuclear fast red.

For NCAM immunostaining, dewaxed and rehydrated tissue sections were preblocked as above, followed by a 1-hour incubation with the primary antibody (500-fold dilution in PBS containing 1% wt/vol fat-free dried milk powder). Washing (2 × 5 minutes in PBS) was followed by incubation with protein A-gold ($OD_{525\ nm} = 0.06$) for 1 hour. Further processing steps were as for polysialic acid. Immunocytochemical controls included the omission of primary antibodies, and incubation of the sections with bacteriophage endoneuraminidase E (2 to 18 hours at 37°C).

Results

The immunohistochemical investigation of the 25 SCLCs demonstrated in all cases the presence of immunoreactivity for polysialic acid (Figure 1a). The immunolabeling was due to the specific binding of MAb 735-gold complexes to polysialic acid, as pretreatment of tumor tissue sections with bacteriophage endoneuraminidase, which specifically hydrolyzes α 2,8-linked polysialic acid, resulted in complete abolition of the staining (Figure 1b). The photochemically silver-intensified immunogold label-

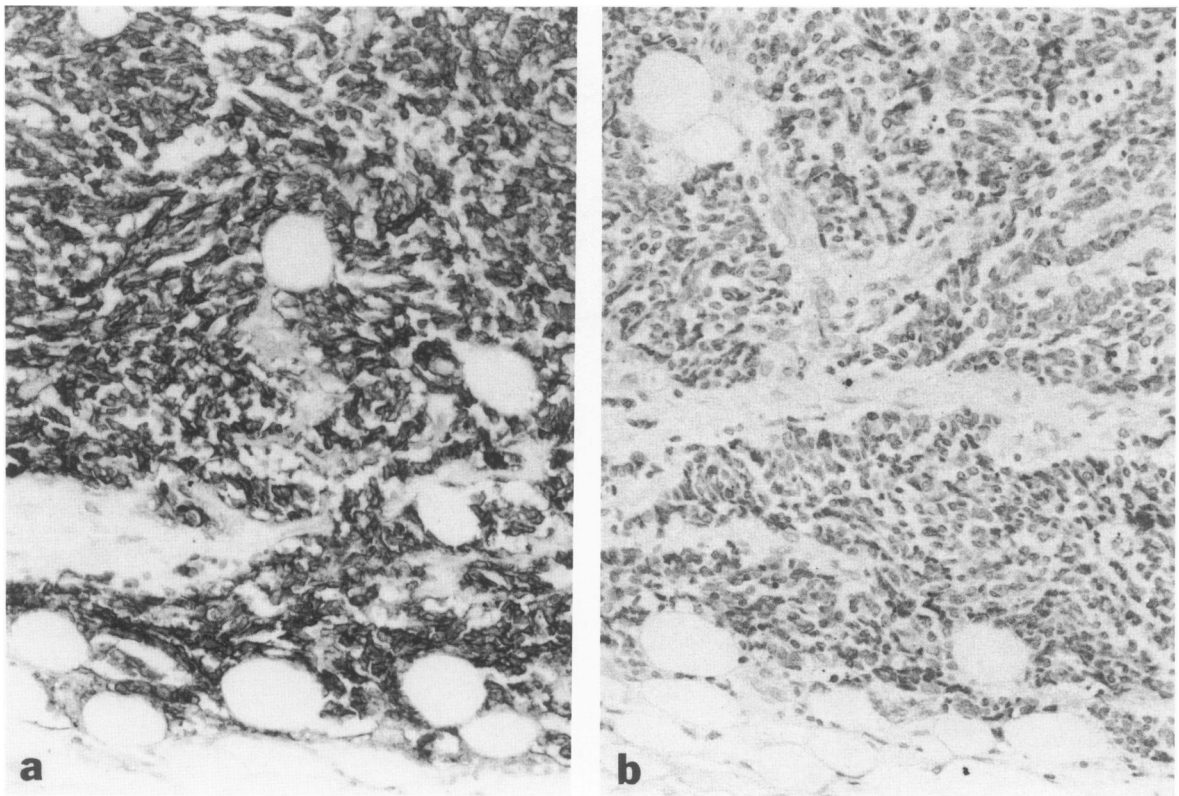

Figure 1. *Immunolocalization of polysialic acid with directly gold-labeled MAb 735 in small-cell lung carcinoma. Positive staining in this and all other micrographs appears in black due to photochemical silver intensification of the immunogold labeling.* **a:** *Intensely polysialic-acid–positive tumor and unstained scant tumor stroma can be seen at low magnification.* **b:** *An adjacent serial section was pretreated with endoneuraminidase to remove polysialic acid, which resulted in abolition of the immunostaining(×180).*

ing appeared as continuous or dense punctate cell surface staining (Figure 1). No labeling was detectable in the scant tumor stroma (Figure 1a), or the normal lung parenchyma, including bronchial respiratory epithelium and glands (Figure 2a). Histologically the SCLCs were composed of the typical fusiform cells with high nuclear cytoplasmic ratio and showing nuclear molding and high mitotic rate. Some SCLCs showed regions composed of spindle cells, which were also positive for polysialic acid. Tumors composed of small round uniform cells also exhibited regions composed of larger fusiform or polygonal cells with larger nuclei and more abundant cytoplasm. They corresponded to the intermediate type of small cell carcinomas of the World Health Organization (WHO) classification[51] or the mixed small cell–large cell variant of the Working Party for Therapy of Lung Cancer–Lung Cancer Study Group (WPL–LCSG) classification[52] and exhibited cell surface immunolabeling for polysialic acid.

Tumor cell groups found in the vicinity of small blood vessels (Figure 2b), in afferent lymphatics, or medullary sinus of lymph nodes also showed immunostaining for polysialic acid. The SCLC were also positively stained by the anti-NCAM polypeptide antibodies (not shown). Bronchial and gastrointestinal carcinoids showed immunostaining for NCAM, confirming data by Schol et al[53] obtained with the SCLC-cluster I MAb 123C3 reactive with an NCAM polypeptide epitope.[54] Generally, however, the bronchial carcinoids as well as the gastrointestinal carcinoids exhibited no detectable immunostaining for polysialic acid (Figure 3). This observation was made for mature carcinoids (Figure 3a) and atypical carcinoids (Figure 3b). In two well-differentiated bronchial carcinoids, a diffuse cytoplasmic labeling of groups of tumor cells could be observed with the antipolysialic acid antibody (Figure 4a). This staining could not be abolished by section pretreatment with endoneuraminidase, however, indicative of nonspecific binding of the IgG–gold complexes. Raising the ionic strength of the PBS to 0.2 mol/l NaCl resulted in the abolition of this nonspecific MAb 735 binding in bronchial carcinoids (Figure 4b), but had no effect on the immunostaining in SCLC.

Discussion

A number of MAbs have been raised against SCLC specimens or cell lines in various laboratories in the

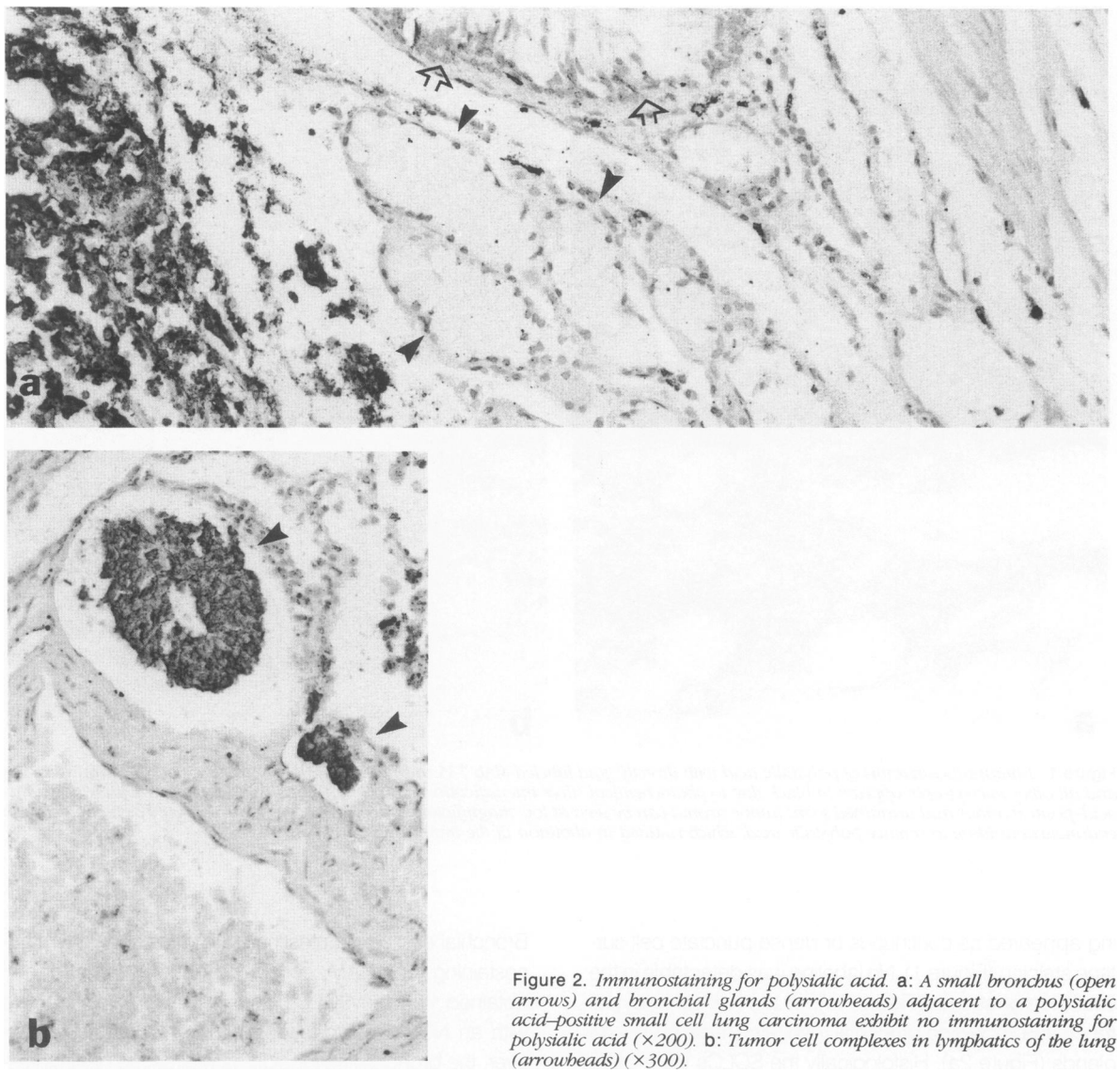

Figure 2. *Immunostaining for polysialic acid.* a: *A small bronchus (open arrows) and bronchial glands (arrowheads) adjacent to a polysialic acid–positive small cell lung carcinoma exhibit no immunostaining for polysialic acid (×200).* b: *Tumor cell complexes in lymphatics of the lung (arrowheads) (×300).*

search for antibodies to aid the classification of lung tumors. Many of these MAbs also exhibited reactivity toward neural tissues and were grouped as SCLC-cluster 1 antibodies.[55] Subsequent immunofluorescence and immunochemical studies on 3T3 cells transfected with a full-length clone of human NCAM showed that they were reactive with NCAM.[54] Monoclonal antibody 123C3, an NCAM-reactive SCLC-cluster 1 antibody, has been reported to stain a whole spectrum of neuroendocrine and non-neuroendocrine cell types in addition to SCLCs and bronchial carcinoids.[53] A major disadvantage of all SCLC-cluster 1 MAbs is that they fail to stain paraffin-embedded tissues.

The present immunohistochemical investigation has demonstrated the presence of NCAM polypeptides in both SCLCs and bronchial carcinoids, as well as carcinoids of the stomach, duodenum, ileum, and colon. These results obtained with a polyclonal NCAM antiserum confirm previous results obtained with the NCAM-reactive SCLC-cluster 1 MAb 123C3.[53] Therefore both tumor types express NCAM polypeptide in addition to biogenic amines, peptide hormones, neurotransmitters, neuron-specific enolase, and so on.[8] In this context it is worth mentioning that NCAM has been detected in muscle,[56] embryonic kidney, and Wilms tumor,[57] as well as being typical of neuroendocrine cell types. A striking feature of NCAM is its content of α 2,8 polysialic acid, which in mammals has been described only on NCAM. In our studies, the MAb 735 has been shown to be a very useful probe for the detection of polysialic acid in paraffin sections.[40–43] Our present results demonstrate that SCLCs can be safely distinguished from bronchial and gastrointestinal carcinoids because only the former contains the highly sialylated form of NCAM. We found that

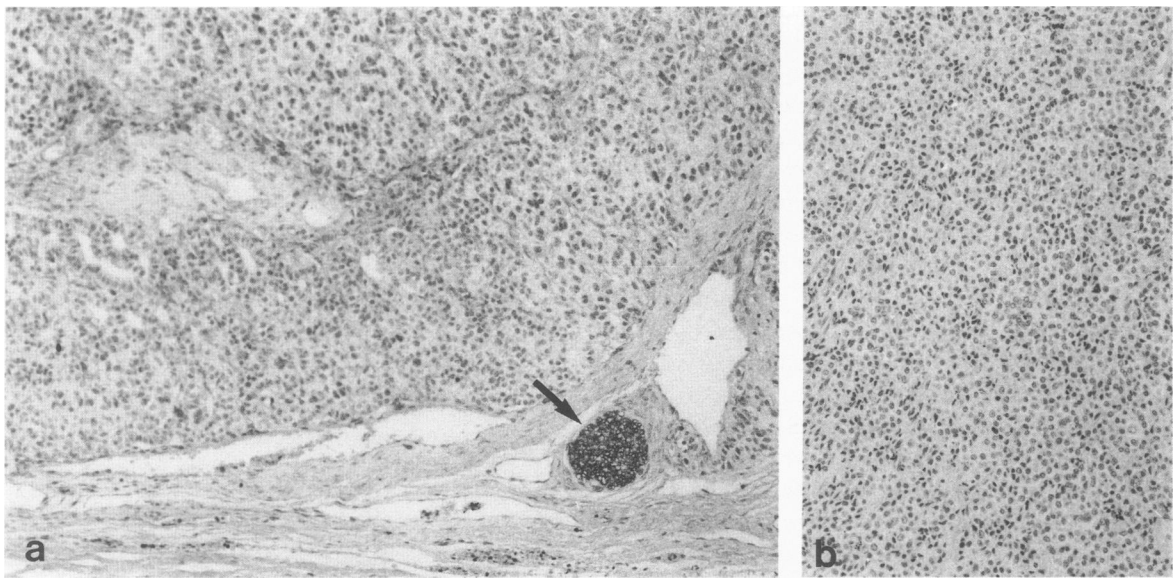

Figure 3. *Carcinoids, immunolocalization of polysialic acid.* **a**: *Mature bronchial carcinoid exhibiting no immunostaining for polysialic acid, whereas a small nerve (arrow) is positively stained (×150).* **b**: *Portion of an atypical bronchial carcinoid negative for polysialic acid (×150).*

SCLCs irrespective of their histologic features were positive for polysialic acid. This indicates that SCLC and its histologic variant of the intermediate type are immunohistochemically identical with respect to this marker. The polysialic acid staining pattern of the combined SCLC, which shows transition into adenocarcinoma or squamous carcinoma,[51,52] remains to be investigated. During the preparation of our manuscript, Kibbelaar et al[58] published data on polysialic acid distribution in lung tumors using the MAb 735. In contrast to our observations, they found granular intracellular staining of bronchial goblet cells as well as positivity in squamous carcinoma (1 of 14 cases), adenocarcinoma (2 of 11 cases), and bronchial carcinoid (1 of 14 cases). Although they reported abolition of polysialic acid immunostaining in sections of SCLC after *Vibrio cholerae* neuraminidase pretreatment of the tissue sections, no such control data were presented on the staining of the other tissues. From our observations on bronchial carcinoids, it became obvious that the observed granular cytoplasmic staining by MAb 735 was not due to presence of polysialic acid. Rather a nonspecific IgG binding probably due to electrostatic interactions as reported by Grube[59] seemed to account for this phenomenon. In our work, such nonspecific antibody binding could be prevented by increasing the ion strength in the PBS. In the absence of appropriate controls, it is possible that the reported staining in normal bronchial goblet cells and the sometimes observed positivity in non-SCLCs may be due to such phenomena. Indeed, in a study on a large number of non-SCLCs of

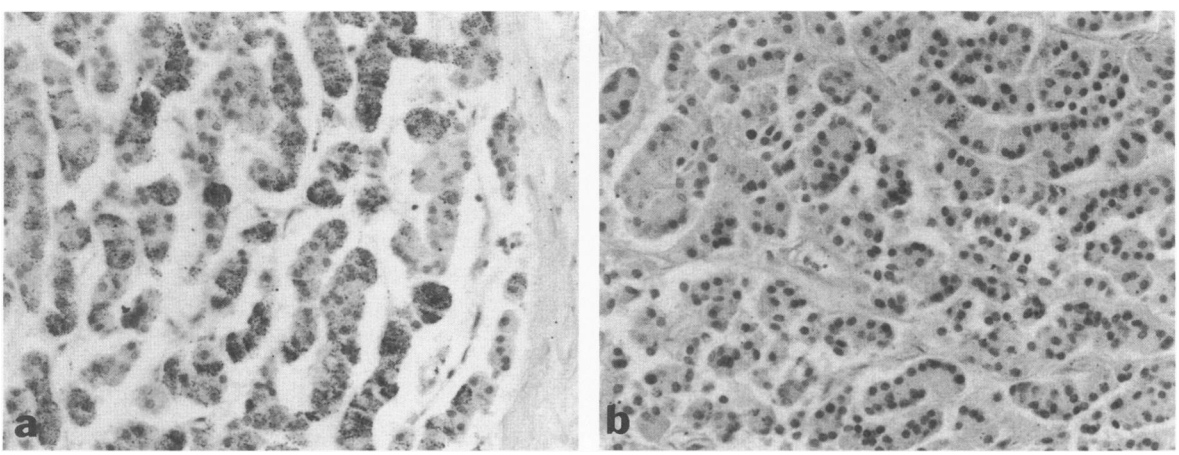

Figure 4. *Bronchial carcinoid.* **a**: *Incubation of sections with gold-labeled MAb 735 diluted in isotonic (0.15 mol/l NaCl) phosphate-buffered saline sometimes resulted in a diffuse cytoplasmic staining that could not be abolished by endoneuraminidase pretreatment (×250).* **b**: *This nonspecific cytoplasmic staining could be prevented by increasing the ion strength from 0.15 mol/l to 0.2 mol/l NaCl (×250).*

various types and degrees of differentiation, we found no polysialic acid immunostaining (manuscript in preparation). These findings once more point to the importance of different controls and in particular show the necessary of applying exoglycosidases and endoglycosidases to enzymatically remove particular carbohydrate moieties if carbohydrate-specific reagents are employed.[48,60]

Our present immunohistochemical data and those obtained for NCAM polypeptide and polysialic acid on other neuroendocrine tumors[30,38,61] demonstrate that these tumors all contain NCAM polypeptide. They differ, however, in the extent of polysialylation of NCAM. Is the presence of cell surface polysialic acid related to the invasive and metastatic potential of these tumors?[42,43,61,62] Obviously there are many highly invasive human tumors that exhibit no cell surface polysialic acid and, conversely, certain normal tissues contain polysialic acid. Therefore the role of NCAM and polysialic acid in invasiveness and metastatic potential in particular tumors deserves further investigation. In conclusion, the present study has demonstrated that SCLCs and bronchial carcinoids contain NCAM, differing in the degree of glycosylation, which allows the immunohistochemical distinction of these neuroendocrine lung tumors with a monoclonal anti-polysialic acid antibody.

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