Expression of Neural Cell Adhesion Molecule-related Sialoglycoprotein in Small Cell Lung Cancer and Neuroblastoma Cell Lines H69 and CHP-212

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ABSTRACT

Monoclonal antibodies (MAbs) 123C3 and 123A8 generated against a membrane preparation of a small cell lung carcinoma (SCLC) specimen recognize not only SCLC and bronchial carcinoids but also a significant portion of non-small cell lung carcinomas (non-SCLC) of various histological types. Together with 13 other monoclonal antibodies, which show preference for SCLC, they have been ranked as SCLC cluster 1 (SC-1) Mabs.

In this study we show that SC-1 MAbs are directed against a restricted number of epitopes, and that SC-1 Mabs and a polyclonal antiserum directed against the neural cell adhesion molecule (NCAM) recognize identical glycoproteins, indicating that SC-1 antigens are closely related to or identical with NCAM. Long polysialic acid units composed of α-(2,8)-linked N-acetylmuramic acid units, which in mammals are found exclusively on NCAM, were present on SC-1 antigens in SCLC. This provides further evidence that SC-1 MAbs recognize NCAM. The SC-1 antigens in the SCLC cell line H69 were present in two forms, NCAM- containing α-(2,8)-polysialic acid units identified by antisera 735, the NCAM-H form, and the less sialylated NCAM-L form. The NCAM-H form consisted of diffusely migrating sialoglycoproteins with a molecular weight of 200,000–250,000, which resolved after neuraminidase treatment into two proteins with molecular weights of 140,000 and 180,000.

Since the NCAM-H form is expressed in the lung tumor type with a poor prognosis, our results suggest that NCAM might be implicated in the invasive behavior of these NCAM-positive lung tumors.

INTRODUCTION

Human small cell lung carcinoma shows neuroendocrine features such as the production of peptide hormones and the presence of marker enzymes, and of neurosecretory granules (1, 2). SCLC accounts for about 25% of the bronchial malignancies and has an extremely poor prognosis. Because of differences in treatment and prognosis between SCLC and non-SCLC, a clear distinction between these two lung tumor types is of great clinical importance. For that purpose, a number of MAbs against SCLC have been produced (3–5), including MAbs 123C3 and 123A8 generated in the Netherlands Cancer Institute (3). The antigens recognized by these SC-1 MAbs have not yet been defined. The tissue distribution patterns of these MAbs were compared at the First International Workshop on SCLC and the MAbs were ranked accordingly (6, 7). Our MAbs 123C3 and 123A8 were, among others, character-

- Received 6/13/89; revised 10/23/89; accepted 10/30/89.
- The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
- This study was partially supported by a grant from Centocor Europe BV, Leiden, The Netherlands.
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- The abbreviations used are: BSA, bovine serum albumin; MAbs, monoclonal antibodies; PBS, phosphate buffered saline; NCAM, neural cell adhesion molecule; NCAM-H, high sialylated NCAM form, recognized by antisera 735 and 123C3; NCAM-L, less sialylated NCAM form, only recognized by antisera 123C3; PSA, polysialic acid; SCLC, small cell lung carcinoma; SC-1, small cell lung carcinoma cluster 1; SDS, sodium dodecyl sulfate.

1 It is assumed that variation in NCAM isoforms influences the functional properties of NCAM molecules (13–16). Because of its role as an adhesion molecule, NCAM, and in particular NCAM isoform modifications in tumor versus normal tissues, might affect invasiveness and thereby influence the malignant behavior of tumor cells.

MATERIALS AND METHODS

Cell Lines, Antibodies. Neuroblastoma cell line CHP-212 was kindly provided by Dr. H. Schlesinger, the University of Pennsylvania, Philadelphia, PA (17). SCLC cell line H69 (18) was a generous gift of Dr. D. Carney, National Cancer Institute, Bethesda, MD. Cell lines were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum and antibiotics. SC-1 MAbs, used in our study (19–22), are depicted in Table 1. MAbs 1, 2 and MAbs 3, 4, 5 (Table 1) were raised in the Netherlands Cancer Institute against a membrane fraction of a SCLC tumor specimen (3) and neuroblastoma cell line CHP-212, respectively. A rabbit antiserum directed against the human NCAM and MAB 735, directed against homopolymers of α-(2,8)-N acetylmuramic acid, were generated by us [respectively, E, B. (23) and D. B-S. (24)]. MAbs 661G10 and 67D11 were raised in the Netherlands Cancer Institute and are directed against the transferrin receptor (25) and MAM-3 (26), respectively.

Immunoassay. Cells (106; H69 or CHD-212) were incubated with excess unlabeled first antibody in PBS-0.2% BSA, for 1 h at room temperature. Subsequently, the cells were incubated with a second 125I-labeled antibody for 15 min. Unbound antibody was washed away with PBS-0.2% BSA, and bound radioactive labeled antibody was measured in a gamma counter. Binding of the labeled antibodies in the...
The binding values were corrected for the percentage inhibition of binding of a nonspecific l2l-labeled MAb, for which MAb 66lGl0 directed against the transferrin receptor (25) was used.

Immunoprecipitation Procedures. Cells were surface labeled with 12l-Iodide and lactoperoxidase and were subsequently lysed in 25 mM Tris-100 mM NaCl-2 mM EDTA pH 8.3-1% Nonidet P-40, in the presence of protease inhibitors: phenylmethylsulfonyl fluoride, trypsin inhibitor, and aprotinin (lysis buffer). Cell lysates were precleared with protein A-Sepharose CL-4B (Pharmacia, Woerden, The Netherlands) and normal mouse serum. Immunoprecipitations were performed with MAbs or polyclonal antiserum, and protein A-Sepharose CL-4B. The specific immunoprecipitates were washed four times in lysis buffer and twice in lysis buffer without Nonidet P-40. Immunoprecipitated material was dissolved in 30 mM Tris-20% glycerol-4% SDS-0.05% bromophenol blue 10% (v/v) β-mercaptoethanol, pH 6.8. After the samples were boiled for 5 min or were kept at 63°C for 10 min (see Fig. 4), the samples were clarified by centrifugation (5 min, 10,000 × g) and analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (27). High molecular weight markers from Bio-Rad (Utrecht, The Netherlands) were used as reference. After electrophoresis, gels were fixed and stained in methanol/acetic acidwater (5:1:5) containing Coomassie Brilliant Blue and destained in methanol/acetic acidwater (75:200:725). The gels were dried and exposed to Kodak X-Omat AR films at −70°C.

Western Blotting. NKI-nbl-3 immunoprecipitates (see "Immunoprecipitation Procedures") were incubated for 10 min at 63°C instead of being boiled for 5 min, separated on a 5% SDS-polyacrylamide gel under reducing conditions, and subsequently transferred to a nitrocellulose filter in Tris/glycine buffer, pH 8.5, at 4°C. Immunostaining was carried out with MAbs (1/100 ascites dilution) in PBS-1% BSA-0.2% Tween 20 using the Protoblot Immunoblotting System (Promega, La Jolla, CA) for 4 hr at 37°C.

Protease Staphylococcus aureus V8 digestions were performed as described by Cleveland et al. (28). Briefly, Mf, 90,000 or 145,000 proteins from immunoprecipitates with 66lGl0 or NKI-nbl-3 and NCAM antiserum, respectively, were excised from the gel and subsequently incubated with various amounts of protease during 30 min at 21°C. Digested peptides were separated on a 15% Laemmli slab gel.

RESULTS

Immunoinhibition Studies. We compared various SC-1 MAbs in an immunoinhibition assay in which the binding of a radioactively labeled MAb is examined in the presence of competing antiserum. As test cells, we used SCLC cell line H69 or neuroblastoma cell line CHP-212. Both cell lines were positive for SC-1 MAbs in an immunofluorescence assay. Inhibition experiments with 12l-I-labeled antibodies 123C3, 123A8, and NKI-nbl-3 and unlabeled MAbs 123C3, 123A8, NKI-nbl-3, NCC-LU-243, and MOC-32 showed complete inhibition (Table 2), indicating that this group of MAbs binds to identical or spatially close epitopes. Similarly, MAbs NKI-nbl-1, NKI-nbl-2, NCC-LU-246, NE-25, NE-150, S-L 11-14, MOC-1, and MOC-21 share the same or closely adjacent epitopes (Table 2). These two groups of MAbs inhibited each other partially in their binding (Table 2). This is probably due to steric hindrance and therefore suggests that these two antigenic determinants are located close to each other on the same polypeptide.

Biochemical Characterization of the SC-1 Antigen. The various SC-1 MAbs were compared in immunoprecipitation studies, using cell surface 125I-labeled CHP-212 cells. MAbs NKI-nbl-3 and NCC-LU-243 precipitated two proteins with molecular weights of 145,000 and 185,000 (Fig. 1, Lanes 3 and 11). After longer film exposure time, both proteins were also identified in immunoprecipitates of the other SC-1 MAbs. All SC-1 MAbs recognized the same protein doublet, the amount of antigen precipitated however, varied among the different SC-1 MAbs. This is probably due to differences in the affinity of the MAbs used. Mab MOC-21 precipitated a third antigen with a

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**Table 1** SCLC cluster 1 monoclonal antibodies used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Isotype</th>
<th>Antigen (Mr)</th>
<th>Ref.</th>
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<tr>
<td>1. 123C3</td>
<td>IgG1</td>
<td>145,000, 185,000</td>
<td>3</td>
</tr>
<tr>
<td>2. 123A8</td>
<td>IgG1</td>
<td>145,000, 185,000</td>
<td>3</td>
</tr>
<tr>
<td>3. NKI-nbl-1</td>
<td>IgG1</td>
<td>145,000, 185,000</td>
<td>3</td>
</tr>
<tr>
<td>4. NKI-nbl-2</td>
<td>IgG1</td>
<td>145,000, 185,000</td>
<td>3</td>
</tr>
<tr>
<td>5. NKI-nbl-3</td>
<td>IgG2</td>
<td>125,000</td>
<td>19</td>
</tr>
<tr>
<td>6. MOC-1</td>
<td>IgG1</td>
<td>125,000</td>
<td>19</td>
</tr>
<tr>
<td>7. MOC-21</td>
<td>IgG2a</td>
<td>25,000</td>
<td>19</td>
</tr>
<tr>
<td>8. MOC-32</td>
<td>IgM</td>
<td>40,000</td>
<td>19</td>
</tr>
<tr>
<td>9. NE-25</td>
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</tr>
<tr>
<td>10. NE-150</td>
<td>IgG1</td>
<td>150,000</td>
<td>20</td>
</tr>
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<td>11. NCC-LU-243</td>
<td>IgG2a</td>
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<td>21</td>
</tr>
<tr>
<td>12. NCC-LU-246</td>
<td>IgG1</td>
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<td>21</td>
</tr>
<tr>
<td>13. S-L 11-14</td>
<td>IgG2a</td>
<td>Glycoprotein</td>
<td>22</td>
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**Table 2** Immunocompetition with the various SC-1 MAbs

<table>
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<tr>
<th>First MAb</th>
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<th>123A8</th>
<th>NKI-nbl-3</th>
<th>NKI-nbl-2</th>
<th>NKI-nbl-1</th>
<th>66lGl0</th>
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<tr>
<td></td>
<td>123C3</td>
<td>123A8</td>
<td>NKI-nbl-3</td>
<td>NKI-nbl-2</td>
<td>NKI-nbl-1</td>
<td>66lGl0</td>
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<tr>
<td>123C3</td>
<td>4</td>
<td>3</td>
<td>97</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>123A8</td>
<td>4</td>
<td>3</td>
<td>98</td>
<td>85</td>
<td>100</td>
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<td>75</td>
<td>100</td>
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</tr>
<tr>
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<td>93</td>
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<td>100</td>
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<td>57</td>
<td>1</td>
<td>100</td>
<td></td>
</tr>
<tr>
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<td>64</td>
<td>75</td>
<td>2</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NCC-LU-246</td>
<td>72</td>
<td>77</td>
<td>78</td>
<td>1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NE-25</td>
<td>66</td>
<td>69</td>
<td>76</td>
<td>1</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>NE-150</td>
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<td>65</td>
<td>79</td>
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<td>100</td>
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<td>6</td>
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</tr>
<tr>
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<td>58</td>
<td>59</td>
<td>58</td>
<td>5</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>MOC-21</td>
<td>55</td>
<td>61</td>
<td>71</td>
<td>8</td>
<td>8</td>
<td>100</td>
</tr>
<tr>
<td>NMS</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
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</tbody>
</table>

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**Fig. 1.** Characterization of SC-1 antigens in CHP-212 cells. Immunoprecipitates of 125-l-cell surface-labeled CHP-212 cells and MAbs NKI-nbl-1 (Lane 1), NKI-nbl-2 (Lane 2), NKI-nbl-3 (Lane 3), 123C3 (Lane 4), 123A8 (Lane 5), NE-25 (Lane 6), NE-150 (Lane 7), NCC-LU-246 (Lane 8), MOC-1 (Lane 9), MOC-21 (Lane 10), NCC-LU-243 (Lane 11), S-L 11-14 (Lane 12), 66lGl0 (Lane 13), and 67D11 (Lane 14).
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molecular weight of 165,000 (Fig. 1, Lane 10); the nature of
this third band still remains to be studied. The control MAb,
66IG10, precipitated a M, 90,000 protein (Fig. 1, Lane 13), as
was expected (25).

Mab 123C3 Recognizes Human NCAM. The tissue distribu­
tion pattern of SC-1 MAbs in human adult and fetal tissues
resembled the pattern of the NCAM, (8). This finding
prompted us to investigate a possible relationship between SC-
1 antigens and NCAM. We therefore used a polyclonal NCAM
antiserum generated against rat brain NCAM, which also rec­
ognizes human NCAM (23), and studied the relationship be­
tween NCAM and SC-1 antigens in three ways: by immunoin­
hibition; by immunoprécipitation and by S. aureus V8 protease
digestion studies.

Immunoinhibition experiments with NCAM antiserum and
125I-labeled CHP-212 or H69 cells, the NCAM antiserum precipitated two proteins, one major protein (M, 145,000) and one M, 185,000 protein (Fig. 2, Lane 5), comparable to the proteins precipitated by SC-1 MAbs (Fig. 2, Lane 1--4). The molecular weight of the M, 145,000 and 185,000 proteins differed only slightly under non-reducing conditions when compared to reducing conditions (a M, 125,000/180,000 protein doublet; data not shown). This is probably due to intrachain disulfide bridges, which have been described for chicken NCAM (9). S. aureus V8 protease digestion of the M, 145,000 protein preparations, which were immu­noprecipitated from CHP-212 cells by MAb NKI-nbl-3 (Fig. 3A) and by NCAM antiserum (Fig. 3B), showed a similar peptide fragment pattern, suggesting that SC-1 MAbs and NCAM antiserum indeed recognize identical proteins. The peptide pattern of the M, 90,000 transferrin receptor protein, which was used as control, showed a quite different pattern (Fig. 3C).

α-(2,8)-Polysialic Acid Units on NCAM Proteins in SCLC.
Another NCAM-specific antiserum used in our study is MAb
735. This MAb recognizes α-(2,8)-linked PSA units with a
chain length of at least 8 residues (29). In mammals, this type
of PSA has thus far been found only on particular forms of
NCAM (8, 29). To determine whether these α-(2,8)-PSA units
are also present on NCAM proteins in SCLC, we analyzed
Western blots of H69 cell preparations with MAb 735. For that
purpose, NCAM proteins were at first immunoprecipitated with
MAb NKI-nbl-3 from H69 cells, separated by SDS-polyacryl­
amide gel electrophoresis, and subsequently transferred to a

![Fig. 2. Identification of antigens recognized by SC-1 MAbs and NCAM
antiserum in CHP-212 cells. Immunoprecipitates of 125I-cell surface-labeled CHP-212 cells with MAb 123C3 (Lane 1), NKI-nbl-1 (Lane 2), NKI-nbl-2 (Lane 3), NKI-nbl-3 (Lane 4), polyclonal rabbit antiserum directed against N-CAM (Lane 5), normal rabbit serum (Lane 6), and control mouse IgG (Lanes 7 and 8).](image)

![Fig. 3. S. aureus V8 peptide maps of the M, 145,000 antigens immunoprecipitated from CHP212 cells with MAbs NKI-nbl-3 (A), NCAM antiserum (B), and a control antigen, the M, 90,000 transferrin receptor, immunoprecipitated by MAb 66IG10 (C). Polypeptides were digested with various amounts of S. aureus V8 protease. Lane 1, 0 µg; Lane 2, 5 µg; Lane 3, 25 µg.](image)

### Table 3 Immunoincompetion with a polyclonal antiserum directed against human NCAM

The binding of a radiolabeled MAb (second MAb-125I) is determined in the presence of NCAM antiserum on live CHP212 cells. Data given are mean values of two experiments, values differed less than 5%.

<table>
<thead>
<tr>
<th>First antibody</th>
<th>Second MAb-125I</th>
<th>% binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCAM</td>
<td>66IG10</td>
<td>100</td>
</tr>
<tr>
<td>NCAM</td>
<td>123C3</td>
<td>7</td>
</tr>
<tr>
<td>NCAM</td>
<td>123A8</td>
<td>3</td>
</tr>
<tr>
<td>NCAM</td>
<td>NKI-nbl-3</td>
<td>1</td>
</tr>
<tr>
<td>NCAM</td>
<td>NKI-nbl-2</td>
<td>31</td>
</tr>
<tr>
<td>NCAM</td>
<td>NKI-nbl-1</td>
<td>30</td>
</tr>
</tbody>
</table>

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NCAM proteins with molecular weights of 145,000 and 185,000 were observed, conditions at which the α-(2,8)-PSA-rich NCAM forms are dissolving the immunoprecipitates at 63°C for 10 min, instead of boiling the samples for 5 min as is usually done. The latter procedure removes the α-(2,8)-PSA units from NCAM proteins (30). MAb NKI-nbl-3 immunostained the NCAM proteins with molecular weights of 145,000 and 185,000, as was to be expected (Fig. 4A, Lane 2). After treatment with V. cholerae neuraminidase, which removes all neuraminic acid, the SC-1 MAbs recognized two antigens with molecular weights of 140,000 and 180,000 (Fig. 4A, Lane 1). A diffuse staining remained at the high molecular weight region in the lane of the untreated NCAM preparation (Fig. 4A, Lane 2) but disappeared after neuraminidase treatment (Fig. 4A, Lane 1). The diffusely migrating proteins in the high molecular weight region therefore represent the NCAM-H proteins. MAb 735 did not recognize the neuraminidase-treated NCAM proteins, as was to be expected (Fig. 4B, Lane 1) but identified only the NCAM-H proteins in the M, 200,000–250,000 range of the gel (Fig. 4B, Lane 2). The unmodified M, 145,000 and 185,000 proteins were not recognized by MAb 735 and therefore represent the NCAM-L proteins that possess no or only very short homopolymers of α-(2,8)-PSA units (maximally 7 residues). We conclude from these experiments that α-(2,8)-PSA units, which thus far in mammals have been found on only particular forms of NCAM, are also present on NCAM proteins in SCLC. H69 SCLC cells apparently contain both NCAM forms, one with the long α-(2,8)-PSA units at a high molecular weight range, and NCAM proteins with molecular weights of 145,000 and 185,000, which do not contain long α-(2,8)-PSA residues (Fig. 4A).

**DISCUSSION**

Monoclonal antibodies 123C3 and 123A8 belong to a group of monoclonal antibodies with very similar reactivities, raised at various institutes throughout the world against SCLC cell lines, SCLC membrane preparations, or neuroblastomas. At a workshop in London in 1987, these antibodies were designated SCLC cluster 1 (6, 7). In this study we demonstrated that these SC-1 MAbs recognize the protein pattern of human NCAM. This finding is based upon similar reaction patterns in immunoinhibition, immunoprecipitation, and S. aureus V8 digestion studies between SC-1 MAbs and a reference polyclonal antiserum which recognizes NCAM (23). We have furthermore shown that all SC-1 MAbs studied, which were raised at different institutes, recognize a restricted set of prominent epitopes on NCAM. All SC-1 MAbs studied fall into two groups, recognizing closely adjacent epitopes on NCAM. The first determinant is recognized by MAb 123C3, 123A8, NKI-nbl-3, NCC-LU-243, and MOC-32; the second determinant by NKI-nbl-1, NKI-nbl-2, NCC-LU-246, NE-25, NE-150, S-L 11-14, MOC-1, and MOC-21 (Table 2). Both groups of MAbs inhibited each other partially in their binding. This is probably due to steric hindrance and therefore suggests that these two antigenic determinants are located close to each other on the same polypeptide. This cross-reactivity of SC-1 MAbs was confirmed by others at the First International Workshop on SCLC (21, 31, 32).

NCAM is a sialoglycoprotein mediating intercellular adhesion by homophilic binding to NCAM on another cell (15). Several NCAM protein isoforms are found on mouse and chicken neural cells, with molecular weights of 120,000, 140,000, and 180,000 (9). All forms are derived from a single copy gene by alternative mRNA splicing and/or posttranslational modifications (9, 33). A major modification is caused by a NCAM characteristic α-(2,8)-polysialylation. Multiple α-(2,8)-PSA units are attached to the NCAM isoforms at certain stages of embryonal development and in particular adult tissues (9). This α-(2,8)-PSA-rich NCAM is recognized by Mab 735 (24), which also detects the SC-1 antigens on SCLC cells (Fig. 4). These results show that α-(2,8)-PSA-rich NCAM is present on SCLC.

In the case of the SCLC cell line H69, both forms of NCAM, the NCAM-H and -L forms, are present (Fig. 4). Fluorescence-activated cell sorter analysis with Mabs 123C3 and 735 showed that both NCAM forms were present on the same cells (data not shown). It is not yet clear whether the absence of long α-(2,8)-PSA units in part of the SCLC-NCAM in H69 cells occurred as a result of tissue culture adaptation. The reverse, however, does not hold; SCLC cells do not acquire long α-(2,8)-PSA units under tissue culture conditions, since these are already present on SCLC tumor samples (34). We found immunoreactivity for antibodies recognizing NCAM in SCLC and some non-SCLC, which had a worse prognosis than negative non-SCLC (35). Whether expression of NCAM on human lung cancer cells results in a more invasive tumor behavior remains to be studied. In the human, NCAM is present on adult neural (36) and skeletal muscle cells (37). In tumors, NCAM expression has been described in Wilms’ tumor, neuroblastoma, and Ewing’s sarcoma (30, 38). NCAMs are expressed in α-(2,8)-PSA-rich NCAM forms in Wilms’ tumor, neuroblastoma, and, as described in this study, also in human SCLC.

One might speculate about the contribution of NCAM to the malignant behavior of tumor cells. For instance, the invasive behavior of these tumors could be attributed to the presence of α-(2,8)-PSA-rich NCAM forms. Polysialylation of NCAM dramatically reduces its adhesive capacity (16) and could therefore...
provoke invasive growth of tumor cells. Particular NCAM isoforms expressed in these tumor cells could also contribute to the malignant behavior of these cells. The SC-1 Mabs recognizing human NCAM now provide reagents to study a possible involvement of NCAM in the invasive behavior of SCLC.

ACKNOWLEDGMENTS

We thank Dr. L. de Ley for generously providing Mabs MOC-1, MOC-21, and MOC-32; Dr. J. C. Laurent for MAbs S-L 11–14; Dr. Ueda for MAbs NE-25 and NE-150; and Dr. Y. Shimosato for MAbs NCC-LU-243 and NCC-LU-246. We thank Drs. W. Mooi, J. Hilgears, H. Ploegh, and P. Borst for valuable advice and critical reading of the manuscript.

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27. Rosier, F., Richler, G., and Laurent, J. C. A group of antibodies which have blocked the growth of small-cell carcinoma of the lung which have amine clonable cultures of small-cell carcinoma of the lung which have amine