Structurally Distinct LewisX Glycans Distinguish Subpopulations of Neural Stem/Progenitor Cells*‡§

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There is increasing evidence that the stem and progenitor cell population that builds the central nervous system is very heterogeneous. Stem cell markers with the potential to divide this cell pool into subpopulations with distinct characteristics are sparse. We were looking for new cell type-specific antigens to further subdivide the progenitor pool. Here, we introduce the novel monoclonal antibody clone 5750. We show that it specifically labels cell surfaces of neural stem and progenitor cells. When 5750-expressing cells were isolated by fluorescence-activated cell sorting from embryonic mouse brains, the sorted population showed increased neurosphere forming capacity and multipotency. Neurospheres generated from 5750-positive cells further subdivide the progenitor pool into subpopulations with distinct characteristics (4–6). The currently available set of antibodies against neural stem and progenitor cells is not sufficient to precisely distinguish between these different subpopulations. Furthermore, intracellular antigens or combinations of transcription factor profiles are useless if it comes to the isolation of living cells, unless the cells are genetically modified to co-express fluorescent proteins such as the GFP (7).

Glycoconjugates have been successfully utilized as specific biomarkers of cell populations at different stages of cellular differentiation. They are mainly expressed on the cell surface. Hence, glycoconjugates are perfectly suitable for isolation methods of living cells such as immunopanning, FACS, and magnetic cell separation. Glycoconjugates comprise proteoglycans, glycoproteins, and glycolipids. Antibodies against glycoconjugates often detect the diverse glycan moieties. An example for an antibody against a glycoconjugate utilized for cell separation is the mAb A2B5, which is routinely used to isolate oligodendrocyte precursor cells (8). Another example is mAb 473HD9 (9), which recognizes a chondroitin sulfate glycosaminoglycan motif (10, 11) that allows enrichment for neural stem cells from cortical embryonic tissue. Another glycan that has been successfully used to isolate neural stem/progenitor cells is the LewisX (LeX) carbohydrate (12, 13). LeX, also known as stage-specific embryonic antigen 1 (SSEA-1) (14) or CD15, is a carbohydrate moiety found on different glycoconjugates. During embryonic development, LeX is expressed by neural progenitor cells, including neural stem cells, neuroblasts, and glioblasts, but not by their more differentiated progenies (12). LeX is expressed on the cell surface of adult neural stem cells (13). Cell surface LeX expression can be used to enrich neural stem and progenitor cells from different CNS regions throughout development. However, the specificity of many cell markers is spatially and temporally restricted. For example, the A2B5 antibody was originally generated against neuronal antigens (15) but is now used to isolate oligodendrocyte precursor cells from the optic nerve. Moreover, in the telencephalon and spinal

During forebrain development, neuroepithelial cells, radial glia cells, and subventricular zone astrocytes constitute the neural stem cell lineage (1, 2). These cells differ by morphology and their antigenic profile. There exist several intracellular marker molecules to identify neural stem cells and their fate-restricted progenitors. The expression of nestin is characteristic for neuroepithelial cells and radial glia. Different subpopulations of radial glia can be distinguished by the expression of the RC2 antigen, the brain lipid-binding protein (BLBP)3 or the glutamate aspartate transporter (GLAST) (3). The progenitor cell population in the embryonic forebrain is very heterogeneous and remains to be further subdivided into subpopulations with distinct characteristics (4–6). The currently available set of antibodies against neural stem and progenitor cells is not sufficient to precisely distinguish between these different subpopulations. Furthermore, intracellular antigens or combinations of transcription factor profiles are useless if it comes to the isolation of living cells, unless the cells are genetically modified to co-express fluorescent proteins such as the GFP (7).

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3 The abbreviations used are: BLBP, brain lipid-binding protein; ChABC, chondroitin ABC lyase; GABAB, ganglionic eminence; GFAp, glial fibrillary acidic protein; GLAST, glutamate aspartate transporter; HNK1, human natural killer antigen 1; LeX, LewisX; L1-CAM, L1 cell adhesion molecule; N-Glyc.F, N-glycosidase F; OPC, oligodendrocyte precursor cell; PDGFR, PDGF receptor; SSEA-1, stage-specific embryonic antigen 1; En, embryonic day n; Pn, postnatal day n; MEM, minimal essential medium; FucT5, fucosyltransferase 9.
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cord, A2B5 labels glial restricted progenitor cells (16, 17). Given this variability, a thorough investigation of the expression pattern is obligatory before an antibody should be used in practical application. Moreover, especially because the heterogeneity of neural stem/progenitor cells seems to be constantly growing, there is a particular need and interest to identify antigens on the cell surface to allow for the isolation of living cells.

In the present study, we were looking for new cell surface antigens specific for neural stem/progenitor cell subpopulations and identified the novel monoclonal antibody clone 5750. We show that it recognizes a cell surface antigen on a subpopulation of neural stem/progenitor cells and can be used to isolate neural stem/progenitor cells from a heterogeneous cell population. mAb 5750 detects glycans containing the LeX motif; however, it is unique among the LeX-detecting antibodies. It labels neural progenitor cells that are distinct from the cell population labeled with anti-LeX mAb clones 487LeX, also referred to as L5 (18, 19), MMA1LeX (20), or SSEA-1LeX (14). This cell-type-specific expression of the different LeX-related glycans can be used to enrich or deplete subpopulations of neural progenitor cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—The following monoclonal antibodies were used: mouse IgG anti-nestin (Millipore, Eschborn, Germany), mouse IgG anti-βIII-tubulin (Sigma), mouse IgM RC2 (Developmental Studies Hybridoma Bank), rat IgM anti-LeX/L5 clone 487 here referred to as 487LeX (18, 19), guinea pig anti-GLAST (Millipore), mouse IgM anti-α-tubulin clone DM1A (Sigma), mouse IgM O4 (21), rat IgG anti-HNK1/L2 clone 412 (22), mouse IgM anti-LeX clone MMA (BD Bioscience, Erembodegem, Belgium) here referred to as MMA1LeX, and mouse IgM anti-LeX clone MC-480 (Developmental Studies Hybridoma Bank) (14) referred to as SSEA-1LeX. The following rabbit polyclonal antibodies were used: anti-glial fibrillary acidic protein (GFAP) (Dako, Hamburg, Germany), anti-BLBP (Millipore), anti-L1-cell adhesion molecule (L1-CAM) (23), KAF14/1 against Tenascin-C (24), anti-platelet-derived growth factor receptor α (PDGFrα) (Santa Cruz, Hamburg, Germany). Secondary antibodies against IgGs or IgMs were conjugated to CY2, CY3, FITC, or horseradish peroxidase (Dianova, Hamburg, Germany). The nuclei were visualized with Hoechst 33258 (Sigma).

**Production of mAb Clone 5750**—For the production of monoclonal antibodies, female LouXSD rats were immunized intraperitoneally and subcutaneously with 50 µg of Tenascin-C purified from postnatal mouse brains (25) emulsified in complete Freund’s adjuvant and twice at 2-week intervals with the same amount of immunogen dissolved in incomplete Freund’s adjuvant. One week later, 50 µg of the antigen in PBS (137 mM NaCl, 3 mM KCl, 6.5 mM Na2HPO4·1.5 mM KH2PO4), pH 7.4, was injected into the tail vein, and fusions were carried out 4 days thereafter by using the mouse myeloma line X-Ag8–653 (Millipore), Belgium) here referred to as MMALeX, and mouse IgM anti-LeX/L5 clone MMA (BD Bioscience, Erembodegem, Belgium) here referred to as MMA1LeX, and mouse IgM anti-LeX clone MC-480 (Developmental Studies Hybridoma Bank) (14) referred to as SSEA-1LeX. The following rabbit polyclonal antibodies were used: anti-glial fibrillary acidic protein (GFAP) (Dako, Hamburg, Germany), anti-BLBP (Millipore), anti-L1-cell adhesion molecule (L1-CAM) (23), KAF14/1 against Tenascin-C (24), anti-platelet-derived growth factor receptor α (PDGFrα) (Santa Cruz, Hamburg, Germany). Secondary antibodies against IgGs or IgMs were conjugated to CY2, CY3, FITC, or horseradish peroxidase (Dianova, Hamburg, Germany). The nuclei were visualized with Hoechst 33258 (Sigma).

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**Animals**—Timed pregnant mice of the NMRI strain were obtained from the department animal facility and housed under standard conditions with access to water and food ad libitum on a normal 12-h light/12-h dark cycle. The day of the vaginal plug was considered as embryonic day 0.5 (E0.5).

**Glycan Array**—The mammalian printed glycan array versions 3.2 and 4.2 were performed according to standard procedures by the Consortium for Functional Glycomics. Different antibody dilutions were tested, each with replicates of three (mAb 5750 1:5–1:100, n = 5; 487LeX 1:100, n = 1).

**Acutely Dissociated Cells**—The tissue of interest from mouse embryos was dissected and enzymatically treated for 20–60 min at 37 °C in MEM with a final concentration of 15 units/ml of papain, 0.12 mg/ml l-cysteine, and 20 µg of DNase I. The enzyme activity was stopped by adding an equal volume ovo- mucoid (1 mg/ml soybean trypsin inhibitor (Sigma), 50 µg/ml BSA V (AppliChem, Darmstadt, Germany), and 40 µg/ml DNase I (Worthington, Freehold, NJ) in L-15 medium. (Sigma). The tissue was gently triturated to a single cell suspension, spun down for 5’ at 200 × g, and resuspended in the appropriate cell culture medium.

**FACS**—Acutely dissociated cortical cells from E14.5 embryos were resuspended in MEM + BSA (MEM (Sigma) supplemented with 0.2% (w/v) BSA V (AppliChem)) and allowed to recover for 15 min. 5750 antibody was added for 20 min to 1 × 10⁶ cells/100 µl whereas the cells were rotated on a horizontal shaker at 150 rpm at room temperature. The cells were washed twice with MEM + BSA. The FITC conjugated anti-rat secondary antibody (Dianova) was added 1:100 diluted in MEM + BSA for 20 min. Subsequently, the cells were washed twice with PBS and passed over a nylon mesh to eliminate cell clumps. Murine embryonic cortical cells were sorted using a BD FACS Vantage SE Cellsorter. Gating parameters were set by side and forward scatter to eliminate debris, dead, and aggregated cells, and by green fluorescence (excitation/emission = 488/530 nm). Parameters for the negative cell population were deduced from cells stained with primary antibody. Initial experiments showed that the goat anti-rat IgM secondary antibody did not show unspecific binding to mouse cells (data not shown).

**Cultivation of Neurospheres**—For clonal analysis, 1250 cells/ml were cultivated in neurosphere medium consisting of DMEM/F-12 (1:1) containing 2% (v/v) B27, 100 units/ml penicillin, and 100 µg/ml streptomycin (all from Sigma), 0.2% (v/v) BSA, and 10 ng/ml EGF (PreproTech) for 1 h each. Single neurospheres were placed on the coated dishes in neurosphere medium containing 1% (v/v)
Cultivation of Glia—Mixed neural cultures were obtained by dissociating mouse postnatal forebrains (P1–P3) as described for acutely dissociated cells. The cells were cultivated at a density of 200,000 cells/mm² in DMEM (Invitrogen) supplemented with 10% (v/v) horse serum (Invitrogen) for 1 week. Primary OPCs and astrocytes were harvested from mixed neural cultures as described (26). Purified OPCs were expanded in DMEM (Invitrogen) containing 1% (v/v) N2 supplement (Invitrogen), 100 µg/ml BSA V (AppliChem), 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen), 10 ng/ml PDGF (Sigma), for 2 days at 37 °C, 7.5% CO₂ on polyornithine (Sigma)-coated dishes. Astrocytes were obtained by growing mixed neural cultures to confluence and incubation with 20 µg/ml cytosine β-D-arabinofuranoside (Sigma) for 1 week.

Complement-mediated Immunocytolysis—Acutely dissociated cortical cells from E14.5 embryos were resuspended in MEM with 0.2% (w/v) BSA. The cells were diluted to a concentration of 1000 cells/ml and then incubated with 0.2% (w/v) horse serum (Invitrogen) for 1 h. The cells were then incubated with 0.05% Trypsin/EDTA (Invitrogen) until the cells detached from the cell culture dish to obtain a single cell suspension. The cells were then diluted in MEM with 0.2% BSA, and immunocytolysis was performed as described for the acutely dissociated cells from E14.5 cortex. Following immunocytolysis, the cells were plated on poly-d-lysine (Sigma) coated cell culture dishes (Nunc, Wiesbaden, Germany) for 24 h in DMEM supplemented with 10% (v/v) horse serum before analysis.

Transfection of COS7 Cells—COS7 cells were routinely cultured on uncoated 10-cm dishes at 37 °C, 6% CO₂ in DMEM supplemented with 10% (v/v) FCS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Lipofection was performed according to the manufacturer’s protocol using the Lipofectamine 2000 reagent (Invitrogen). The cells were transfected with a fucosyltransferase 9 (Fut9) expression plasmid, containing full-length mouse Fut9 cloned into pcDNA3.1 or the empty pcDNA3.1 vector (Invitrogen) as a negative control. To identify transfected cells by GFP fluorescence, the cells were cotransfected with pEGFP-N1 (Invitrogen; ratio pcDNA3.1/pEGFP-N1 5:1). Immunocyto- and Immunohistochemistry—Immunocytochemical staining was performed as outlined previously (27). For staining of acutely dissociated cells, cells from embryonic tissue were resuspended in neurosphere medium and plated on laminin-coated dishes for 2 h before immunocytochemical analysis was performed. The cells from postnatal tissue were grown in DMEM (Invitrogen) 10% FCS on laminin for 24 h before analysis. For simultaneous staining of mAb 5750 with mAbs 487LeX and MMA1LeX, 5750 antibody was linked to NHS-PEO4-Biotin (AppliChem), and stainings were performed sequentially using streptavidin Cy3 (Dianova) conjugate. For immunohistochemistry, E13.5 or E14.5 embryos were removed, and immersion was fixed for 8 h with 4% (w/v) paraformaldehyde in PBS at 4 °C. Tissues were cryoprotected overnight with 20% (w/v) sucrose in PBS, embedded in tissue-freezing medium (Jung, Nussloch, Germany), and frozen on dry ice. The sections (14–16 µm) were cut on a cryostat (Leica, Solms, Germany). Before immunostaining, cryosections were rehydrated in blocking solution (PBS/10% FCS) for 2 h at room temperature in a humidified slide chamber. Primary antibodies were diluted in blocking solution and incubated overnight at 4 °C. The sections were washed three times for 5 min with PBS containing 0.1% BSA (PBS/A). Secondary antibodies were diluted in PBS/A and applied for 2 h in the presence of Hoechst (1:100,000). After three final washing steps with PBS, the cryosections were mounted with glass coverslips in Immumount.

Glycanase Digestion—L1-CAM, Tenascin-C, and Phos- phacan were purified from mouse brains as previously described (23, 24). 300 µg of affinity-purified protein were adjusted to a final concentration of 0.4% (w/v) SDS and 2% (v/v) β-mercaptoethanol and a volume of 40 µl in PBS. The samples were boiled for 5 min at 95 °C. After cooling on ice, the denatured samples were diluted 1:1 with N-glycosidase F buffer (150 mM Na₂HPO₃, pH 8, 50 mM EDTA, 3% (v/v) Nonidet P-40) before adding 0.5 unit of N-glycosidase F EC 3.2.2.18 (Roche Applied Science). Deglycosylation was performed at 37 °C for 4–5 h. If indicated, chondroitinase ABC (ChABC; EC 4.2.2.4) digestion was performed prior to N-glycan removal. Up to 300 µg of purified protein were diluted in 40 mM Tris-HCl, pH 8, 40 mM sodium acetate in a total volume of 30 µl. 5 µl of ChABC (50 milliunits/ml) were added, and the sample was incubated for 4–5 h at 37 °C.

Western Blotting—Cultured cells or brain tissue was homogenized in protein lysis buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% (v/v) Triton X-100, 0.1% (w/v) deoxycholate, 0.1% (w/v) SDS) and cleared at 16,000 g for 15 min. After SDS-PAGE, the proteins were transferred to PVDF membranes (Roth, Karlsruhe, Germany). Before immunostaining, cryosections were rehydrated in blocking solution (PBS/10% FCS) for 2 h at room temperature, the signals were detected by enhanced chemoluminescence (Thermo Fisher Scientific, Bonn, Germany).

Documentation and Data Analysis—The images were acquired with the Axioplan2 (Zeiss, Jena, Germany) and processed with Photoshop CS2 (Adobe). For quantifications of acutely dissociated cells, a minimum of 300 Hoechst-positive nuclei were counted per antibody in at least three independent experiments. Statistical significance was assessed using the two-tailed Student’s t test. The p values are given as * for p ≤ 0.05, ** for p ≤ 0.01, and *** for p ≤ 0.001.
RESULTS

The Monoclonal Antibody Clone 5750 Selectively Labels Neural Stem and Progenitor Cells—We were interested whether mAb 5750 could be used as a cell type-specific marker for CNS cells. When we used mAb 5750 for immunohistochemical staining on cryosections of an E13.5 mouse embryo, we found a striking and highly specific staining only in areas of the dorsal forebrain and the nasal epithelium (Fig. 1A). We further observed some weaker staining in the brain stem (Fig. 1A). However, staining was absent in most body parts (Fig. 1A). To clarify which cells mAb 5750 labels in the brain, we co-labeled markers against neural precursors and neurons, the two cell types that constitute the forebrain at E13.5. The staining pattern of mAb 5750 overlapped with that of the broad precursor cell marker nestin (Fig. 1B). However, 5750 immunoreactivity strongly accumulated at the ventricular surface (Fig. 1C). Together, this indicates that mAb 5750 labels undifferentiated cells but no neurons. We went further and performed a more detailed analysis of the expression of the 5750 epitope by quantification of the number of 5750-immunopositive cells from single cell suspensions of acutely dissociated forebrains. Here, our data showed that at E10.5, 31.34 ± 5.79% (n = 4) of all Hoechst dye-labeled cells in the forebrain were positive for the 5750 epitope. In the embryonic cortex, the number of 5750-positive cells decreased between E14.5 and E18.5 from 21.78 ± 3.53% at E14.5 to 10.70 ± 3.36% at E18.5 (n = 5, p ≤ 0.001). The neural precursor cell markers nestin and RC2 labeled twice as many cells at the investigated time points (Fig. 1D and supplemental Table S1). At E14.5 and E18.5, the number of 5750-positive cells was significantly lower in ganglionic eminence (GE) than in the cortex, ranging between 3 and 4% (E14.5, 3.10 ± 0.49%; E18.5, 4.02 ± 1.41%; p ≤ 0.01; Fig. 1E and supplemental Table S1). Note that we did not distinguish between cortex and GE at E10.5. Because the number of 5750 immunopositive cells is reduced in GE, the overall number of 5750-positive cells in the cortex at E10.5 may be even higher (Fig. 1D).

Based on these initial observations, we were interested whether mAb 5750 exclusively labels neural stem/progenitor cells and hence could be used as a cell type-specific marker. Therefore, we performed double immunocytochemical stainings with mAb 5750 and well characterized cell type-specific markers on neural stem/progenitor cell cultures that were grown as free floating neurospheres (28). Double staining with 5750 and the progenitor cell marker nestin, BLBP, or GLAST on cryosectioned neurospheres showed a strong overlap of the labeled cell populations (Fig. 1, F–H). There was considerably less overlap with markers such as the astrocytic marker GFAP (Fig. 1I) or the neuronal βIII-tubulin (Fig. 1I). When neurospheres were cultured under adherent, differentiating conditions, double-immunostaining revealed that not all nestin-positive cells were co-labeled with mAb 5750 (Fig. 1K). In the light of these results, we conclude that mAb 5750 labels a subpopulation of nestin-positive progenitors.

5750 Surface Labeling Allows to Accumulate or Deplete Neural Stem/Progenitor Cells—Having shown that 5750-expressing cells represent a neural stem/progenitor cell population, we
investigated whether the mAb 5750 also provides a useful tool to enrich for sphere forming cells from primary tissue. The expression of the 5750 epitope on the cell membrane allows the isolation of living cells by cell sorting techniques. Therefore, we tried to isolate neural stem/progenitor cells from the embryonic cortex by fluorescence-activated cell sorting after staining of acutely dissociated cortical cells with mAb 5750 (Fig. 2). Gates were set to isolate cells with a high expression of the 5750 epitope and a 5750-negative cell population devoid of 5750 immunoreactivity. Immunocytochemical analysis of the sorted cells showed that the 5750-positive cell population was enriched in nestin-positive precursor cells as compared with the immunodepleted cell population or the unsorted control (Fig. 2). The mAb 5750-positive cell population (5750) shows a 3-fold increased neurosphere forming capacity compared with the unsorted control (control) and 32-fold to the immunodepleted cell population (negative). D, Neurospheres obtained from 5750-positive cells are multipotent and retain multipotency over several passages. Immunocytochemical analysis of differentiated neurospheres shows that βIII-tubulin-positive neurons (red), GFAP-positive astrocytes (blue), and O4-positive oligodendrocytes (green) are present. The data are expressed as the means ± S.D. (*, p < 0.05; **, p < 0.01; *** p < 0.001). F, Passage: βIII, βIII-tubulin. Scale bar, 50 μm.

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The mAb 5750 Detects a LeX Epitope That Is Present on Several Cell Surface and Extracellular Matrix Molecules—Because the mAb 5750 turned out to be a useful marker to label and enrich for neural stem/progenitor cells, we went on to identify which molecules and structural motif the antibody detects. The mAb 5750 was generated during an immunization with purified Tenascin-C. Therefore, we first tested whether the 5750 epitope is present on Tenascin-C. Fig. 3A shows that mAb 5750

FIGURE 2. FACS sorting using mAb 5750 enriches for self-renewing, multipotent neural precursor cells. E14.5 cortices were dissociated to single cell suspensions, stained with mAb 5750 and FITC-conjugated secondary antibody, and subjected to FACS analysis. A, the diagram shows the gate settings of one representative experiment of three for the FACS analysis. A 5750-positive cell population expressing high levels of the 5750 epitope (5750) and a 5750-negative cell population (negative) was isolated. B, Immunocytochemical analysis of the sorted cells shows that the 5750-positive cell population (5750) contains mostly nestin-positive precursor cells, whereas the majority of βIII-tubulin-positive neurons are found in the 5750-negative cell population (negative). Unsorted cells were used as a control (control). C, the 5750-positive cell population (5750) shows a 3-fold increased neurosphere forming capacity compared with the unsorted control (control) and 32-fold to the immunodepleted cell population (negative). D, Neurospheres obtained from 5750-positive cells are multipotent and retain multipotency over several passages. Immunocytochemical analysis of differentiated neurospheres shows that βIII-tubulin-positive neurons (red), GFAP-positive astrocytes (blue), and O4-positive oligodendrocytes (green) are present. The data are expressed as the means ± S.D. (*, p < 0.05; **, p < 0.01; *** p < 0.001). F, Passage: βIII, βIII-tubulin. Scale bar, 50 μm.

FIGURE 3. The mAb 5750 detects a glycan on Tenascin-C, L1-CAM, and Phosphacan. A and B, immunoaffinity-purified Tenascin-C (A) or L1-CAM (B) were treated either with (+) or without (−) N-glycosidase F (N-Glyc.F) to remove N-linked glycans. The silver stainings confirm the purity and integrity of the respective proteins. Western blot analysis with polyclonal antibodies against the respective proteins (pk Tnc and pk L1) visualizes migration of the core protein in the gel. Note that both in silver stain and after detection with polyclonal antibodies, the detected bands shift down after N-Glyc.F treatment. N-Glyc.F activity was controlled by the removal of the HNK1 epitope. Probing with mAb 5750 shows that the epitope is present on both Tenascin-C and L1-CAM and disappears following N-Glyc.F treatment. C, immunooaffinity-purified Phosphacan was subjected to SDS-PAGE after treatment (+) with N-Glyc.F and/or in combination with ChABC. By Western blotting, 5750 immunoreactivity on Phosphacan is not altered neither after treatment with N-Glyc.F, ChABC, or both enzymes. Note the shift in migration after ChABC treatment. Blotting for HNK1 shows successful N-Glyc.F digestion.
detects affinity-purified Tenascin-C. However, Tenascin-C was not the only protein detected by mAb 5750. In addition, mAb 5750 detected purified L1-CAM (Fig. 3B) and Phosphacan (Fig. 3C). Because mAb 5750 labels multiple proteins, we tested whether the 5750 epitope may be a glycan moiety. Cleavage of N-linked glycans from purified Tenascin-C and L1-CAM with N-glycosidase F diminished 5750 immunoreactivity (Fig. 3, A and B). The analysis suggests that mAb 5750 is directed against an internal tandem repeat of LeX, whereas mAb 487LeX binds terminal LeX, also on a shorter spacer. Phosphacan was transfected with a GFF control plasmid (GFP) or in combination with a plasmid coding for Fut9 (Fut9 + GFP). The 5750 epitope was only detectable on Fut9-transfected COS7 cells, indicating that α1–3-linked fucose produced by Fut9 is sufficient for binding of mAb 5750. RFU, relative fluorescent units; Fuc, fucose; Gal, galactose; Sp0, -CH2CH2NH2; Sp8, -CH2CH2CH2NH2. Scale bar, 50 μm.

FIGURE 4. The 5750 antibody binds to carbohydrates containing the LeX motif. A, a total of 511 structurally defined glycans was tested for binding to mAb 5750 using a standardized glycan array. The graph shows the results of one representative experiment of 5. The numbers indicate the glycans on array version 4.2 that showed high affinity for mAb 5750. Their structural compositions are depicted in C and D. Note that all of the ligands contain the basic LeX motif. The error bars are indicated in gray. B, glycan array version 4.2 performed with the LeX-directed mAb 487LeX. Note that the detected glycans overlap only to some extent with those detected by mAb 5750. C–E, structural composition of the glycans that showed high affinity exclusively for mAb 5750 (C), for both mAbs 5750 and 487LeX (D), and for 487LeX only (E). The analysis suggests that mAb 5750 is directed against an internal tandem repeat of LeX, whereas mAb 487LeX binds terminal LeX, also on a shorter spacer. F, COS7 cells were transfected with a GFP control plasmid (GFP) or in combination with a plasmid coding for Fut9 (Fut9 + GFP). The 5750 epitope was only detectable on Fut9-transfected COS7 cells, indicating that α1–3-linked fucose produced by Fut9 is sufficient for binding of mAb 5750. RFU, relative fluorescent units; Fuc, fucose; Gal, galactose; Sp0, -CH2CH2NH2; Sp8, -CH2CH2CH2NH2. Scale bar, 50 μm.

To obtain more information on the structural composition of the glycan detected by MAb 5750, glycan microarrays were performed. The MAb 5750 was screened against 511 different oligosaccharides. MAb 5750 reacted exclusively with glycans containing the LeX motif (Fig. 4, A–E). MAb 5750 showed a strong affinity for poly-N-acetyl lactosamine chains containing tandem LeX repeats. A terminal LeX motif is not obligatory for antibody binding. Moreover, only low affinity was observed for short, basic LeX motifs, and no reactivity was seen for sulfated LeX or the basic sialyl LeX motif (Fig. 4 and supplemental Figs. S1 and S2). Different monoclonal LeX antibodies have already been published. Hence, we also assayed the monoclonal antibody clone 487LeX known to recognize LeX-related carbohydrates (18). In contrast to mAb 5750, mAb 487LeX showed a high affinity for terminal LeX on poly-N-acetyl lactosamine backbones and short, basic LeX motifs (Fig. 4, A–E). This indicates that the 5750 epitope is unique and differs from the epitope recognized by mAb 487LeX. In an independent approach, we confirmed that the α1,3-linked fucose is essential for 5750 antibody binding. COS7 cells generally do not express the 5750 epitope and show no immunoreactivity after staining with mAb 5750. However, they are capable of protein glycosylation. We transfected COS7 cells with a plasmid encoding for Fut9, which catalyzes the addition of fucose to N-acetylgalactosamine on poly-N-acetyl lactosamine chains, thereby synthesizing the distal Lewis X motif (29). The expression of Fut9 was sufficient for the synthesis of the 5750 epitope by COS7 cells as seen by the 5750 immunoreactivity of the transfected cells.
This shows that α1–3-linked fucose is essential for binding of mAb 5750. In summary, our investigations concerning the molecular composition of the 5750 epitope revealed that mAb 5750 detects glycans containing the LeX motif, with a high affinity toward internal tandem LeX repeats on poly-N-acetyl lactosamine chains.

**Each LeX mAb Clone Detects a Discrete LeX-related Carbohydrate Epitope Expressed by Different Cell Populations**—The data that we obtained so far revealed that mAb 5750 recognizes a LeX related structure, present on different proteins. When comparing the expression pattern of the epitopes detected by the two anti-LeX antibodies 5750 and 487LeX, both showed indeed a similar staining pattern at E14.5 in vivo. Both LeX-related epitopes are expressed primarilly in the embryonic cortex, whereas immunoreactivity in the medial and lateral GE was reduced (Fig. 5, B–D). When we analyzed mouse brain lysates from various developmental stages by Western blot, we could detect several protein bands with mAb 487LeX in the range between 100 and 250 kDa, whereas the 5750 epitope was found predominantly only on a high molecular mass protein of far above 250 kDa (Fig. 5E). These differences in the pattern of 5750 versus 487LeX epitope-carrying proteins led to the hypothesis that both LeX antibodies may label distinct cellular populations. However, the quantification of acutely dissociated cells from E10.5, E14.5, and E18.5 forebrains revealed no significant differences in the number of immunopositive cells (Fig. 5F and supplemental Table S1). Because mAb 487LeX detected many additional proteins at postnatal stages, we also analyzed the number of LeX-positive cells at P2 in the forebrain. Here, 61.29 ± 6.79% of the cells were 487LeX immunopositive, whereas only 12.74 ± 0.59% were immunopositive for the 5750 epitope (Fig. 5G; p ≤ 0.01, n = 3). Immunohistochemical stainings of coronal sections of the postnatal forebrain demonstrated a high 487LeX immunoreactivity throughout the brain, whereas 5750 immunoreactivity was confined to the lateral ventricle and the medio-ventral forebrain (Fig. 5, H and I). These observations strongly support the assertion that the 487LeX epitope and the 5750 epitope are structurally distinct. Furthermore, at least at postnatal stages, they are expressed by different cell populations.
In an alternative approach to demonstrate that different LeX antibodies target distinct subpopulations of cells, 5750- or 487LeX-expressing cells were depleted by complement-mediated immunocytolysis using acutely dissociated E14.5 cortical cells (Fig. 6). Thereafter, the cells were cultivated under neurosphere-forming conditions at a clonal density of 1250 cells/ml. Immunodepletion of the 5750-positive cell population resulted in a reduction of neurosphere formation by 85% (5750-depleted, 15.30 ± 2.11%, p = 0.01, n = 4; Fig. 6E) in comparison with an untreated control (set at 100%). This was comparable with the reduction seen with mAb 487LeX (487LeX-depleted 15.74 ± 20.65%, p = 0.001, n = 6; Fig. 6E). This result supports the conclusion that both LeX-detecting antibodies label the sphere-forming stem/progenitor cell population at this developmental stage. However, only a minor decrease in neurosphere forming capacity was observed when we depleted the 5750-positive cell population from embryonic GE (5750-depleted, 77.45 ± 21.58%; p > 0.05, n = 6; Fig. 6F). This correlates with the small number of 5750-positive cells in GE at E14.5. Depletion of 487LeX-positive cells from GE resulted in a neurosphere reduction by 42% (487LeX-depleted, 58.47 ± 19.79%; p = 0.01, n = 6; Fig. 6F). Different from the embryonic cortex, the stem/progenitor cell populations in the GE can apparently be distinguished by a divergent expression profile of the cell surface antigens detected by mAbs 5750 and 487LeX.

The conclusion that different LeX antibodies label distinct cell populations at postnatal stages was further supported by immunocytochemical stainings of primary mixed neural cultures using the 5750- or 487LeX antibodies (Fig. 7A). These cultures contain astrocytes, OPCs, neural stem/progenitor cells, and microglia (30, 31). When double immunocytochemistry was performed with mAb 487LeX and 5750 on these cultures, the mAb 487LeX labeled many bipolar shaped cells, presumably OPCs. MAb 5750 was most prominently expressed by cells with a flat morphology (Fig. 7B). The comparison of mAb 5750 with the anti-LeX antibody clones MMA-LeX (20) and SSEA-1LeX (14) revealed that, in contrast to the 487LeX labeling, all of the antibodies labeled cells with flat morphology rather than bipolar cells (Fig. 7, C and D). However, the 5750- and MMA-LeX-labeled cell populations, as well as the 5750- and the SSEA-1LeX-labeled cell populations, did not overlap entirely (Fig. 7, C and D). Next, we compared protein lysates from these mixed neural cultures with cultivated astrocytes and OPCs. The Western blot analysis using three different anti-LeX clones, 5750, 487LeX, and MMA-LeX, revealed that, although high molecular mass proteins were labeled similarly by all three antibodies, notable differences were apparent in the proteins detected below 250 kDa (Fig. 7E).

We further investigated whether the differential expression of the 5750 and the 487LeX epitope on different neural cell types can be used to selectively deplete a cell population from a mixed culture. Therefore, we performed immunocytolysis with the mAbs 5750 and 487LeX on the mixed neural cell cultures (Fig. 7, F–I). The prominent expression of the 487LeX glycan on OPCs resulted in the depletion of PDGFRα-positive cells after immunocytolysis with mAb 487LeX. Immunocytolysis with mAb 5750 had no effect on the PDGFRα-positive cell population, although the depletion of 5750-positive cells was confirmed by Western blot and immunostaining. This reinforces the notion that mAbs 487LeX and 5750 target different cell populations.

Hence, we conclude that each anti-LeX antibody clone detects a discrete LeX-related glycan. These distinct LeX-containing glycans are expressed by different subpopulations of cells. This feature can be used to enrich or deplete different neural cell types.

**DISCUSSION**

In this study we introduce the novel mAb 5750. We show that it detects a cell surface epitope on neural stem/progenitor cells and can be used to enrich for neurosphere forming cells by FACs. Therefore, it serves as a new tool for the isolation and investigation of neural progenitor subtypes. At the structural level, we identified the LeX glycan motif as the 5750 epitope. MAb 5750 is unique among the antibodies that detect LeX. It labels a subpopulation of cells that differs from cell population(s) labeled with published anti-LeX antibodies. We further show that different anti-LeX antibody clones can be used to label and subsequently sort for different cell populations.

Our data strongly support that the mAb 5750 detects a unique epitope that is structurally different from the epitopes detected by other anti-LeX antibodies. This can be explained by the fact that carbohydrates in the vicinity of the LeX motif have a strong influence on the binding affinity of the 5750 antibody. The mAb 5750 shows a high affinity for LeX glycans containing internal tandem LeX motifs on a long poly-N-acetyl lactosamine backbone but not for the short LeX motif alone. It is
therefore conceivable that mAb 5750 detects only a minority of the LeX glycans. Similar observations have previously been published for the LeX-detecting antibody SSEA-1LeX (14). SSEA-1LeX recognizes the LeX carbohydrate only when it is present on a long poly-N-acetyl lactosamine backbone, whereas mAb clones 5750, MMA-LeX, and SSEA-1-LeX label cells with flat soma, devoid of processes. However, 5750 overlaps only to some extent with MMA-LeX or SSEA-1-LeX. E, protein lysates from mixed neural cultures (MNC), astrocyte-enriched cultures (Astros), or oligodendrocyte precursor cell enriched cultures (OPC) were analyzed by Western blot with different anti-LeX mAbs. The protein pattern detected in the Western blot is unique for each anti-LeX antibody clone. MAb 487-LeX detects additional proteins on OPCs that are only very weakly or not at all detectable with mAbs 5750 and MMA-LeX (asterisks). The arrowhead indicates the top of the resolving gel. F, mixed neural cultures were incubated with complement-active guinea pig serum alone (control) and in the presence of mAbs 487-LeX or 5750 to deplete the respective cell population. The Western blot shows that 487-LeX immunodepletion leads to a complete elimination of the PDGFRα-positive cell population, whereas 5750 immunodepletion had no effect on PDGFRα-positive cells. The efficiency of the immunocytolysis was verified by blotting for 487-LeX and 5750. G-I, immunofluorescent images of immunodepleted mixed neural cultures, as described for F, stained for 5750, 487-LeX (red), and PDGFRα (green), as indicated. The cell nuclei are labeled in blue. Note that 487-LeX immunodepletion targets OPCs, whereas 5750 immunodepletion does not.

**FIGURE 7. Different anti-LeX monoclonal antibody clones are unique and enable to distinguish between cell types.** A, mixed neural cultures were cultivated as shown, by dissociating postnatal forebrain tissue and cultivation in the presence of horse serum (HS). B–D, double labeling of mixed neural cultures with mAb 5750 (red) and anti-LeX antibody clones 487-LeX (B), MMA-LeX (C), or SSEA-1-LeX (D) in green. The cell populations labeled with the different anti-LeX mAbs overlap to some extent but are not identical. Note that mAb 487-LeX strongly labels process-bearing cells with small somata, whereas mAb clones 5750, MMA-LeX, and SSEA-1-LeX detect cells with flat soma, devoid of processes. However, 5750 overlaps only to some extent with MMA-LeX or SSEA-1-LeX. E, protein lysates from mixed neural cultures (MNC), astrocyte-enriched cultures (Astros), or oligodendrocyte precursor cell enriched cultures (OPC) were analyzed by Western blot with different anti-LeX mAbs. The protein pattern detected in the Western blot is unique for each anti-LeX antibody clone. MAb 487-LeX detects additional proteins on OPCs that are only very weakly or not at all detectable with mAbs 5750 and MMA-LeX (asterisks). The arrowhead indicates the top of the resolving gel. F, mixed neural cultures were incubated with complement-active guinea pig serum alone (control) and in the presence of mAbs 487-LeX or 5750 to deplete the respective cell population. The Western blot shows that 487-LeX immunodepletion leads to a complete elimination of the PDGFRα-positive cell population, whereas 5750 immunodepletion had no effect on PDGFRα-positive cells. The efficiency of the immunocytolysis was verified by blotting for 487-LeX and 5750. G-I, immunofluorescent images of immunodepleted mixed neural cultures, as described for F, stained for 5750, 487-LeX (red), and PDGFRα (green), as indicated. The cell nuclei are labeled in blue. Note that 487-LeX immunodepletion targets OPCs, whereas 5750 immunodepletion does not. NPC, neural precursor cell; α-tub., α-tubulin. Scale bar, 50 μm.
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carbohydrate chain attached to a given protein/lipid. Our Western blot analyses have indicated that there definitively exist differences in the proteins labeled with mAb 5750 and 487LeX, respectively. Irrespective of which of the discussed possibilities is correct, there remains no doubt that different LeX antibodies vary in their binding pattern, and this provides each clone with unique properties.

The position of LeX within a carbohydrate chain obviously accounts for variations in the binding patterns of the different LeX antibody clones. These differences also support the idea that different LeX clones may also be of functional relevance and usefulness to distinguish between cellular subpopulations. Our study showed that the LeX glycans detected by mAb 487LeX are prominently expressed by oligodendrocyte precursor cells, and this feature could be used to deplete this cell population from mixed neural cell cultures. In addition to this property, the mAb 487LeX is one of few anti-LeX antibodies that labels neurons such as human NT2N neurons (39). These two facts reinforce the notion that mAb 487LeX labels more differentiated cell types rather than solely neural progenitor cells. This may be due to a broader binding specificity of mAb 487LeX, allowing the antibody to bind to the LeX motif on short as well as long backbones. The LeX motif on a long poly-N-acetyl lactosamine backbone, as detected by the mAbs 5750 and SSEA1LeX, seems more confined to the undifferentiated stem cells. In this respect, mAb 5750 is more suitable for the investigation of stem cells than mAb 487LeX and other anti-LeX mAbs labeling differentiated cell types. Future studies will be needed to investigate whether the LeX motif on a long poly-N-acetyl lactosamine backbone can indeed be considered a characteristic of neural stem cells, whereas the short terminal LeX motif is a characteristic of differentiated neural cells.

However, the expression of the 5750 epitope is not characteristic for all neural stem cells, in general. We showed that LeX is expressed by the majority of neural stem/progenitor cells in the embryonic cortex but to a lesser extent on neural stem/progenitor cells of the GE. Therefore, the LeX antibody clone 5750 can be used to distinguish between subpopulations of neural stem/progenitor cells. The stem and progenitor cell population in the embryonic forebrain is heterogeneous (4–6). Stem cell markers with the potential to subdivide the neural progenitor cell pool into populations with distinct characteristics are sparse. Therefore, mAb 5750 could be used to investigate the difference between these two stem cell populations and the functional role of the LeX glycan for cortical cells. Besides cell aggregation and migration (36, 40–42), LeX has been implicated to play a role in growth factor responses such as Wnt and FGF signaling (12, 43). However, despite its selective expression, the function of the 5750 epitope and other LeX glycans for cortical development remains elusive.

In conclusion, our experiments demonstrate that the structurally different LeX glycans can be used for the isolation and characterization of subpopulations of neural progenitor cells and their progenies. Our findings further show that the full potential of glycoconjugates has not been tapped regarding their attractiveness as biomarkers of cell populations at different stages of differentiation. The mAb 5750 is an example that glycan antigens are useful for sustained characterization and functional investigation of the different progenitor cell populations. Here, the specificity of the individual LeX clones may prove useful when trying to subdivide the heterogeneous stem and progenitor cell population into further subgroups. Finally, this study provides new insights into the complexity and variability of glycan expression by neural stem and progenitor cells.

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