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The Le^x Carbohydrate Sequence Is Recognized by Antibody to L5, a Functional Antigen in Early Neural Development

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Abstract: The L5 antigenic determinant was previously suggested to be a carbohydrate epitope present on murine cell recognition molecules in the developing brain and to be an early neural marker in the chick embryo. Here, we show that L5 immunoreactivity is associated with complex-type *N*-glycosidic oligosaccharides. To identify the carbohydrate structure recognized by the L5 antibody, we investigate its binding to N-linked oligosaccharides derived from L5 glycoproteins and to known glycans. Results of mass spectrometric analyses of L5-positive neoglycolipids prepared from L5 glycoproteins are consistent with those for *N*-glycans containing a 3-fucosyl *N*-acetyllactosamine sequence. We also investigate L5 binding to structurally defined, lipid-linked oligosaccharides based on the blood group type I and II backbones. Chromatogram binding assays, ELISA, and inhibition studies show that the antibody reacts strongly with carbohydrate chains presenting the 3-fucosyl *N*-acetyllactosamine sequence [Lewis^x (Le^x) or X-hapten] also recognized by anti-SSEA-1 and anti-CD15. Histochemical studies with different antibodies recognizing the Le^x sequence show partially overlapping patterns of immunoreactivity during early neural development in the chick embryo. Therefore, we suggest that the epitope recognized by L5 antibody is closely related to those for anti-SSEA-1 and anti-CD15. **Key Words:** CD15—Chick embryo—L5—Lewis^x—Neural induction—SSEA-1.
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Among the monoclonal antibodies raised to glycoproteins of the murine nervous system, the L5 antibody has been shown to recognize neurons and astrocytes in the cerebellum (Streit et al., 1990, 1993). The L5 antigenic determinant is expressed on multiple glycoproteins, the recognition molecule L1, Thy-1, the chondroitin sulphate proteoglycan astrochondrin, and several components not yet characterized (Streit et al., 1990). Based on results of in vitro experiments, it was proposed that the L5 epitope might participate in the outgrowth of astrocyte processes on extracellular matrix (Streit et al., 1993).

In the early chick embryo, the L5 antigen appears to be expressed initially by cells that are competent to respond to neural inducing signals. During neural induction it becomes rapidly restricted to and up-regulated in the developing nervous system (Roberts et al., 1991; Streit et al., 1995). Hensen's node (the source of neural inducing signals), and hepatocyte growth factor/scatter factor, which is expressed in the node, are both able to maintain and enhance L5 expression in areas of the embryo that would otherwise lose immunoreactivity rapidly. Furthermore, the L5 antibody interferes with neural induction in vivo (Roberts et al., 1991), suggesting that the L5 epitope itself plays a role during early neural development.

Initial studies indicated that the L5 antigenic determinant is a carbohydrate, as immunoreactivity of the murine cell recognition molecule L1 is lost after digestion with a preparation of endoglycosidase F (Streit et al., 1990). To gain insight into the mechanisms of how the L5 antigen participates in neural development, we

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Abbreviations used: BSA, bovine serum albumin; DFLNnH, difucolacto-*N*-neohexaose; DHPE, L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine; DiLe^x, biantennary *N*-glycosidic oligosaccharides containing Le^x on two antennae; FpLNH, fuco-*p*-lacto-*N*-hexaose; HRP, horseradish peroxidase; Le^x, Lewis^x; Le^{x}Tri, Lewis^x-trisaccharide; Le^{x}Tri, Lewis^x-trisaccharide; LNDFH-I and LNDFH-II, lacto-*N*-difucohexaose I and II, respectively; LNFP-I, LNFP-II, and LNFP-III, lacto-*N*-fucopentaose I, II, and III, respectively; LNnDFH-II, lacto-*N*-neodifucohexaose II; LNnT, lacto-*N*-neotetraose; LNT, lacto-*N*-tetraose; LSIMS, liquid secondary ion mass spectrometry; MFLNH, monofucolacto-*N*-hexaose; MonoLe^x, biantennary *N*-glycosidic oligosaccharides containing Le^x on one antenna; PBS, phosphate-buffered saline; 3SLNFP-II, 3′-sialyllacto-*N*-fucopentaose II; 3SLNFP-III and 6SLNFP-III, 3′ and 6′-sialyllacto-*N*-fucopentaose III ceramides, respectively; SuLe^{x}Tetra, sulphated Lewis^x-tetrasaccharide.

have investigated the antigenic determinant recognized by the antibody. We report that the L5 antibody recognizes an epitope closely related to those for anti-SSEA-1 and anti-CD15.

MATERIALS AND METHODS

Antibodies

The rat monoclonal antibody L5 was prepared and purified from culture supernatant as described previously (Streit et al., 1990). The mouse IgM anti-SSEA-1 antibody, clone MC-480 (Solter and Knowles, 1978), was purchased from the Developmental Studies Hybridoma Bank and the mouse IgM AC4 (Dodd and Jessell, 1985) was a kind gift from Dr. T. M. Jessell. In ELISA, we used alkaline phosphatase- (Dianova, Hamburg, Germany) or horseradish peroxidase (HRP)-coupled (Calbiochem) antibodies to rat IgM. The latter were also used as secondary antibodies in chromatogram binding assays and immunohistochemistry with L5. For immunohistochemical staining with anti-SSEA-1 and AC4, HRP-coupled antibodies to mouse IgM were purchased from Sigma.

Glycoproteins and glycopeptides

L1 glycoprotein (Rathjen and Schachner, 1984) was purified from the soluble fraction of early postnatal mouse brain and was kindly provided by Dr. B. Fuss (UCLA, U.S.A.).

L5-positive glycoproteins from mouse brain were purified as described previously (Streit et al., 1990). L5 glycoproteins were isolated from freshly frozen pig brain using the following procedure. Brain tissue (50 g) was homogenized in 75 ml 50 m*M* Tris-HCl, 150 m*M* NaCl, 1 m*M* EDTA, pH 8.0, containing the following protease inhibitors: trypsin inhibitors from soybean (10 μg/ml) and turkey egg white (20 μg/ml), iodoacetamide (0.1 m*M*), phenylmethylsulfonyl fluoride (1 m*M*), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; 10 μg/ml), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK; 10 μg/ml), pepstatin (1 μ*M*), and leupeptin (1 μ*M*). Chloroform/methanol 1:2 (vol/vol) (900 ml) was added and the suspension stirred for 1–2 h at room temperature. The pellet was recovered by filtration through Whatman 3 MM filters, washed with ethanol twice, and dried yielding ~5 g of delipidated protein (10% of starting wet weight of the tissue). The proteins were then solubilized in 20 m*M* Tris-HCl, 1 m*M* EDTA, 150 m*M* NaCl, 1% deoxycholate, pH 8.0, containing the protease inhibitor cocktail described above for 4 h at 4°C. Insoluble material was removed by centrifugation at 100,000 *g*. The supernatant was passed over a Sepharose 4B column connected in series with an L5 monoclonal antibody affinity column (Streit et al., 1990) equilibrated in 20 m*M* Tris-HCl, 150 m*M* NaCl, 0.1% deoxycholate, pH 7.6. After washing with 5 volumes each of 20 m*M* Tris-HCl, 450 m*M* NaCl, 0.1% deoxycholate, pH 7.6, and equilibration buffer, the L5 monoclonal antibody column was eluted with 100 m*M* diethanolamine, 20 m*M* Tris, 1 m*M* EDTA, 0.1% deoxycholate, pH 11.5. The eluate was immediately neutralized with 1 *M* Tris-HCl, pH 6.8, extensively dialyzed against water and freeze dried. This procedure resulted in ~50 μg of purified L5 glycoproteins.

Enzymatic deglycosylation of affinity-purified L5 glycoproteins with endo-β-*N*-acetylglucosaminidase D from *Diplococcus pneumoniae* (EC 3.2.1.96; 2 mU/10 μg of protein), peptide *N*-glycohydrolase F free endo-β-*N*-acetylglucosaminidase F from *Flavobacterium meningosepticum* (EC 3.2.1.96; 20 mU/10 μg of protein), endo-β-*N*-acetylglucosaminidase H from *Streptomyces plicatus* (15 mU/10 μg of protein), peptide *N*-glycohydrolase F from *Flavobacterium meningosepticum* (EC 3.2.2.18; 0.5 U/10 μg of protein), neuraminidase from *Clostridium perfringens* (EC 3.2.1.18; 1 mU/10 μg of protein), or chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4; 10 mU/10 μg of protein) was performed according to the manufacturer's instructions (Boehringer Mannheim, Germany). To stop the reaction, samples were heated to 100°C for 5 min.

Glycopeptides from Thy-1 glycoprotein were obtained as described (Parekh et al., 1987) and were kindly provided by Dr. A. Williams (Oxford, U.K.).

RNase B, hen ovalbumin, fetal calf serum fetuin, human transferrin, human immunoglobulin G, and α_1-acid glycoprotein were purchased from Sigma.

Oligosaccharides

Lacto-*N*-tetraose (LNT), lacto-*N*-neotetraose (LNnT), lacto-*N*-fucopentaose I (LNFP-I), lacto-*N*-fucopentaose II (LNFP-II), and lacto-*N*-fucopentaose III (LNFP-III), lacto-*N*-difucohexaose I (LNDFH-I), lacto-*N*-neodifucohexaose II (LNnDFH-II), lacto-*N*-difucohexaose II (LNDFH-II) were purchased from Dextra Laboratories (Reading, U.K.); fuco-*p*-lacto-*N*-hexaose (FpLNH), monofucolacto-*N*-hexaose (MFLNH), and difucolacto-*N*-neohexaose (DFLNnH) were from Biocarb Chemicals (Lund, Sweden). Lewis^x-trisaccharide (Le^xTri) was from Dextra. Lewis^a-trisaccharide (Le^aTri) was a gift from Prof. A. Lubineau (Orsay, France); sialyl Lewis^a-pentasaccharide (3SLNFP-II) was a gift from Dr. G. Strecker (Lille, France); sulphated Lewis^x-tetrasaccharide (SuLe^{x}Tetra) was a gift from Dr. K. C. Nicolaou (Scripps Institute, California, U.S.A.).

N-linked oligosaccharides were obtained from glycoproteins by hydrazinolysis; these included RNase B [high mannose-type oligosaccharides (Liang et al., 1980)], transferrin [biantennary complex-type oligosaccharides (Spik et al., 1975)], fetuin [mainly triantennary complex-type oligosaccharides (Takasaki and Kobata, 1986)], α_1-acid glycoprotein [tri- and tetraantennary complex-type oligosaccharides including outer chains containing the sialyl Lewis^x (Le^x) sequence (Yoshima et al., 1981)], ovalbumin [hybrid-type oligosaccharides (Yamashita et al., 1983)], human IgG [biantennary complex-type oligosaccharides with and without bisecting *N*-acetylglucosamine (Mizuochi et al., 1982)], L5 glycoproteins (multiantennary complex-type), and Thy-1 glycopeptides [site 1, Asn^{23}, high mannose-type and site 2, Asn^{74}, biantennary complex-type oligosaccharides containing fucose residues (Parekh et al., 1987; Williams et al., 1993)]. Asialo oligosaccharides were prepared by digestion with *Arthrobacter ureafaciens* neuraminidase (Boehringer Mannheim) using conditions suggested by the supplier after they were released by hydrazinolysis. Neutral oligosaccharides were obtained after passing over either a DEAE A-25 Sephadex column (Pharmacia, Milton Keynes, U.K.) or through a double-bed column of AG50W X12 and AG3 4A ion-exchange resins (Bio-Rad, Hemel Hempstead, U.K.). Biantennary *N*-glycosidic oligosaccharides containing Le^x on one antenna (MonoLe^x) or two antennae (DiLe^x) were prepared from a recombinant glycoprotein and were kindly provided by Dr. D. Cumming (Genetics Institute, Boston, MA, U.S.A.).

Lipid-linked oligosaccharides

Neoglycolipids were prepared by conjugation of oligosaccharides to L-1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE; Fluka Chemicals Ltd., Gillingham, U.K.) and purified according to Feizi et al. (1994). The glycosphingolipids 3′-sialyllacto-*N*-fucopentaose III ceramide (3SLNFP-III) and 6′-sialyllacto-*N*-fucopentaose III ceramide (6SLNFP-III) were synthesized chemically, and were gifts from Drs. A. Hasegawa and M. Kiso (Gifu University, Japan).

Chromatogram binding assay

Neoglycolipids and glycolipids were chromatographed on aluminum-backed silica gel 60 high-performance TLC plates (Merck, Poole, U.K.) using chloroform/methanol/water solvent mixtures by volume as indicated in the figure legends. Lipids were visualized by primulin staining (Feizi et al., 1994) before overlay assays. Dried plates were treated with 0.01% Plexigum P-28 in *n*-hexane (Cornelius Chemical Co., Romford, U.K.) for 1 min, air dried again, and blocked in 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.4, for 2 h at room temperature. The plates were then incubated with monoclonal L5 antibody diluted 1:50 in 1% BSA in PBS for another 2 h at room temperature, washed three times in PBS for 5 min each, and incubated in HRP-coupled secondary antibody (1:2,000) for one more hour. After washing, the plates were processed for detection with ECL (Amersham International plc, Amersham, U.K.) according to the manufacturer's instructions; in general, exposure times of 15–30 s were used.

ELISA and inhibition assays

L5 glycoproteins (20 μg/ml in 100 m*M* $NaHCO_3$) before and after enzymatic deglycosylation were coated on flexible microtitre plates (Falcon, Cockeysville, MD, U.S.A.) overnight at 4°C. After blocking for 2 h with 3% BSA in PBS, the wells were washed briefly in PBS, incubated with L5 monoclonal antibody (1:50) diluted in 1% BSA in PBS for 2 h, washed five times in PBS, and incubated with secondary antibody coupled to alkaline phosphatase (1:5,000) for another hour. Color reaction was performed with 1 mg/ml *p*-nitrophenol phosphate (Sigma) in 20 m*M* Tris-HCl, pH 9.6, 0.5 m*M* $MgCl_2$, and 0.5 m*M* $ZnCl_2$ and absorbance measured at 405 nm.

Neoglycolipids were dissolved in methanol at concentrations indicated in the figure legends, applied to flexible microtitre plates, and air dried. Incubation steps were performed as described above, except in some cases where HRP-coupled secondary antibodies were used. In these cases the colour reaction was developed with 4 m*M* *o*-phenylenediamine, 0.004% H_2O_2 in 20 m*M* citric acid, and 50 m*M* Na_2HPO_4, pH 5.0, and measured at 492 nm.

Appropriate antibody concentrations for inhibition studies were determined by titration of L5 antibody binding to constant amounts of coated neoglycolipids or proteins. Concentrations giving 60–70% binding were chosen. For inhibition assays, the antibody was preincubated for 2 h with soluble oligosaccharides before ELISA, which was then performed as described above.

Mass spectrometry

The L5-positive neoglycolipid band derived from L5 glycoproteins was cut out from the TLC plate with its aluminum backing and subjected directly to liquid secondary ion mass spectrometry (LSIMS) after addition of solvent (3 μl; chloroform/methanol/water, 25:25:8 by volume) and matrix (tetramethylurea/diethanolamine/*m*-nitrobenzyl alcohol, 2:2:1 by volume). A negative ion mass spectrum was obtained at 1,000 resolving power on a VG Analytical ZAB2-E mass spectrometer with a DEC 3300/Opus data system using a caesium gun operated at 35 keV and emission current of 0.5 μA.

Whole-mount immunostaining of embryos

Fertile hens' eggs were obtained from Spafas (Massachusetts) and incubated at 38°C until they had reached the desired stage of development. Stages were assessed according to Hamburger and Hamilton (1951). Whole-mount immunostaining was performed as described previously (Streit et al., 1995).

RESULTS

Demonstration of L5 immunoreactivity on complex-type *N*-glycosidic oligosaccharides

When total L5 glycoproteins isolated from pig brain are digested with endoglycosidases with different specificities, we find that L5 immunoreactivity in ELISA is markedly decreased after digestion with peptide *N*-glycosidase F (data not shown) showing a reduction in antibody binding to <10% of that observed with the untreated glycoproteins. This is consistent with our previous results using endoglycosidase F containing peptide *N*-glycosidase F (Streit et al., 1990). In contrast, digestion with peptide *N*-glycosidase F free endoglycosidase F has no effect. Digestion with endoglycosidases D or H, or with chondroitinase ABC, does not alter L5 immunoreactivity. There is a modest increase of antibody binding (~15%) after neuraminidase treatment. These results are consistent with L5 immunoreactivity being associated predominantly with complex-type *N*-glycosidic oligosaccharides that are neuraminidase resistant and partly masked by sialic acid.

Evidence for a relationship between L5 and Le^x

To analyze L5-positive oligosaccharides further, N-linked oligosaccharides were released from L5 glycoproteins by hydrazinolysis. Neoglycolipids were prepared from the neutral and acidic oligosaccharide fractions and tested for L5 antibody binding by the chromatogram overlay assay (Fig. 1). No immunoreactivity is associated with the acidic oligosaccharides (not shown), but two prominent immunoreactive bands are detected in the neutral fraction of oligosaccharides derived from pig brain glycoproteins, and one prominent and one weakly positive band in a similar fraction derived from mouse brain. The chromatographic mobilities of the main immunoreactive neoglycolipids from L5 glycoproteins are in the same range as those derived from complex-type biantennary asialooligosaccharide chains from transferrin. Two immunoreactive bands are also observed in neutral oligosaccharides of α_1-acid glycoprotein (see below).

When analyzed by LSIMS, the main immunoreactive band derived from murine L5 oligosaccharides

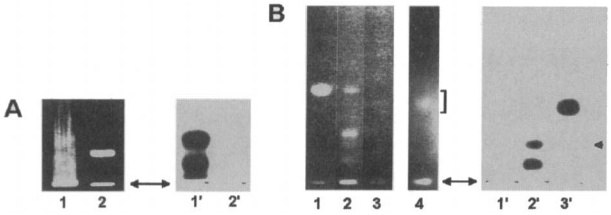
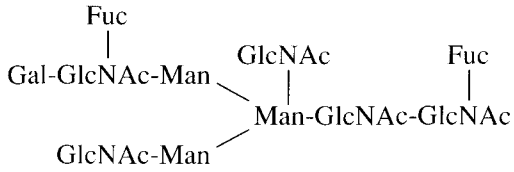


FIG. 1. L5 antibody binding to N-linked oligosaccharides derived from L5 glycoproteins. N-linked oligosaccharides were released from total L5 glycoproteins purified from pig (**A**) and mouse brain (**B**), transferrin (A and B), and α_1-acid glycoprotein (B). The neutral oligosaccharides were converted to neoglycolipids and resolved by TLC (solvent systems, chloroform/methanol/water 55:45:10 in A, 80:75:20 in B). After primulin staining (lanes 1 and 2 in A; lanes 1–4 in B), the chromatograms were probed with L5 antibody (lanes 1′ and 2′ in A; lanes 1′–3′ in B). There are two major L5 immunoreactive bands in oligosaccharides derived from pig brain (A: lane 1′), and one prominent and one very weak (arrowhead) L5 immunoreactive band in those derived from mouse brain (B: lane 3′). Neoglycolipids from transferrin (A: lanes 2, 2′; B: lanes 1, 1′) and α_1-acid glycoprotein (B: lanes 2, 2′) were applied for comparison of mobility and L5 immunoreactivity. Lane 4 in B shows neoglycolipids derived from murine L5 glycoproteins separated by preparative TLC; the area corresponding to the main L5 positive band (square bracket) was analyzed by mass spectrometry. Double-headed arrows indicate the origin.

(Fig. 1) yielded a quasimolecular ion M-H at *m/z* 2,620 and an ion resulting from dehydration (*m/z* 2,602); the latter is characteristic of a lipid-linked *N*-glycosidic oligosaccharide with a core *N*-acetylglucosamine (Mizuochi et al., 1989; Feizi et al., 1994). The deduced composition for these ions is five *N*-acetylhexosamines, four hexoses, and two deoxyhexoses, which is consistent, for example, with the following sequence:



An *N*-glycosidic oligosaccharide of this type, with a bisecting *N*-acetylglucosamine, would be predicted to be resistant to digestion by endoglycosidase F (Trimble and Tarentino, 1991).

A relationship of the L5 epitope to Le^x is suggested by the results of chromatogram binding experiments with neoglycolipids from several *N*-glycosylated proteins (Fig. 2). The L5 antibody reacts with neoglycolipids derived from asialo α_1-acid glycoprotein, which contain complex-type tri- and tetraantennary oligosaccharides with the Le^x sequence in their outer chains (also see Fig. 1). It also recognizes neoglycolipids derived from complex-type oligosaccharides containing outer arm fucose at Asn^{74} of Thy-1 but not those of oligomannose-type from Asn^{23}. The antibody does not bind to oligosaccharides derived from other glycoproteins lacking Le^x-related sequences, i.e., human IgG, ribonuclease B, and fetuin (Fig. 2) and ovalbumin (not shown).

Structural requirements for L5 antibody binding

We performed chromatogram binding assays using a series of structurally defined, lipid-linked oligosaccharides based on Galβ1–4GlcNAc and Galβ1–3GlcNAc, which constitute backbones of the Le^x and Le^a antigens, respectively (Table 1, Fig. 3). Among the oligosaccharide sequences examined, a marked preference is revealed for the Le^x antigen sequence; L5 antibody binds strongly to the Le^x-active LNFP-III and LNnDFH-II but not to the nonfucosylated backbone LNnT (Fig. 3A). Under the standard assay condition using 50 pmol of neoglycolipid per lane, there is almost undetectable binding to the Le^a active LNFP-II (Fig. 3A). This observation is confirmed in ELISA using immobilized LNFP-II (Fig. 4A) and in inhibition assays (see below).

In chromatogram binding assays, no L5 immunoreactivity is observed with the Le^b active sequence LNDFH-I and the blood group H-active sequence LNFP-I, nor with FpLNH, which has an internal Le^x sequence (Fig. 3A and Table 1). Furthermore, the sialyl LNFP-II (3SLNFP-II), sialyl LNFP-III (3 and 6SLNFP-III), and SuLe^{x}Tetra are L5 negative (Table 1). These data suggest that the L5 antibody recognizes terminal Le^x sequences and that binding is inhibited by additional moieties attached to the terminal galactose.

Further details of L5 specificity are obtained by comparison of various branched oligosaccharides in chromatogram binding assays (Fig. 3B). The intensity of L5 antibody binding to the branched DFLNnH sequence is equivalent to that to LNFP-III, whereas L5 binding to MFLNH and to the Mono- and DiLe^x oligosaccharides of *N*-glycosidic-type is weaker than to LNFP-III, which indicates a preference for fucose 3–linked to the terminal Galβ1–4GlcNAcβ1–3 sequence rather than to the Galβ1–4GlcNAcβ1–6 or the Galβ1–4GlcNAcβ1–2 sequences.

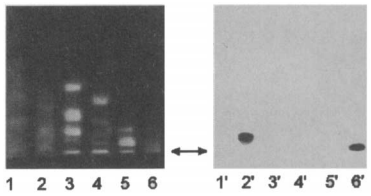


FIG. 2. L5 antibody binding to *N*-glycosidic, complex-type oligosaccharides containing fucose. N-linked oligosaccharides were released from glycopeptides derived from Asn^{23} (lanes 1, 1′) and Asn^{74} (lanes 2, 2′) of Thy-1 glycoprotein, human IgG (lanes 3, 3′), RNase B (lanes 4, 4′), fetuin (lanes 5, 5′), and α_1-acid glycoprotein (lanes 6, 6′); after desialylation, neutral oligosaccharides were linked to lipids and resolved by TLC (solvent system, chloroform/methanol/water 55:45:10). Double-headed arrow indicates origin. After primulin staining (lanes 1–6), chromatograms were probed with L5 antibody (lanes 1′–6′). The antibody binds strongly to neoglycolipids derived from Asn^{74} Thy-1 and α_1-acid glycoprotein.

TABLE 1. *Structurally defined lipid-linked oligosaccharides tested for L5 antibody binding in the chromatogram binding assay*

Designation	Sequence	Binding
LNT	Galβ1–3GlcNAcβ1–3Galβ1–4Glc	–
LNnT	Galβ1–4GlcNAcβ1–3Galβ1–4Glc	–
LNFP-I	Galβ1–3GlcNAcβ1–3Galβ1–4Glc \| 1,2 Fucα	–
LNFP-II	Galβ1–3GlcNAcβ1–3Galβ1–4Glc \| 1,4 Fucα	(+)
LNFP-III	Galβ1–4GlcNAcβ1–3Galβ1–4Glc \| 1,3 Fucα	+++
Le^{a}Tri	Galβ1–3GlcNAc \| 1,4 Fucα	–
Le^{x}Tri	Galβ1–4GlcNAc \| 1,3 Fucα	–
LNDFH-I	Galβ1–3GlcNAcβ1–3Galβ1–4Glc \| 1,2 \| 1,4 Fucα Fucα	–
LNDFH-II	Galβ1–3GlcNAcβ1–3Galβ1–4Glc \| 1,4 \| 1,3 Fucα Fucα	(+)
LNnDFH-II	Galβ1–4GlcNAcβ1–3Galβ1–4Glc \| 1,3 \| 1,3 Fucα Fucα	+++
FpLNH	Galβ1–3GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc \| 1,3 Fucα	–
3SLNFP-II	Galβ1–3GlcNAcβ1–3Galβ1–4Glc \| 2,3 \| 1,4 NeuAcα Fucα	–
3SLNFP-III	Galβ1–4GlcNAcβ1–3Galβ1–4Glc \| 2,3 \| 1,3 NeuAcα Fucα	–
6SLNFP-III	Galβ1–4GlcNAcβ1–3Galβ1–4Glc \| 2,6 \| 1,3 NeuAcα Fucα	–
SuLe^{x}Tetra	Galβ1–4GlcNAcβ1–3Gal | 3 | 1,3 HSO_3 Fucα	–
MFLNH	Fucα | 1,3 Galβ1–4GlcNAcβ1–6 \ Galβ1–4Glc / Galβ1–3GlcNAcβ1–3	++

TABLE 1—*Continued*

Designation	Sequence	Binding
DFLNnH	Fucα | 1,3 Galβ1–4GlcNAcβ1–6 \ Galβ1–4Glc / Galβ1–4GlcNAcβ1–3 \| 1,3 Fucα	+++
MonoLe^x	Fucα | 1,3 Galβ1–4GlcNAcβ1–2Manα1–6/3 \ Manβ1–4GlcNAcβ1–4GlcNAc / Galβ1–4GlcNAcβ1–2Manα1–3/6	+
DiLe^x	Fucα | 1,3 Galβ1–4GlcNAcβ1–2Manα1–6 \ Manβ1–4GlcNAcβ1–4GlcNAc / Galβ1–4GlcNAcβ1–2Manα1–3 \| 1,3 Fucα	++

Oligosaccharides were conjugated to DHPE except for 3SLNFP-III and 6SLNFP-III, which were used as chemically synthesized glycosphingolipids; then they were separated by TLC and probed with L5 antibody. Binding is indicated by the following: −, no binding at 50–500 pmol/lane; (+), negligible binding at 50–100 pmol/lane but unequivocal binding at higher levels; +, moderate binding; ++, medium binding; and +++, strong binding at 50–100 pmol/lane.

The Le^{x}Tri neoglycolipid, however, does not react with L5 antibody under the assay conditions (Fig. 3A and Table 1); this may be due to the chemical modification of the *N*-acetylglucosamine (ring opening) that results from linkage to the lipid (Stoll et al., 1988). In contrast, free Le^{x}Tri is consistently more active than the pentasaccharide LNFP-III in the inhibition experiments performed in ELISA using immobilized LNFP-III (Fig 4B) and L5-positive glycoprotein L1 (Fig. 4C). In the same assay, the free oligosaccharide LNFP-II and Le^{a}Tri are very poor inhibitors of L5 binding (Fig. 4B). 3-Fucosyllactose is ~30 times less potent as an inhibitor compared with Le^x, indicating the importance of the *N*-acetylglucosamine. These re-

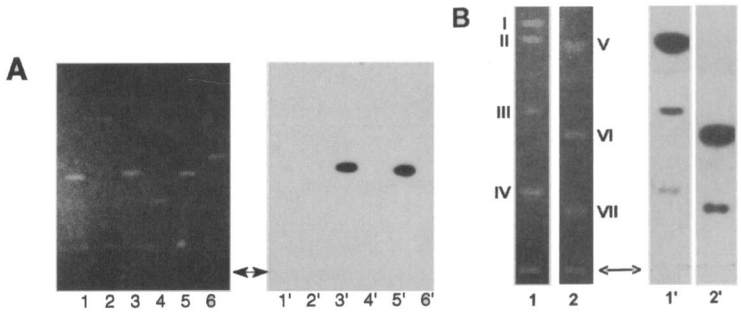


FIG. 3. L5 antibody recognition of oligosaccharides containing the terminal Le^x sequence. Neoglycolipids (~50 pmol/lane) from structurally defined oligosaccharides were resolved by TLC, stained with primulin (A, lanes 1–6; B, lanes 1 and 2), and overlaid with L5 antibody (A, lanes 1′–6′; B, lanes 1′ and 2′). **A:** LNFP-II (lanes 1, 1′), Le^{x}Tri (lanes 2, 2′), LNFP-III (lanes 3, 3′), FpLNH (lanes 4, 4′), LNnDFH-II (lanes 5, 5′), LNnT (lanes 6, 6′). Note very weak L5 positivity of LNFP-II (lanes 1, 1′). Solvent system, chloroform/methanol/water 55:45:10 (vol/vol). **B:** Neoglycolipids from the following oligosaccharides were applied: Lanes 1, 1′: LNnT (band I), LNFP-III (band II), MFLNH (band III), MonoLe^x (band IV); lanes 2, 2′: LNFP-II (band V), DFLNnH (band VI), and DiLe^x (band VII). Solvent system, chloroform/methanol/water 80:75:20 (vol/vol). Note that MFLNH, *N*-MonoLe^x, and *N*-DiLe^x show much weaker L5 immunoreactivity than LNFP-III and DFLNnH.

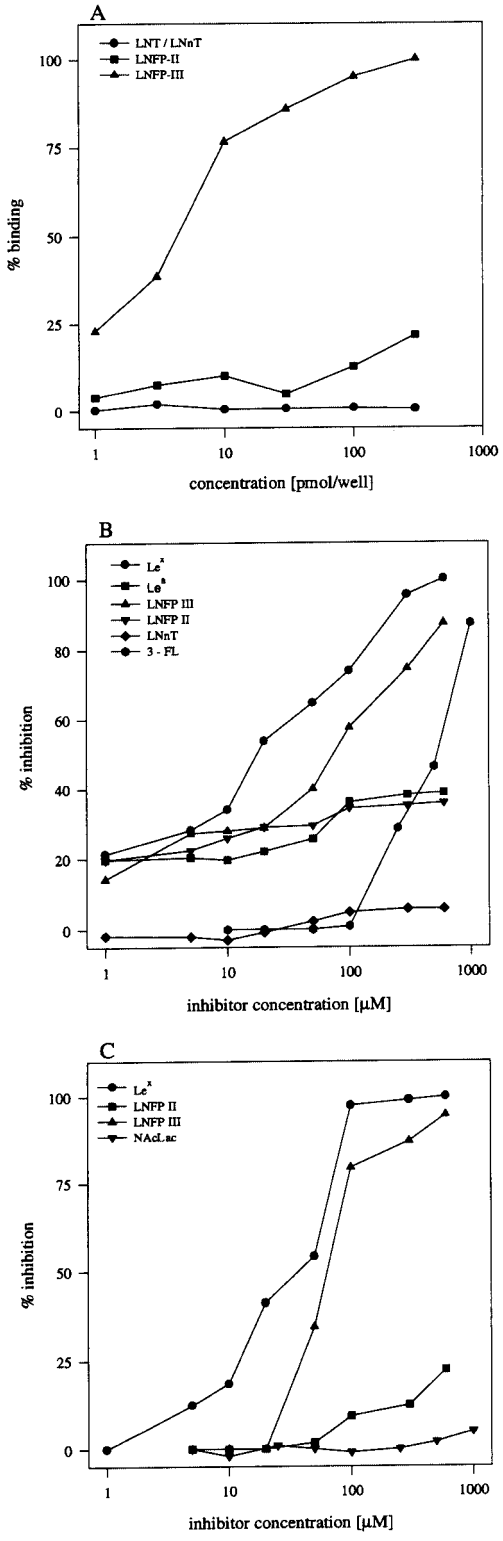


FIG. 4. Binding of L5 antibody to neoglycolipids and glycoproteins and inhibition of binding by Le^x-related compounds. **A:** Binding curves. Different amounts of neoglycolipids of LNT, LNnT, LNFP-II, and LNFP-III were coated on ELISA plates and tested for L5 immunoreactivity. Maximal binding was set to 100%. Graph shows data from one representative experiment of three performed independently in triplicate. Standard deviation in each experiment was <2% for all values. **B:** Inhibition of L5 antibody binding to immobilized LNFP-III by soluble carbohydrates. LNFP-III neoglycolipids (25 pmol/well) were coated on ELISA plates and probed with L5 antibody, preincubated with Le^x, Le^a, LNFP-III, LNFP-II, LNnT, and 3-FL (3-fucosyllactose) at various concentrations before binding assay. Graph shows results of one representative experiment of three performed independently in triplicate. Binding of L5 antibody in the absence of any inhibitor was set to 100%; standard deviation was <2% for all data points in each experiment. **C:** Inhibition of L5 antibody binding to the cell recognition molecule L1 by soluble carbohydrates. L1 (0.5 μg/ml) was coated on ELISA plates and probed with L5 antibody, preincubated with increasing concentrations of Le^x, LNFP-II, LNFP-III, and *N*-acetyllactosamine before binding assay. Graph shows results of one representative experiment of two performed independently in triplicate. Binding of L5 antibody in the absence of any inhibitor was set to 100%; standard deviation was <2.5% for each value in each experiment.

sults support the notion that the L5 antibody reacts with the Le^x sequence, as present on the defined structures tested, and on the glycoprotein L1 and only shows negligible affinity for Le^a-active sequences.

Expression of Le^x in the chick embryo

We compared the patterns of immunoreactivity of the L5 antibody with those of two other antibodies known to recognize the Le^x sequence, anti-SSEA-1 (Solter and Knowles, 1978; Gooi et al., 1981) and AC4 (Dodd and Jessell, 1985), by whole-mount immunostaining of chick embryos (Fig. 5). All three antibodies give identical staining patterns at stages 3–4^+ (Fig. 5A, D, and G). At later stages of development, the three antibodies stain the elevating neural plate and forming neural tube strongly (Fig. 5B, C, E, F, H, and I). However, some differences can be seen elsewhere at these stages. L5 immunoreactivity is confined to the developing neural tissue; but anti-SSEA-1 and AC4, in addition, stain the developing area vasculosa where blood vessel formation begins, and the primordial germ cells (which can also be identified by alkaline phosphatase staining; Fig. 5K and L). This is consistent with a previous report (Yoshinaga et al., 1991) that SSEA-1 is a marker for primordial germ cells in the mouse embryo.

DISCUSSION

In this study we present evidence that the L5 antibody recognizes the blood group-related antigen $Lewis^x$ (also termed Le^x or X-hapten), as do antibodies anti-SSEA-1 and anti-CD15. In overlay assays and ELISA, the L5 antibody binds strongly to neoglycolipids prepared from oligosaccharides that present the terminal Le^x sequence, such as *N*-glycosidic chains derived from glycoproteins or LNFP-III. In inhibition studies, Le^xTri is shown to be the most potent inhibitor of L5 antibody binding to immobilized LNFP-III or to L5-positive glycoproteins such as L1. The binding experiments allow the following conclusions to be

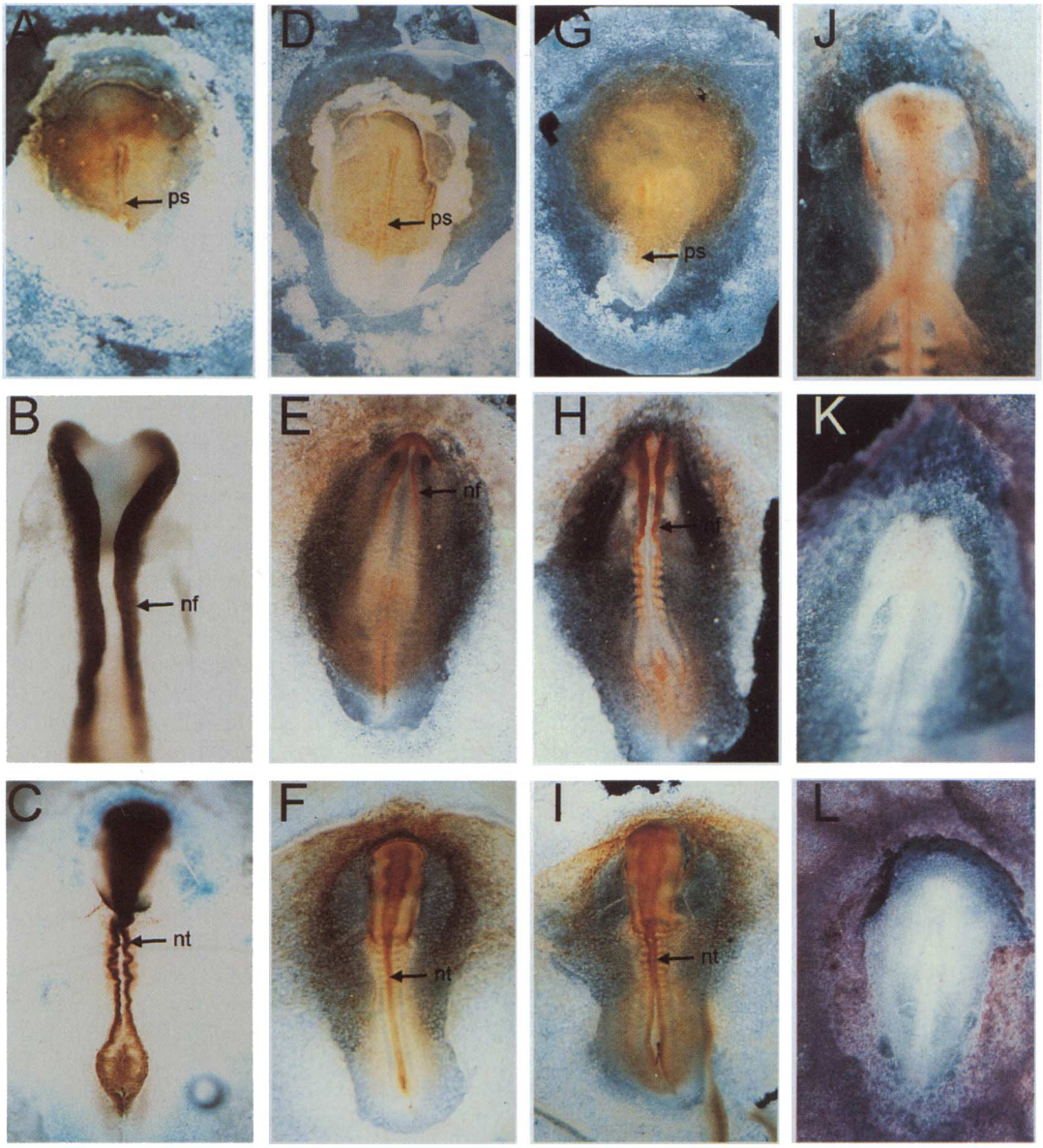


FIG. 5. Expression patterns of the Le^x sequence in whole-mount immunostained chick embryos. Chick embryos of different stages were stained with the antibodies L5 (A–C), anti-SSEA-1 (D–F), and AC4 (G–J) or stained for alkaline phosphatase (K and L). **A, D,** and **G:** Stage $3^+/4$. All three antibodies stain the area pellucida surrounding the anterior primitive streak (ps); immunoreactivity extends into the extraembryonic area opaca. **B, E,** and **H:** Stage 7/8. Strong immunoreactivity with all three antibodies is observed in the elevating neural plate and neural folds (nf). **C, F,** and **I:** Stage 10/11. L5 immunoreactivity (C) is confined to the closing neural tube (nt), whereas SSEA-1 (F) and AC4 (I), in addition, stain the developing area vasculosa. **J:** Higher magnification of the same embryo as in I viewed from the ventral side. The primordial germ cells, visible as scattered individual cells in the head region of the embryo, are AC4 positive. **K:** Stage 9 embryo stained for alkaline phosphatase, which identifies primordial germ cells. **L:** Stage 5 embryo stained for alkaline phosphatase; primordial germ cells are located mainly at the anterior margin of the area pellucida, fewer at the lateral margins.

drawn: First, fucosylation of the subterminal *N*-acetylglucosamine and the presence of an unsubstituted terminal galactose are essential features for antibody recognition, because nonfucosylated structures, and those with modification of the galactose with additional fucose, sialic acid or sulphate, and internal Galβ1–4(Fucα1–3)GlcNAc sequences are all L5 negative. This is further supported by the fact that neuraminidase digestion of brain-derived L5 glycoproteins increases their L5 immunoreactivity, probably by unmasking Le^x determinants. Second, *N*-acetylglucosamine appears to be another important component of the epitope, because in inhibition experiments 3-fucosyllactose proves to be a relatively poor inhibitor of L5 antibody binding. Third, Le^xTri appears to comprise the entire antigenic determinant with none of the inter-

nal saccharides (as present in LNFP-III) being involved, because the trisaccharide is the most potent inhibitor of L5 antibody binding to neoglycolipids and glycoproteins. In this respect, the L5 antibody resembles anti-SSEA-1 and differs from several CD15 antibodies that are more strongly inhibited by LNFP-III than by Le^xTri (Gooi et al., 1985). The results of mass spectrometric analyses of L5-positive neoglycolipids derived from L5 glycoproteins isolated from brain support the above conclusions.

We find a very weak interaction of the L5 antibody with the type 1 structure LNFP-II, an isomer of LNFP-III. Moreover, inhibition studies show that neither Le^a-Tri nor LNFP-II interfere with L5 antibody binding to LNFP-III in a concentration-dependent manner. We therefore suggest that the Le^x determinant is likely to represent the L5 epitope as it is presented on glycoproteins. The stronger binding to LNFP-III (which contains an extended linear backbone) compared with the Le^x located on *N*-glycosidic oligosaccharides (Mono-Le^x and DiLe^x), raises the possibility that glycoproteins with the Le^x sequence at the periphery of extended backbone sequences of the poly-*N*-acetyllactosamine type are important carriers of the L5 epitope. The Le^x epitope was shown to be an abundant component of the N-linked glycans of rat brain, where it occurs mainly in the sialylated form (Krusius and Finne, 1978). The slight increase in L5 reactivity after neuraminidase treatment may suggest that this epitope is only partly sialylated in the L5 glycoproteins.

The Le^x antigen has attracted much attention for some years as its expression is highly regulated during development and because it appears to be involved in a variety of cell interactions. Le^x constitutes the stage-specific embryonic antigen 1, SSEA-1 (Gooi et al., 1981), which appears in the eight- to 16-cell stage of the mouse embryo (Solter and Knowles, 1978; Pennington et al., 1985; Fenderson et al., 1986), where it, or a related sequence, may be involved in compaction of the blastomeres (Bird and Kimber, 1984; Fenderson et al., 1984; Rastan et al., 1985). Whether this is mediated by a lectin–oligosaccharide interaction as originally proposed (Gooi et al., 1981; Rastan et al., 1985), or a carbohydrate–carbohydrate interaction (Eggens et al., 1989; Hakomori, 1992), has not yet been established. Localization studies correlated Le^x expression and pluripotency of cells (Fox et al., 1981), which is also suggested by its disappearance from embryonal carcinoma cells upon differentiation (Gooi et al., 1981; Pennington et al., 1985; Brown et al., 1993).

The Le^x determinant, associated with glycolipids or glycoproteins, is also found in the developing and adult CNS of a variety of organisms (Lagenaur et al., 1982; Yamamoto et al., 1985; Mai and Reiffenberger, 1988; Mai and Schönlau, 1990; Bartsch and Mai, 1991; Marani and Mai, 1992; Satoh and Kim, 1994), showing considerable differences in distribution patterns in different species. Some of this variability might be due to the antibodies used for detection, which appear to differ in their fine specificities and tend to give variable results in similar systems (compare Gooi et al., 1985; Dodd and Jessell, 1986; Marani and Mai, 1992; Satoh and Kim, 1994). One common feature emerging from most of the studies is the expression of the epitope on astrocytes, which was also found for the L5 epitope in the murine cerebellum (Streit et al., 1990), where it was proposed to participate in the formation of astrocytic processes on extracellular matrix (Streit et al., 1993). Extensive process outgrowth of astrocytes is not only observed during normal development, but also after injury of the CNS; these reactive astrocytes were shown to reexpress the SSEA-1 antigen strongly (Gocht and Löhler, 1993). In this context, it is noteworthy that CD15 expression in immortalized astrocytes is particularly concentrated at contact sites and processes (Stark et al., 1992), supporting the notion that Le^x might indeed mediate interactions between astrocytes and the extracellular matrix.

In the chick embryo, various antibodies have been used to localize Le^x/CD15 during development, again showing different expression patterns (Dodd and Jessell, 1986; Scott, 1993), none of which matches L5 expression exactly. Our results indicate that anti-SSEA-1 (Solter and Knowles, 1978; Gooi et al., 1981) and AC4 (Dodd and Jessell, 1985, 1986; Yamada et al., 1991) are most closely related to the L5 antibody. Their patterns of immunoreactivity in the early embryo are almost identical, showing the presence of the Le^x sequence predominantly in the developing nervous system. The differences at later stages might be due to distinct fine specificities or different susceptibility of the three antibodies to substitutions. The exact motifs recognized by them are still unknown.

During embryogenesis, striking changes in the expression of other carbohydrate antigens have been described (Dodd and Jessell, 1986; Canning and Stern, 1988; Thorpe et al., 1988; Loveless et al., 1990), some of which can be correlated with specific developmental processes. Appearance of the neural plate coincides with the loss of long-chain linear and branched type 2 antigens i (Den) and I (Step) (Thorpe et al., 1988) and the type 1 structure recognized by FC10.2 (Loveless et al., 1990); whereas, at the same time, short branched chains such as sialosyl I (Ma) appear to accumulate in the presumptive neural plate. In a similar manner, lectin binding properties of the neuroectoderm change dramatically during neurulation (Currie et al., 1984; Takahashi and Howes, 1986; Takahashi, 1988, 1992).

Up to now, very little has been known about the biological properties of these glycoconjugates during embryogenesis. Our previous studies showed that the L5 carbohydrate is present at a very early stage in neural development and it was suggested that L5 may be a marker for cells competent to respond to neural inducing signals (Streit et al., 1995) and that it plays an important role during the early steps of neural development, because antibodies to the epitope interfere with neural induction or neural plate formation (Rob-

erts et al., 1991). The exact mechanism of L5 action still remains unclear. That we were able to identify the L5 epitope as an Le^x-related sequence, which is known to participate in cell–cell interactions, raises the possibility that the L5 epitope is also involved in interactions between cells of the developing neural ectoderm. This may involve binding of a complementary ligand on the cell surface.

Underscoring the biological role of carbohydrate moieties during early neural development, it is of interest that transgenic mice deficient in *N*-acetylglucosaminyl transferase I show defects in development of neuroepithelial cells and closure of the neural tube (Ioffe and Stanley, 1994; Metzler et al., 1994). In the future, it will be important to determine whether the defects in neural development are due to the lack of complex-type N-linked chains per se or to the lack of expression of the Le^x determinant.

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REFERENCES

Bartsch D. and Mai J. K. (1991) Distribution of the 3-fucosyl-*N*-acetyl-lactosamine (FAL) epitope in the adult mouse brain. *Cell Tissue Res.* **263,** 353–366.

Bird J. M. and Kimber S. J. (1984) Oligosaccharides containing fucose linked α(1-3) and α(1-4) to *N*-acetylglucosamine cause decompaction of mouse morulae. *Dev. Biol.* **104,** 449–460.

Brown D. G., Warren V. N., Påhlsson P., and Kimber S. J. (1993) Carbohydrate antigen expression in murine embryonic stem cells and embryos. I. Lacto and neo-lacto determinants. *Histochem. J.* **25,** 452–463.

Canning D. R. and Stern C. D. (1988) Changes in the expression of the carbohydrate epitope HNK-1 associated with mesoderm induction in the chick embryo. *Development* **104,** 643–655.

Currie J. R., Maylié-Pfenninger M.-F., and Pfenninger K. H. (1984) Developmentally regulated plasmalemmal glycoconjugates of the surface and neural ectoderm. *Dev. Biol.* **106,** 109–120.

Dodd J. and Jessell T. M. (1985) Lactoseries carbohydrates specify subsets of dorsal root ganglion neurons projecting to the superficial dorsal horn of rat spinal cord. *J. Neurosci.* **5,** 3278–3294.

Dodd J. and Jessell T. M. (1986) Cell surface glycoconjugates and carbohydrate-binding proteins: possible recognition signals in sensory neurone development. *J. Exp. Biol.* **124,** 225–238.

Eggens I., Fenderson B. A., Toyokuni T., Dean B., Stroud M., and Hakomori S. (1989) Specific interaction between Le^x and Le^x determinants. *J. Biol. Chem.* **264,** 9476–9484.

Feizi T., Stoll M. S., Yuen C. T., Chai W., and Lawson A. M. (1994) Neoglycolipids: probes of oligosaccharide structure, antigenicity, and function. *Methods Enzymol.* **230,** 484–519.

Fenderson B. A., Zehavi U., and Hakomori S.-I. (1984) A multivalent lacto-*N*-fucopentaose III-lysyllysine conjugate decompacts preimplantation mouse embryos, while the free oligosaccharide is ineffective. *J. Exp. Med.* **160,** 1591–1596.

Fenderson B. A., Holmes E. H., Fukushi Y., and Hakomori S.-I. (1986) Coordinate expression of X and Y haptens during murine embryogenesis. *Dev. Biol.* **114,** 12–21.

Fox N., Damjanov I., Martinez-Hernandez A., Knowles B. B., and Solter D. (1981) Immunohistochemical localisation of early embryonic antigen (SSEA-1) in postimplantation mouse embryos and fetal and adult mice. *Dev. Biol.* **83,** 391–398.

Gocht A. and Löhler J. (1993) Microenvironmental changes during axonal regrowth in the optic nerve of the myelin deficient rat. Immunocytochemical and ultrastructural observations. *J. Neurocytol.* **22,** 461–479.

Gooi H. C., Feizi T., Kapadia A., Knowles B. B., Solter D., and Evans M. J. (1981) Stage-specific embryonic antigen involves α1-3 fucosylated type 2 blood group chains. *Nature* **292,** 156–158.

Gooi H. C., Hounsell E. F., Edwards A., Majdic O., Knapp W., and Feizi T. (1985) Differences in the fine specificities of monoclonal (class A) antibodies to human myeloid cells. *Clin. Exp. Immunol.* **60,** 151–158.

Hakomori S.-I. (1992) Le^x and related structures as adhesion molecules. *Histochem. J.* **24,** 771–776.

Hamburger V. and Hamilton H. L. (1951) A series of normal stages in the development of the chick embryo. *J. Morphol.* **88,** 49–92.

Ioffe E. and Stanley P. (1994) Mice lacking *N*-acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential role for complex or hybrid N-linked carbohydrates. *Proc. Natl. Acad. Sci. USA* **91,** 728–732.

Krusius T. and Finne J. (1978) Characterization of a novel sugar sequence from rat brain glycoproteins containing fucose and sialic acid. *Eur. J. Biochem.* **84,** 395–403.

Lagenaur C., Schachner M., Solter D., and Knowles B. B. (1982) Monoclonal antibody against SSEA-1 is specific for a subpopulation of astrocytes in mouse cerebellum. *Neurosci. Lett.* **31,** 181–184.

Liang C. J., Yamashita K., and Kobata A. (1980) Structural study of the carbohydrate moiety of bovine pancreatic ribonuclease B. *J. Biochem. (Tokyo)* **88,** 51–58.

Loveless W., Bellairs R., Thorpe S. J., Page M., and Feizi T. (1990) Developmental patterning of the carbohydrate antigen FC10.2 during early embryogenesis in the chick. *Development* **108,** 97–106.

Mai J. K. and Reiffenberger G. (1988) Distribution of the carbohydrate epitope 3-fucosyl-*N*-acetyl-lactosamine (FAL) in the adult human brain. *J. Chem. Anat.* **1,** 255–285.

Mai J. K. and Schönlau C. (1990) Distribution of FAL epitopes in developing interlaminar and laminar regions of the monkey and human dorsal lateral geniculate nucleus. *Soc. Neurosci. Abstr.* **16,** 1289.

Marani E. and Mai J. K. (1992) Expression of the carbohydrate epitope 3-fucosyl-*N*-acetyl-lactosamine (CD15) in the vertebrate cerebellar cortex. *Histochem. J.* **24,** 852–868.

Metzler M., Gertz A., Sarkar M., Schachter H., Schrader J. W., and Marth J. D. (1994) Complex asparagine-linked oligosaccharides are required for morphogenic events during post-implantation development. *EMBO J.* **13,** 2056–2065.

Mizuochi T., Taniguchi T., Shimizu A., and Kobata A. (1982) Structural and numerical variations of the carbohydrate moiety of immunoglobulin G. *J. Immunol.* **129,** 2016–2020.

Mizuochi T., Loveless R. W., Lawson A. M., Chai W., Lachmann P. J., Childs R. A., Thiel S., and Feizi T. (1989) A library of oligosaccharide probes (neoglycolipids) from *N*-glycosylated proteins reveals that conglutinin binds to certain complex-type as well as high mannose-type oligosaccharide chains. *J. Biol. Chem.* **264,** 13834–13839.

Parekh R. B., Tse A. G. D., Dwek R. A., Williams A. F., and Rademacher T. W. (1987) Tissue-specific N-glycosylation, site-specific oligosaccharide patterns and lentil lectin recognition of rat Thy-1. *EMBO J.* **6,** 1233–1244.

Pennington J. E., Rastan S., Roelcke D., and Feizi T. (1985) Saccharide structures of the mouse embryo during the first eight days of development. *J. Embryol. Exp. Morphol.* **90,** 335–361.

Rastan S., Thorpe S. J., Scudder P., Brown S., Gooi H. C., and

Feizi T. (1985) Cell interactions in preimplantation embryos: evidence for involvement of saccharides of the poly-*N*-acetyllactosamine series. *J. Embryol. Exp. Morphol.* **87,** 115–128.

Rathjen F. G. and Schachner M. (1984) Immunocytological and biochemical characterization of a new neuronal cell surface component (L1 antigen) which is involved in cell adhesion. *EMBO J.* **3,** 1–10.

Roberts C., Platt N., Streit A., Schachner M., and Stern C. D. (1991) The L5 epitope: an early marker for neural induction in the chick embryo and its involvement in inductive interactions. *Development* **112,** 959–970.

Satoh J. and Kim S. U. (1994) Differential expression of Lewis^x and sialyl-Lewis^x antigens in fetal human neural cells in culture. *J. Neurosci. Res.* **37,** 466–474.

Scott S. A. (1993) Ontogeny, characterization and trophic dependence of AC4/anti-SSEA-1-positive sensory neurons in the chick. *Dev. Brain Res.* **75,** 175–184.

Solter D. and Knowles B. B. (1978) Monoclonal antibodies defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA* **75,** 5565–5569.

Spik G., Bayard B., Fournet B., Strecker G., Bouquelet S., and Montreuil J. (1975) Studies on glycoconjugates. LXIV. Complete structure of two carbohydrate units of human serotransferrin. *FEBS Lett.* **50,** 296–299.

Stark A., Strapper N. J., Sondermann H., and Mai J. K. (1992) Retinoic acid increases CD15 expression in immortalized astrocytes. *Histochem. J.* **24,** 827–832.

Stoll M. S., Mizuochi T., Childs R. A., and Feizi T. (1988) Improved procedure for the construction of neoglycolipids having antigenic and lectin-binding activities, from reducing oligosaccharides. *Biochem. J.* **256,** 661–664.

Streit A., Faissner A., Gehrig B., and Schachner M. (1990) Isolation and biochemical characterization of a neural proteoglycan expressing the L5 carbohydrate epitope. *J. Neurochem.* **55,** 1494–1506.

Streit A., Nolte C., Rásony T., and Schachner M. (1993) Interaction of astrochondrin with extracellular matrix components and its involvement in astrocyte process formation and cerebellar granule cell migration. *J. Cell Biol.* **120,** 799–814.

Streit A., Stern C. D., Théry C., Ireland G. W., Aparicio S., Sharpe M. J., and Gherardi E. (1995) A role for HGF/SF in neural induction and its expression in Hensen's node during gastrulation. *Development* **121,** 813–824.

Takahashi H. (1988) Changes in peanut lectin binding sites on the neuroectoderm during neural tube formation in the bantam chick embryo. *Anat. Embryol.* **178,** 353–358.

Takahashi H. (1992) The masking effect of sialic acid on Con A, PNA and SBA ectoderm binding sites during neurulation in the bantam chick embryo. *Anat. Embryol.* **185,** 389–400.

Takahashi H. and Howes R. I. (1986) Binding pattern of ferritin-labeled lectins (RCA I and WGA) during neural tube closure in the bantam embryo. *Anat. Embryol.* **174,** 283–288.

Takasaki S. and Kobata A. (1986) Asparagine-linked sugar chains of fetuin: occurrence of tetrasialyl triantennary sugar chains containing the Galβ1-3GlcNAc sequence. *Biochemistry* **25,** 5706–5715.

Thorpe S. J., Bellairs R., and Feizi T. (1988) Developmental patterning of carbohydrate antigens during early embryogenesis of the chick: expression of antigens of the poly-*N*-acetyllactosamine series. *Development* **102,** 193–210.

Trimble R. B. and Tarentino A. L. (1991) Identification of distinct endoglycosidase (endo) activities in *Flavobacterium meningosepticum*: endo F1, endo F2 and endo F3. Endo F1 and endo H hydrolyze only high mannose and hybrid glycans. *J. Biol. Chem.* **266,** 1646–1651.

Williams A. F., Parekh R. B., Wing D. R., Willis A. C., Barclay A. N., Dalchau R., Fabre J. W., Dwek R. A., and Rademacher T. W. (1993) Comparative analysis of the *N*-glycans of rat, mouse and human Thy-1. Site-specific oligosaccharide patterns of neural Thy-1, a member of the immunoglobulin superfamily. *Glycobiology* **3,** 339–348.

Yamada T., Placzek M., Tanaka H., Dodd J., and Jessell T. (1991) Control of cell pattern in the developing nervous-system: polarizing activity of the floor plate and notochord. *Cell* **64,** 635–647.

Yamamoto M., Boyer A. M., and Schwarting F. A. (1985) Fucose-containing glycolipids are stage- and region-specific antigens in developing embryonic brain of rodents. *Proc. Natl. Acad. Sci. USA* **82,** 3045–3049.

Yamashita K., Ueda I., and Kobata A. (1983) Sulfated asparagine-linked sugar chains of hen egg albumin. *J. Biol. Chem.* **258,** 14144–14147.

Yoshima H., Matsumoto A., Mizuochi T., Kawasaki T., and Kobata A. (1981) Comparative study of the carbohydrate moieties of rat and human plasma alpha 1-acid glycoproteins. *J. Biol. Chem.* **256,** 8476–8480.

Yoshinaga K., Muramatsu H., and Muramatsu T. (1991) Immunohistochemical localization of the carbohydrate antigen 4C9 in the mouse embryo: a reliable marker of mouse primordial germ cells. *Differentiation* **48,** 75–82.