Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 2248–2252, April 1986
Neurobiology

Selective expression of endogenous lactose-binding lectins and lactoseries glycoconjugates in subsets of rat sensory neurons

(carbohydrate-binding proteins/carbohydrate differentiation antigens/spinal cord/neuronal development)

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Communicated by Gerald D. Fischbach, November 14, 1985

ABSTRACT **Cell-surface glycoconjugates and endogenous lectins have been implicated in cellular interactions that contribute to embryonic development. Functional subsets of primary sensory neurons in mammalian dorsal root ganglia (DRG) have been shown recently to express specific cell-surface oligosaccharide structures. We report here that endogenous lectins with affinity for sensory neuron glycoconjugates are also synthesized by subsets of DRG neurons and are present in the dorsal horn of the developing spinal cord. The distribution of two endogenous lactose-binding lectins, RL-14.5 and RL-29 (subunit M_rs of 14,500 and 29,000, respectively), was examined by immunoblotting and by immunocytochemistry in embryonic and postnatal rat DRG and spinal cord. The two lectins appear soon after the formation of the DRG and are present in the cell bodies and terminals of subsets of DRG neurons that also express cytoplasmic and cell-surface lactoseries glycoconjugates. RL-14.5 and RL-29 are present in overlapping, but not coincident, subsets of DRG neurons that project to the superficial dorsal horn of the spinal cord. In addition, RL-14.5, but not RL-29, is expressed in spinal motoneurons from embryonic day 14. The preferential localization of lactoseries glycoconjugates and lactose-binding lectins in the DRG and the dorsal horn of the spinal cord suggests that these complementary molecules contribute to the development and function of primary sensory neurons.**

Primary sensory neurons in the dorsal root ganglion (DRG) mediate the transfer of cutaneous sensory information to the dorsal horn of the spinal cord. The spinal integration of sensory input is achieved, in part, by the projection of sets of DRG neurons conveying distinct sensory modalities to segregated laminar domains in the dorsal horn (1) and by the formation of sensory connections with appropriate classes of dorsal horn neurons (2). Specificity in sensory projections is apparent from the time that afferent fibers first enter the dorsal horn (3, 4) and may be generated by selective interactions between sensory axons and spinal cord cells.

Studies to characterize molecules involved in the organization of afferent projections to the spinal cord have identified a series of developmentally regulated cell-surface glycoconjugates that are restricted to subsets of DRG neurons projecting to the dorsal horn (5–7). Lactoseries glycoconjugates are expressed in the cytoplasm and on the surface of small-diameter DRG neurons and their central terminals in laminae I and II of the dorsal horn (5, 6), whereas DRG neurons with central terminals in laminae III and IV express globoseries glycoconjugates (7). Similar or identical lacto- and globoseries glycoconjugates are expressed as differentiation antigens on preimplantation embryos and have been implicated in embryonic cell adhesion (8, 9).

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Cell-surface glycoconjugates may mediate cellular interactions by associating with complementary carbohydrate-binding proteins (lectins) (10). Developmentally regulated lactose-binding lectins (LBLs) expressed in a number of tissues, including embryonic brain (11, 12) and spinal cord (13), can interact with lactoseries glycoconjugates (14, 15) and therefore represent potential ligands for lactoseries determinants on DRG neurons. To investigate this possibility, antibodies directed against rat LBLs isolated from lung (16) have been used to determine whether this class of lectin is present in DRG and spinal cord. We report here that two rat LBLs [RL-14.5 and RL-29 (subunit M_rs of 14,500 and 29,000, respectively)] are selectively expressed in subsets of developing and mature DRG neurons that have central terminals restricted to the superficial dorsal horn of the spinal cord. Furthermore, there is a striking overlap in the distribution of lactoseries glycoconjugates and LBLs within DRG and spinal cord, suggesting that these two classes of molecules may interact during the development of sensory neurons.

METHODS

Identification of LBLs in Rat DRG and Spinal Cord. Purification, by affinity and ion-exchange chromatography, of two distinct rat LBLs with subunit M_rs of 14,500 and 29,000 and the generation of antibodies specific to each lectin have been described (16). The lectins are referred to as RL-14.5 and RL-29. To obtain soluble extracts containing RL-14.5 and RL-29, DRG and spinal cord tissues were homogenized with phosphate-buffered saline (pH 7.2) (PBS) containing 0.3 M lactose, 4 mM 2-mercaptoethanol, and 2 mM EDTA, as described (16). Crude membrane fractions were obtained by centrifugation (100,000 × *g*, 60 min) of DRG homogenates prepared as described above. For immunoblotting, soluble and membrane fractions were diluted to a final protein concentration of 1–4 μg/μl in 2% $NaDodSO_4$/0.75 M 2-mercaptoethanol/10% glycerol in 0.06 M Tris·HCl (pH 6.8) and boiled for 10 min. Proteins were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis in a Hoeffer SE-200 apparatus using a 3% stacking gel and 12.5% running gel and transferred to nitrocellulose paper by electrophoretic blotting at 12 V, 200–250 mA, for 45 min at 22°C. Nitrocellulose sheets were then processed by peroxidase-antiperoxidase immunochemical procedures (17) and allowed to react with

Abbreviations: SSEA, stage-specific embryonic antigen; DRG, dorsal root ganglion; RL-14.5 and RL-29, rat lectins with subunit M_rs of 14,500 and 29,000, respectively; mAb, monoclonal antibody; LBL, lactose-binding lectin; E, embryonic day; P, postnatal day.

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0.05% diaminobenzidine (Wako, Osaka, Japan) activated with 0.002% hydrogen peroxide.

Immunocytochemistry. Neonatal and adult rats were anesthetized and perfused intracardially with 5 ml (neonatal) or 50 ml (adult) of ice-cold saline followed by perfusion with 50 ml (neonatal) or 500 ml (adult) of ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). DRG and spinal cord were removed and placed in 30% sucrose overnight at 4°C. Ten- to 15-μm cryostat sections were collected onto gelatin/chrome alum-subbed slides. DRG and spinal cord from gestational day 12–21 rat embryos were fixed by immersion in 4% paraformaldehyde and subsequently processed as described above. Cryostat sections of DRG and spinal cord and of whole embryos were rinsed in PBS and incubated in either rabbit anti-RL-14.5 or rabbit anti-RL-29 antibodies diluted 1:800 in PBS containing 0.1% Triton X-100 and 1% heat-inactivated normal goat serum, for 18–24 hr at 4°C. After washing, sections were incubated in rhodamine-coupled goat anti-rabbit immunoglobulin (Tago, Burlingame, CA) diluted 1:200, washed, "coverslipped," and examined by fluorescence microscopy. Immunoreactive sensory neurons were divided into two classes on the basis of intensity of immunofluorescent labeling. The significance of two levels of expression of lectin in sensory neurons is not clear. However, the number of neurons in each category was found to be constant between experiments, indicating that these populations are consistent and not the result of experimental variations in fixation conditions or antibody penetration.

The immunocytochemical localization of carbohydrate antigens in DRG neurons was performed as described (5–7) using mouse and rat monoclonal antibodies (mAbs) against lactoseries (A5, LD2, and LA4) and globoseries (anti-SSEA-3 and anti-SSEA-4, where SSEA indicates stage-specific embryonic antigen) epitopes (Table 2) and appropriate fluorescein isothiocyanate-labeled second antibodies. Dual fluorochrome immunocytochemical localization of LBLs and carbohydrate antigens was performed on adult rats using second antibodies selective for rabbit or mouse immunoglobulins.

RESULTS

Identification of RL-14.5 and RL-29 in DRG and Spinal Cord. To determine whether RL-14.5 and RL-29 are present in rat DRG and spinal cord, soluble and membrane proteins isolated from neonatal rat DRG and spinal cord were separated by $NaDodSO_4$/polyacrylamide gel electrophoresis and transferred, electrophoretically, to nitrocellulose paper. Immunostaining with antibodies specific for RL-14.5 and RL-29 revealed that DRG and spinal cord from embryonic, neonatal, and adult rats contained immunoreactive proteins with apparent M_rs of 14,500 and 29,000. These proteins were the only detectable immunoreactive species in soluble extracts of DRG and spinal cord (Fig. 1 *a* and *b*). As reported previously with this class of lectin (11), the majority of RL-14.5 and RL-29 immunoreactivity was recovered in soluble form. DRG homogenates or membrane fractions extracted from DRG and spinal cord in the presence of 0.3 M lactose contained only small amounts of the two lectins, with no other detectable immunoreactive bands (not shown).

Immunoblotting techniques were also used to determine whether there are changes in the apparent molecular weight or levels of LBLs during the development of DRG and spinal cord. RL-14.5 was detected in DRG on embryonic day (E) 14 and continued to be expressed at subsequent stages of development, with highest levels of immunoreactivity detected from E 20 to postnatal day (P) 5 (Fig. 1*b*). Lactose-inhibitable hemagglutinating activity detected in soluble extracts of chicken spinal cord is also expressed maximally at an equivalent stage of neuronal development (13). RL-29 immunoreactivity was first detected in DRG on E 16 and

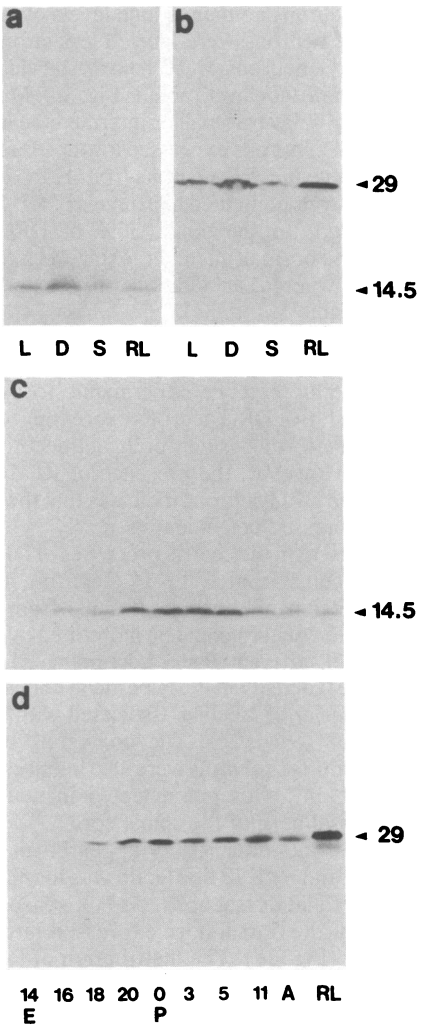

FIG. 1. Identification and developmental regulation of LBLs in rat DRG and spinal cord. (*a* and *b*) Immunoblot analysis of soluble extracts from neonatal rat DRG (D), spinal cord (S), and lung (L) probed with antibodies specific for RL-14.5 (*a*) and RL-29 (*b*). Twenty micrograms of protein was added to each lane of the gel. DRG, spinal cord, and lung contain a single band reactive with each antibody, with electrophoretic mobilities identical to the affinity-purified rat lung lectins (RL). The same two bands were detected, at much lower intensity, in membrane preparations from neonatal DRG and spinal cord loaded at the same protein concentration (not shown). (*c* and *d*) Expression of RL-14.5 and RL-29 in soluble extracts of rat DRG during embryonic and postnatal development. Twenty-five micrograms of protein was added to each lane of the gel. Numbers under each lane refer to the embryonic (E) and postnatal (P) age of rats from which DRG extracts were prepared. A, extract prepared from adult rat DRG. Arrowheads indicate approximate molecular weights (shown as $M_r \times 10^{-3}$) of immunoreactive bands calculated from protein standards of known molecular weight.

continued to be expressed at subsequent stages of development (Fig. 1*d*). These results indicate that from early stages of embryonic development, rat DRG and spinal cord contain proteins with the same subunit size and immunoreactivity as the two characterized LBLs from rat lung (16).

Localization of RL-14.5 and RL-29 in DRG and Spinal Cord During Development. The cellular localization of RL-14.5 and RL-29 was determined by immunofluorescence. The distribution of both proteins was highly restricted within the rat nervous system, with prominent labeling in spinal and cranial sensory neurons.

In the DRG, RL-14.5 was detected by E 13 and continued to be expressed by a subset of neurons at subsequent stages of embryonic and postnatal development (Fig. 2*a*). On P 0,

30–40% of DRG neurons contained high levels of RL-14.5 and a further 5–10% of neurons were labeled less strongly. In the adult, 46% of DRG neurons were heavily labeled and 17% exhibited intermediate labeling (Table 1, Fig. 3*a*). Most neurons that expressed RL-14.5 were small or intermediate in diameter, although some larger neurons expressed low levels of the lectin. RL-29 was detectable in DRG neurons from E 16 (Fig. 2*b*). By P 0, 40–50% of neurons contained high levels of RL-29 and 10% were lightly labeled. In the adult, 56% of DRG neurons, predominantly those of small diameter, exhibited intense RL-29 immunoreactivity, whereas an additional 10% expressed lower levels of RL-29 (Table 1, Fig. 3*c*).

Analysis of the size distribution of labeled cell bodies suggests that most small DRG neurons contained RL-14.5 and RL-29. Dual-label immunofluorescence experiments to evaluate, directly, the relationship of DRG neurons expressing RL-14.5 and RL-29 were not performed since both anti-lectin sera were raised in rabbits. However, the presence of RL-14.5 but not RL-29 in some large DRG neurons indicates that the two lectins are not always expressed coincidently.

RL-14.5 was present in the central processes of DRG neurons in the dorsal funiculus from E 13–14 (Fig. 2*a*). From E 15 onward, the projection of sensory afferents from the dorsal funiculus into the dorsal horn could be monitored by expression of RL-14.5. At P 0, afferent fibers containing RL-14.5 were distributed at highest density in the superficial dorsal horn, with a much lower intensity of labeling associated with afferents in laminae III and IV (Fig. 2*c*). The collaterals of afferents projecting into the dorsal columns were also intensely labeled at P 0 (Fig. 2*c*). RL-14.5 was not detected in neuroblasts or neurons in the dorsal horn of the spinal cord.

Afferent fibers containing RL-29 appeared in the dorsal funiculus on E 17, and by E 18 this lectin was found in afferent fibers in the superficial dorsal horn. At P 0, sensory fibers in laminae I and II of the dorsal horn expressed intense RL-29 immunoreactivity (Fig. 2*d*). The distribution of RL-29 in the DRG and spinal cord was more restricted than that of RL-14.5 and was absent from deeper laminae of the dorsal horn (Fig. 2*d*). The intensity of RL-14.5 and RL-29 immunoreactivity in the dorsal horn of adult rats was much lower than that at day P 0 and was restricted to laminae I and II. RL-14.5 and RL-29, therefore, appear to be present at maximal levels in sensory neurons over the period that afferent fibers establish appropriate projections in the dorsal horn.

Table 1. Expression of LBLs in adult rat DRG neurons

Lectin	*n*	% DRG neurons: Intensely labeled	Lightly labeled	Unlabeled
RL-14.5	4736	46	17	37
RL-29	9887	56	10	33

n, Number of DRG neuronal profiles analyzed.

The distribution of these two lectins in other regions of the developing spinal cord was also restricted. RL-14.5 was detected in motoneurons on E 13, with the intensity of labeling increasing during later embryonic and early postnatal stages (Fig. 2*c*). In contrast, RL-29 was not present in motoneurons (Fig. 2*d*). From E 16 to P 0, radial glial fibers in the ventrolateral funiculus expressed RL-29 but not RL-14.5 (Fig. 2*d*). In early embryos, RL-29 was also detected in cells and fibers that project radially from the basal plate region of spinal cord (not shown).

In supraspinal regions of the embryonic, neonatal, and adult central nervous system, RL-14.5 and RL-29 immunoreactivity was concentrated in areas known to receive primary afferent input from neurons located in cranial sensory ganglia. Immunoreactive fibers and terminals were present in the dorsal horn of the medulla, in the nucleus of the solitary tract, and in the gracile nucleus. Motoneurons in brainstem nuclei also expressed RL-14.5 but not RL-29 immunoreactivity. RL-14.5 and RL-29 were undetectable, or expressed at very low levels, in cerebral cortex, hippocampus, thalamus, cerebellum, and olfactory bulb.

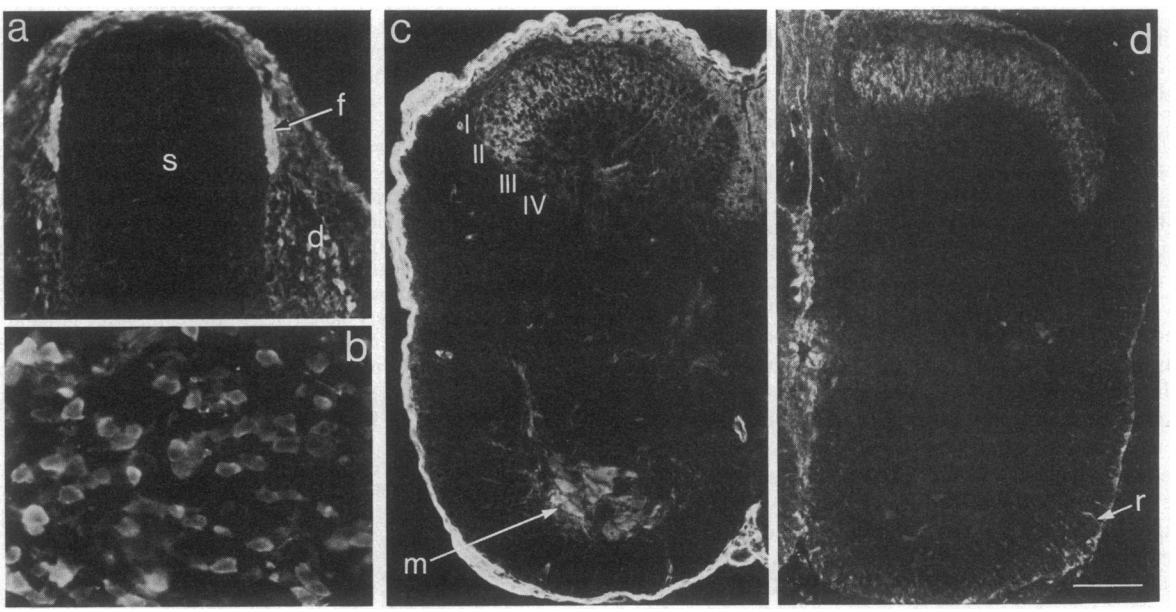

FIG. 2. Immunocytochemical localization of LBLs in rat DRG and spinal cord during development. (*a*) Localization of RL-14.5 in the DRG and dorsal funiculus of E 14 rat spinal cord. Immunoreactive sensory fibers are not detectable in the dorsal horn of the spinal cord at this stage. Labeling of connective tissue elements surrounding the spinal cord is also apparent. d, DRG; f, dorsal funiculus; s, spinal cord. (*b*) Localization of RL-29 in E 20 rat DRG. At this stage 40–50% of neurons contain the lectin. (*c*) Localization of RL-14.5 in the spinal cord on P 0. An intense band of immunoreactive sensory fibers is present in laminae I and II, with much less intense labeling in laminae III and IV. The collaterals of primary afferents in the dorsal columns and the cell bodies and processes of motoneurons (m) in the ventral horn are also heavily labeled. Roman numerals indicate dorsal horn laminae. (*d*) Localization of RL-29 in the spinal cord on P 0. Immunoreactive sensory afferents are restricted to laminae I and II. Light staining of radial glial fibers (r) in the ventral white matter can be observed. (Scale bar: 150 μm in *a*; 28 μm in *b*; 100 μm in *c* and *d*.)

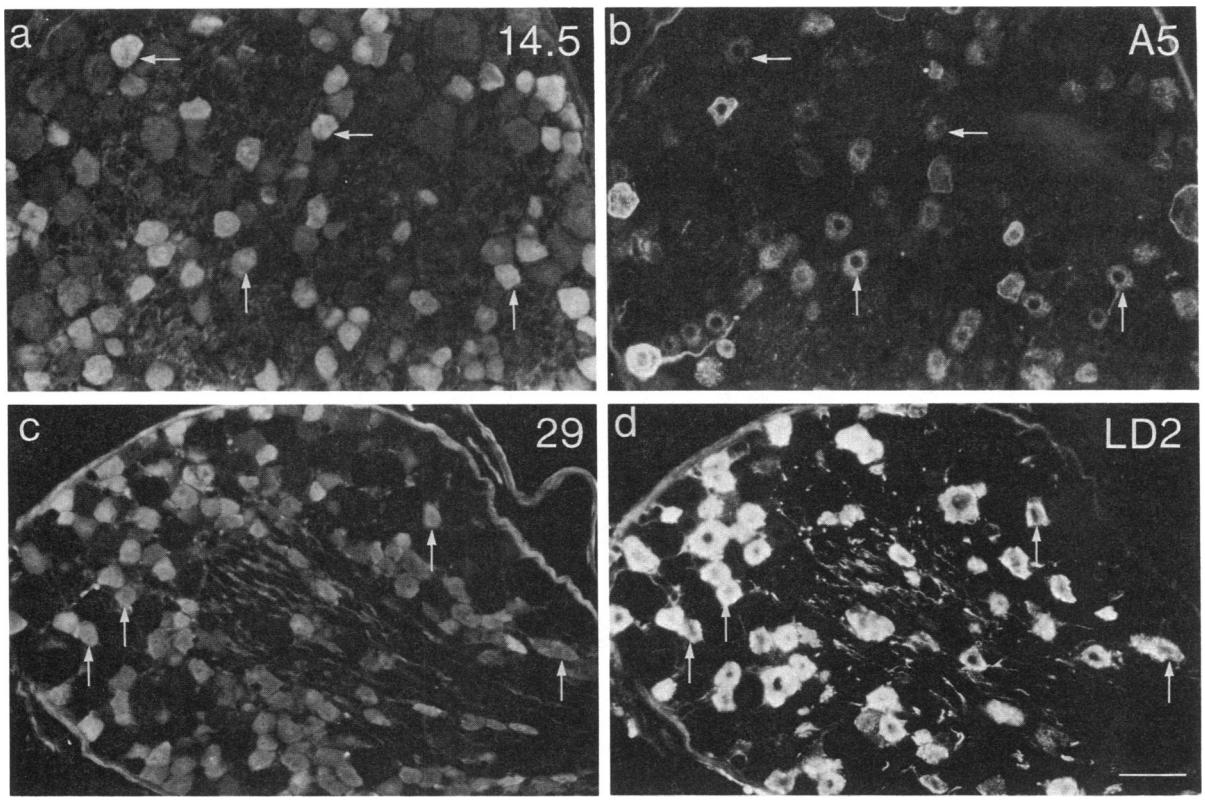

FIG. 3. Overlap in expression of LBLs and sensory neuron-specific lactoseries carbohydrate epitopes in adult rat DRG neurons. (*a* and *b*) Localization of RL-14.5 (*a*) and the lactoseries epitope recognized by mAb A5 (*b*) on the same section of DRG using dual-color immunofluorescence labeling. Extensive, but not perfect, overlap is apparent between neurons that express RL-14.5 and A5-reactive antigens. (*c* and *d*) Localization of RL-29 (*c*) and the LD2 lactoseries epitope (*d*) on the same DRG section. Most DRG neurons labeled by mAb LD2 also contain RL-29. Arrows in *a* and *b* and in *c* and *d* identify the same cells in each pair of micrographs. (Scale bar: 60 μm in *a* and *b*; 50 μm in *c* and *d*.)

Overlap in Expression of LBLs and Carbohydrate Antigens. Double-label immunocytochemistry revealed a substantial overlap between populations of DRG neurons that expressed LBLs and lactoseries carbohydrate epitopes (see Table 3). mAbs A5, LD2, and LA4 are directed against distinct carbohydrate epitopes, each of which is derived from a common lactosyl backbone sequence that is associated with glycolipids and possibly glycoproteins on DRG neurons (6) (Table 2). A5-, LD2- and LA4-reactive glycoconjugates are expressed on partially overlapping neuronal populations that constitute the majority of DRG neurons projecting to the superficial dorsal horn (6). Between 72% and 98% of lactoseries-immunoreactive neurons expressed RL-14.5 and RL-29, depending on the particular epitope examined (Fig. 3, Table 3). Moreover, DRG neurons that expressed these three epitopes constituted >75% of the total number of DRG neurons expressing either RL-14.5 or RL-29.

The incidence of overlap between neurons that express lactoseries glycoconjugates and LBLs may be greater than that indicated with mAbs used in these experiments. DRG neurons that expressed LBLs but not the carbohydrate epitopes detected by mAbs A5, LD2, and LA4 (Table 3) may synthesize other complex lactoseries structures. Indeed, separate subsets of small DRG neurons have been shown to express several type 1 [Gal(β1-3)GlcNAc] lactoseries structures (6), but the intensity of labeling of these structures in DRG neurons was not sufficient to permit a double-label analysis.

The overlap between lectins and globoseries epitopes, detected with available mAbs, was much less prevalent than that with lactoseries epitopes (Table 3). We have shown previously that three distinct subsets of DRG neurons are defined by the SSEA-3 and SSEA-4 globoseries determinants (7). SSEA-3^+/SSEA-4^- DRG neurons are small and appear to project to lamina I, whereas SSEA-3^+/SSEA-4^+ and SSEA-3^-/SSEA-4^+ DRG neurons are large and terminate in laminae III and IV (5–7). Small (SSEA-3^+/SSEA-4^-) DRG

Table 2. Carbohydrate epitopes recognized by mAbs

Lactoseries	
Backbone structure	Gal(β1-$^{3}_{4}$)GlcNAc-R
mAb A5 epitope	Gal(β1-4)GlcNAc-
mAb LD2 epitope*	Gal(α1-3)Gal(β1-4)GlcNAc-R′
mAb LA4 epitope*	Gal(α1-3)Gal(β1-4)GlcNAc-R″
Globoseries	
Backbone structure	GalNAc(β1-3)Gal(α1-4)Gal(β1-R)
mAb anti-SSEA-4 epitope	NeuAc(α2-3)Gal(β1-3)GalNAc(β1-
mAb anti-SSEA-3 epitope	R-GalNAc(β1-3)Gal(α1-4)Gal(β1-

*mAbs LD2 and LA4 recognize closely related but distinct epitopes on partially overlapping subsets of DRG neurons (6). Clarification of the structural difference in these epitopes, here designated by R′ and R″, requires further investigation.

Table 3. Correlation of lectin and carbohydrate antigen expression in DRG neurons

Carbohydrate antigen (defined by mAb)	% total neurons labeled	Spinal termination (lamina)	% DRG neurons containing: Carbohydrate antigens that also express lectins*		% DRG neurons containing: Lectins* that also express carbohydrate antigens	
			RL-14.5	RL-29	RL-14.5	RL-29
Lactoseries						
$A5^+$	42	I, II	82	91	57	59
$LD2^+$	23	II (outer)	72	84	26	31
$LA4^+$	35	II (inner)	94	98	54	53
Globoseries						
SSEA-3^+/SSEA-4^-	4	I	23	40	3	7
SSEA-3^-/SSEA-4^+ } SSEA-3^+/SSEA-4^+	9	III, IV	23	<1	4	<1

For details of the carbohydrate specificity of mAbs and neuronal subsets identified by each antibody, see Table 2 and ref. 6. Spinal termination is defined according to the lamination scheme of Rexed (18).
*Numbers calculated from combined totals of neurons exhibiting light and intense lectin immunoreactivity.

neurons exhibited considerable overlap with RL-29, whereas larger (SSEA-3^-/SSEA-4^+ or SSEA-3^+/SSEA-4^+) DRG neurons showed virtually no overlap (Table 3).

These results indicate that LBLs are located preferentially in DRG neurons that project to the superficial dorsal horn. Although the majority of these DRG neurons expresses lactoseries glycoconjugates, a minor population expresses globoseries determinants. At present it is not clear whether globoseries-immunoreactive neurons that project to the superficial dorsal horn also synthesize lactoseries structures.

DISCUSSION

The results described above indicate that two soluble LBLs previously isolated and characterized from rat lung (16) are expressed in neural cells and are particularly prominent in primary sensory neurons. Moreover, within the DRG, these lectins are localized in functionally distinct subsets of primary sensory neurons. Both lectins can be detected in the DRG soon after final mitotic division of sensory neurons and are restricted to central sensory axons that terminate in the superficial dorsal horn. The expression of these lectins in sensory terminals appears to be developmentally regulated, in that RL-14.5 and RL-29 immunoreactivity decreases at later stages of postnatal development. The developmental regulation of other LBLs has been demonstrated in avian central nervous system (11–13).

There is substantial overlap between subsets of DRG neuronal cell bodies that contain RL-14.5 and/or RL-29 and those that express sensory neuron-specific lactoseries glycoconjugates (5–7). Furthermore, the coincident distribution of RL-14.5, RL-29, and lactoseries antigens in the superficial dorsal horn indicates that many sensory terminals contain both LBLs and lactoseries glycoconjugates and provides additional evidence for a preferential association of the lectins with sensory neurons that express lactoseries carbohydrate determinants. This overlap suggests that lectins and complementary glycoconjugates interact in or on the sensory neurons that synthesize them.

Glycoconjugates that contain lactoseries structures have been implicated in the intercellular adhesion of neural (19) and preimplantation embryonic (20, 21) cells. Developmentally regulated lectins have also been shown to participate in ordered cell migration and cellular aggregation (22). A role for RL-14.5 and RL-29 in cellular interactions in the spinal cord is supported by evidence that LBLs similar to those detected in DRG neurons are externalized and associated with membrane-bound or extracellular glycoconjugates on other cell types (23–25). In preliminary studies, the release of RL-14.5 and RL-29 from neonatal DRG neurons, maintained in dissociated cell culture, has been detected (unpublished data), suggesting that LBLs may be localized extracellularly, in the vicinity of sensory terminals in the developing spinal cord.

The identification of LBLs and complementary lactoseries glycoconjugates in developing primary sensory neurons should make it possible to examine in more detail whether these molecules participate in the organization of sensory projections in the developing spinal cord.

We thank P. N. Hamilton for preparing DRG cultures. This project was supported by grants from the National Institutes of Health, the National Science Foundation, the National Multiple Sclerosis Society, The McKnight Foundation, and the Muscular Dystrophy Association. T.M.J. was supported by grants from Dupont and the Rita Allen Foundation and is an Investigator of the Howard Hughes Medical Institute. L.J.R. is in receipt of a predoctoral fellowship from the National Institutes of Health.

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