The Novel Carbohydrate Epitope L3 Is Shared by Some Neural Cell Adhesion Molecules

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Abstract. The monoclonal L3 antibody reacts with an *N*-glycosidically linked carbohydrate structure on at least nine glycoproteins of adult mouse brain. Three out of the L3 epitope-carrying glycoproteins could be identified as the neural cell adhesion molecules L1 and myelin-associated glycoprotein, and the novel adhesion molecule on glia. Expression of the L3 carbohydrate epitope is regulated independently of the protein backbone of these three glycoproteins. Based on the observation that out of three functionally characterized L3 epitope-carrying glycoproteins three fulfill the operational definition of an adhesion molecule, we would like to suggest that they form a new family of adhesion molecules that is distinct from the L2/HNK-1 carbohydrate epitope family of neural cell adhesion molecules. Interestingly, some members in each family appear to be unique to one family while other members belong to the two families.

We have recently shown that the neural cell adhesion molecules L1 and N-CAM (for reviews see Edelman and Thiery, 1985) share a common carbohydrate structure that is recognized by the monoclonal antibodies L2 and HNK-1 (Kruse et al., 1984). This epitope consists of a 3'-sulphated glucuronic acid that is part of an unusual glycolipid (Chou et al., 1985, 1986; Noronha et al., 1986). Based on the observation that out of two functionally identified cell surface molecules carrying the L2/HNK-1 epitope, two were indeed cell adhesion molecules, we formulated the hypothesis that all members of this family are cell adhesion molecules. The myelin-associated glycoprotein (MAG)[1] has been hypothesized (for review see Quarles, 1983/1984), but only recently shown to fulfill the operational definition of a neural cell adhesion molecule since it mediates neuron-oligodendrocyte and oligodendrocyte-oligodendrocyte adhesion (Poltorak, M., R. Sadoul, G. Keilhauer, C. Landa, T. Fahrig, and M. Schachner, manuscript submitted for publication). As a fourth prominent member of the family, the J1 glycoprotein could be shown to be involved in neuron-astrocyte adhesion (Kruse et al., 1985). The number of members in this family is hard to estimate at present but appears to be >10. The physiological role of the shared L2/HNK-1 carbohydrate epitope has been suggested to be important since it is common to functionally important molecules (Schachner et al., 1985), and there are first hints that this epitope is itself involved in cell adhesion (Keilhauer et al., 1985; McGarry et al., 1985). Interestingly, only a subpopulation of a particular adhesion molecule was found to express the epitope (Kruse et al., 1984; Poltorak, M., R. Sadoul, G. Keilhauer, C. Landa, T. Fahrig, and M. Schachner, manuscript submitted for publication; our unpublished observations) indicating that the carbohydrate structure is regulated independently of the protein backbone.

The question now is whether other families exist whose members are combined by common features. These are most likely to exist at the protein level, but it would also appear possible that they are based on other shared carbohydrate structures. Here we report of the existence of a new family of cell adhesion molecules characterized by the L3 carbohydrate epitope that is expressed by a novel cell adhesion molecule on glia (AMOG) (Antonicek et al., 1987), by other, yet unidentified glycoproteins in the nervous system, and by two members of the L2/HNK-1 family, L1 and MAG, but not by N-CAM and J1.

Materials and Methods

Animals

NMRI and nude mice were used for all experiments as indicated in the accompanying paper (Antonicek et al., 1987).

Antibodies

Monoclonal L3 antibody was obtained from female Lou × Sprague Dawley F1 hybrid rats (4–6-wk old) immunized with "total L2" (Kruse et al., 1984) (50 μg/injection) as described previously for monoclonal L1 antibodies (Rathjen and Schachner, 1984). Splenocytes of animals with high titers were chosen for fusion with the mouse myeloma clone Ag8-653 (Kearney et al., 1979) according to the method of de St. Groth and Scheidegger (1980). Fusions were screened as described (Antonicek et al., 1987). Monoclonal L3 antibody is an IgM as determined by gel filtration or SDS PAGE. L3 antibody was prepared either as ascites in nude mice or from serum-free culture supernatants.

Antibodies to glial fibrillary acidic protein, cell adhesion molecule L1, L2 carbohydrate epitope, 04 antigen, and fibronectin were obtained and used as described (Antonicek et al., 1987).

1. *Abbreviations used in this paper*: AMOG, a novel cell adhesion molecule on glia; MAG, myelin-associated glycoprotein.

Analytical Procedures

SDS PAGE analysis was performed with 7% acrylamide slab gels according to Laemmli (1970). Staining of gels by reduction of silver ions was carried out according to Oakley et al. (1980). Protein determinations were carried out according to Lowry et al. (1951) or with a micromethod according to Bradford (1976). BSA served as standard.

Western blot analysis of proteins isolated by SDS PAGE was carried out according to Towbin et al. (1979) as indicated (Antonicek et al., 1987).

Isolation of Cell Adhesion Molecules by Immunoaffinity Chromatography

Monoclonal L1, N-CAM, MAG, and L2 antibodies and polyclonal J1 antibodies were used for preparation of antibody columns as described (Antonicek et al., 1987; Rathjen and Schachner, 1984). Antigens were isolated from detergent lysates of crude membrane fraction from adult mouse brain in the presence of protease inhibitors as described (Antonicek et al., 1987; Kruse et al., 1984; Poltorak, M., R. Sadoul, G. Keilhauer, C. Landa, T. Fahrig, and M. Schachner, manuscript submitted for publication).

Enzymatic Digest with Endoglycosidase F

Endoglycosidase F removes *N*-glycosidically linked oligosaccharide chains at the di-*N*-acetylchitobiosyl-linkage leaving an *N*-acetylglucosamine residue at the asparagin residue of the protein (Elder and Alexander, 1982). Endoglycosidase F treatment of immunoaffinity-purified L1 was performed as described by Kruse et al. (1984).

Cell Culture

Monolayer cultures of trypsin-dissociated cerebella from 6-d-old mice were prepared and maintained for 2 and 14 d in vitro as described previously (Schnitzer and Schachner, 1981). Pure cultures of Schwann cells were prepared from sciatic nerves of 1-d-old mice by trypsin-collagenase dissociation, plating on laminin, and removal of fibroblast-like cells by immunocytolysis with Thy-1 and MESA-1 antibodies. Neurons from dorsal root ganglia of 1-d-old mice were prepared by trypsin treatment of ganglia, plating on rat-tail collagen, and using 5'-fluoro-2'-deoxyuridin (FUdR) (10^{-5} M) to remove nonneural cells. Cultures of dorsal root ganglia were used for indirect immunofluorescence after 13 d of maintenance in vitro after FUdR treatment and after 3 d of maintenance in vitro without treatment with FUdR. The N2A clone of C1300 neuroblastoma was maintained as described (Pollerberg et al., 1985).

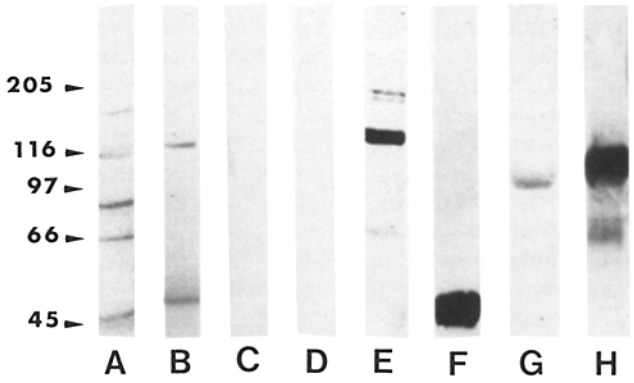

Figure 1. Identification of L3 carbohydrate epitope-expressing glycoproteins from adult mouse brain by Western blot analysis after separation by SDS PAGE using L3 antibodies (*A–G*). (*A*) Crude membrane fraction; (*B*) total L2 consisting of all members of the L2/HNK-1 family expressing the epitope; (*C*) rest L2 consisting of all members of the L2/HNK-1 family except for L1 and N-CAM which were removed by immunoaffinity chromatography; (*D*) immunoaffinity-purified N-CAM; (*E*) immunoaffinity-purified L1; (*F*) immunoaffinity-purified AMOG; (*G*) immunoaffinity-purified MAG; (*H*) immunoaffinity-purified MAG shown by the reducing silver method. Lanes *F–H* were loaded with equal amounts of antigens.

Biosynthetic Labeling of Glycoproteins in Cerebellar Cell Culture

Cultures of cerebellar cells were labeled with [^{35}S]methionine as described (Faissner et al., 1985; Antonicek et al., 1987). Cultures were labeled with [^{35}S]methionine in the absence and presence of tunicamycin (1.5 μg/ml; Sigma Chemical GmbH, Munich, FRG), also as described in these publications.

Immunoprecipitation

Immunoprecipitations were performed as described (Faissner et al., 1985; Antonicek et al., 1987) with the following modification: Monoclonal L3 antibody and polyclonal L1 antibodies were reacted with live, washed monolayer cultures of early postnatal mouse cerebellum at concentrations used for indirect immunofluorescence (10–20 μg/ml in Hank's balanced salt solution) for 20 min in the CO_2 incubator. Cultures are then washed two times with Hank's balanced salt solution, detergent-treated with 1% NP-40, centrifuged for 1 h at 100,000 *g* and 4°C, and the resulting supernatants used for immunoprecipitation with MARK-1 antibody (Bazin, 1982) for L3 antibody and protein A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) for polyclonal L1 antibodies as described before (Antonicek et al., 1987; Faissner et al., 1985).

Immunocytological Procedures

Indirect immunofluorescence on viable and ethanol-treated cell cultures was performed as described by Schnitzer and Schachner (1981).

Results

Monoclonal L3 antibody was obtained by immunization of rats with a glycoprotein fraction obtained by immunoaffinity chromatography on a monoclonal L2 antibody column (Kruse et al., 1984) from detergent lysates of crude membrane fraction from adult mouse brain. The glycoproteins that are specifically retained by this monoclonal antibody column have been designated total L2 and include the L2 epitope–carrying fractions of the four presently characterized neural cell adhesion molecules L1, N-CAM, J1, and MAG.

Identification of the L3 Epitope-carrying Glycoproteins

Several glycoproteins of adult mouse brain were recognized in detergent lysates of a crude membrane fraction from adult mouse brain as shown by Western blot analysis (Fig. 1 *A*). The more prominent bands migrated with apparent molecular masses of ~170, 130, 120, 80, 70, and 50 kD. In the total L2 glycoprotein fraction monoclonal L3 antibodies reacted with two bands at 140 and 55 kD (Fig. 1 *B*). "Rest L2", which consists of all glycoproteins carrying the L2 epitope (total L2) except for L1 and N-CAM and which contains predominantly the L2 epitope-carrying fraction of J1 and MAG (Kruse et al., 1984, 1985), was not recognized by monoclonal L3 antibody (Fig. 1 *C*). Immunoaffinity-purified N-CAM was also not recognized by monoclonal L3 antibody (Fig. 1 *D*). L3 antibody reacted with immunoaffinity-purified L1 (Fig. 1 *E*), the AMOG (Fig. 1 *F*) and MAG (Fig. 1 *G*). For this experiment, MAG was immunoaffinity purified with a monoclonal antibody that reacts with a protein epitope on MAG (Poltorak, M., R. Sadoul, G. Keilhauer, C. Landa, T. Fahrig, and M. Schachner, manuscript submitted for publication). L3 antibody did not react with all carbohydrate variants of the protein backbone of MAG that were isolated by the monoclonal MAG antibody column (Fig. 1 *G*, and compare with broad band in Fig. 1 *H*). J1, immunoaffinity-purified from adult mouse brain by monoclonal L2 or polyclonal J1 antibody columns representing the L2/HNK-1 epitope-positive

and total J1 glycoprotein fraction, respectively, was not recognized by L3 antibody (not shown).

These experiments suggested that the L3 antibody reacts with at least nine glycoprotein bands from adult mouse brain among which three could be identified as the adhesion molecules L1, MAG, and AMOG. The unidentified bands are those at 170, 130, 120, 80, 70, and 55 kD in the crude membrane and total L2 fractions, assuming that the 50-kD band in the crude membrane fraction and the 140-kD band in total L2 are AMOG and L1, respectively. It seems as if not all carbohydrate variants of MAG express the L3 epitope and that the L2 epitope carrying glycoproteins of MAG do not express the L3 epitope. On the other hand, L3 antibody reacted with both the L2-positive and L2-negative fractions of the L1 glycoproteins that were obtained by immunoaffinity isolation of purified L1 with an L2 antibody column (not shown). It is noteworthy that L1 and MAG are not among the more prominent glycoproteins in the adult mouse brain, since they could not be detected by L3 antibody in the crude membrane fraction. Alternatively, the L3 epitope–positive subpopulation of MAG and L1 is proportionately small.

Identification of the L3 Epitope as Carbohydrate Determinant

To verify whether the antigenic determinant recognized by monoclonal L3 antibody is localized in the carbohydrate or protein part of the glycoproteins, immunoaffinity-purified L1 was treated with endoglycosidase F which is known to remove *N*-glycosidically linked carbohydrate chains (Elder and Alexander, 1982). After treatment with endoglycosidase F, a reduction in apparent molecular mass of the bands at 200, 180, and 140 kD is seen (Fig. 2 *C*, and compare with Fig. 2 *A*). While the endoglycosidase F–treated L1 was not recognized by monoclonal L3 antibodies (Fig. 2 *B*) the untreated L1 was (Fig. 2 *A*). As could be seen from the reaction of endoglycosidase F–treated L1 with polyclonal L1 antibodies (Fig. 2 *C*), the L1 chains were shifted to slightly lower molecular mass ranges. The 70–80-kD component of L1 is not visible in this experiment (compare with Fig. 1 *E*).

To further verify the L3 antigenic determinant as carbohydrate, monolayer cultures of early postnatal mouse cerebella were treated with tunicamycin, which inhibits the formation of *N*-glycosidically linked complex carbohydrate chains (Struck and Lennarz, 1977). Cultures were biosynthetically labeled with [^{35}S]methionine, immunoprecipitated, and analyzed after SDS PAGE by fluorography (Fig. 3). A modified

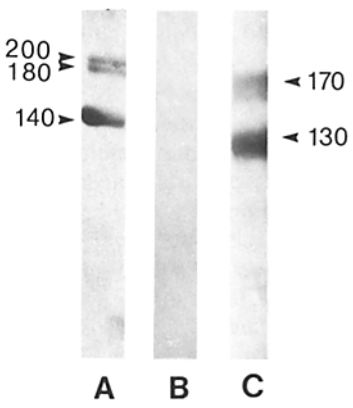

Figure 2. Identification of the L3 epitope as carbohydrate determinant by Western blot analysis. (*A*) Immunoaffinity-purified L1 treated with L3 monoclonal antibody; (*B*) immunoaffinity-purified L1 after digest with endoglycosidase F, treated with monoclonal L3 antibody; (*C*) immunoaffinity-purified L1 after digest with endoglycosidase F (as in *B*), treated with polyclonal L1 antibodies.

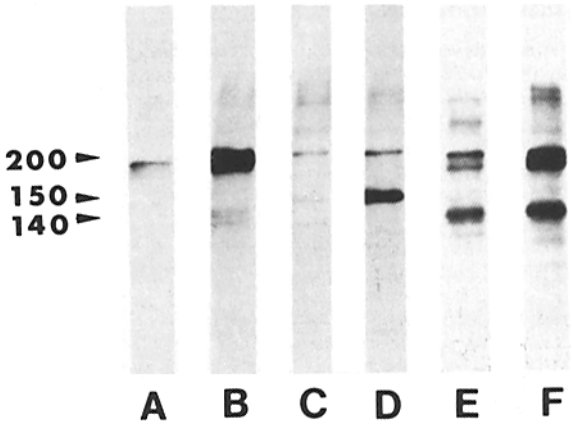

Figure 3. Identification of L3 epitope–carrying glycoproteins in monolayer cultures of early postnatal mouse cerebellum biosynthetically labeled with [^{35}S]methionine as analyzed by immunoprecipitation, separation by SDS PAGE, and fluorography. (*A*, *C*, and *E*) Immunoprecipitates with monoclonal L3 antibody; (*B*, *D*, and *F*) immunoprecipitates with polyclonal L1 antibodies. (*A* and *B*) untreated cultures; (*C* and *D*) cultures treated with 1.5 μg/ml tunicamycin; (*E* and *F*) cultures treated with 5 μg/ml trypsin. High molecular mass bands in *B–F* are nonspecifically precipitated.

version of previously described immunoprecipitation procedures (Faissner et al., 1985) had to be used since the L3 epitope was no longer seen by the antibody after cells had been solubilized in the nonionic detergent NP-40. Since live monolayer cultures were found to label well by immunofluorescence with L3 antibodies (see Fig. 4), L3 antibody was incubated with the live cells before detergent solubilization and immunoprecipitation by second antibody. This modified immunoprecipitation procedure was also used with polyclonal L1 antibodies for control. As has been observed previously (Faissner et al., 1985), polyclonal L1 antibodies reacted in biosynthetically labeled cultures only with the 200-kD form of L1 (Fig. 3 *B*). L3 antibody also recognized a band with the same apparent molecular mass as L1 (Fig. 3 *A*, and compare with Fig. 3 *B*). It could be shown that this high molecular mass band or its doublet (at 200 and 180 kD), which is sometimes observed after biosynthetic labeling in vitro, was indeed L1, since trypsin digestion of live cells yielded the characteristic degradation product at 140 kD (Faissner et al., 1985), both for polyclonal L1 and monoclonal L3 antibodies (Fig. 3, *E* and *F*). These experiments indicated that L1 is the major antigen in these cultures, 90% of which consist of neurons (Schnitzer and Schachner, 1981). Since L1 is known to be a neuronal marker in these cultures the predominant detection of L1, but not of MAG or AMOG, is understandable. When these cultures were treated with tunicamycin, polyclonal L1 antibodies precipitated a major band at 150 kD and a minor one at 200 kD, as observed previously (Faissner et al., 1985). The persistence of the 200-kD band under tunicamycin is most likely due to the incomplete action of the drug at the concentration of 1.5 μg/ml (Fig. 3 *D*), which was low enough to assure good viability of cells. L3 antibody still recognized the 200-kD band, but did not recognize the 150-kD band (Fig. 3 *C*). These experiments are compatible with the notion that the L3 epitope is localized on *N*-glycosidically linked carbohydrate chains.

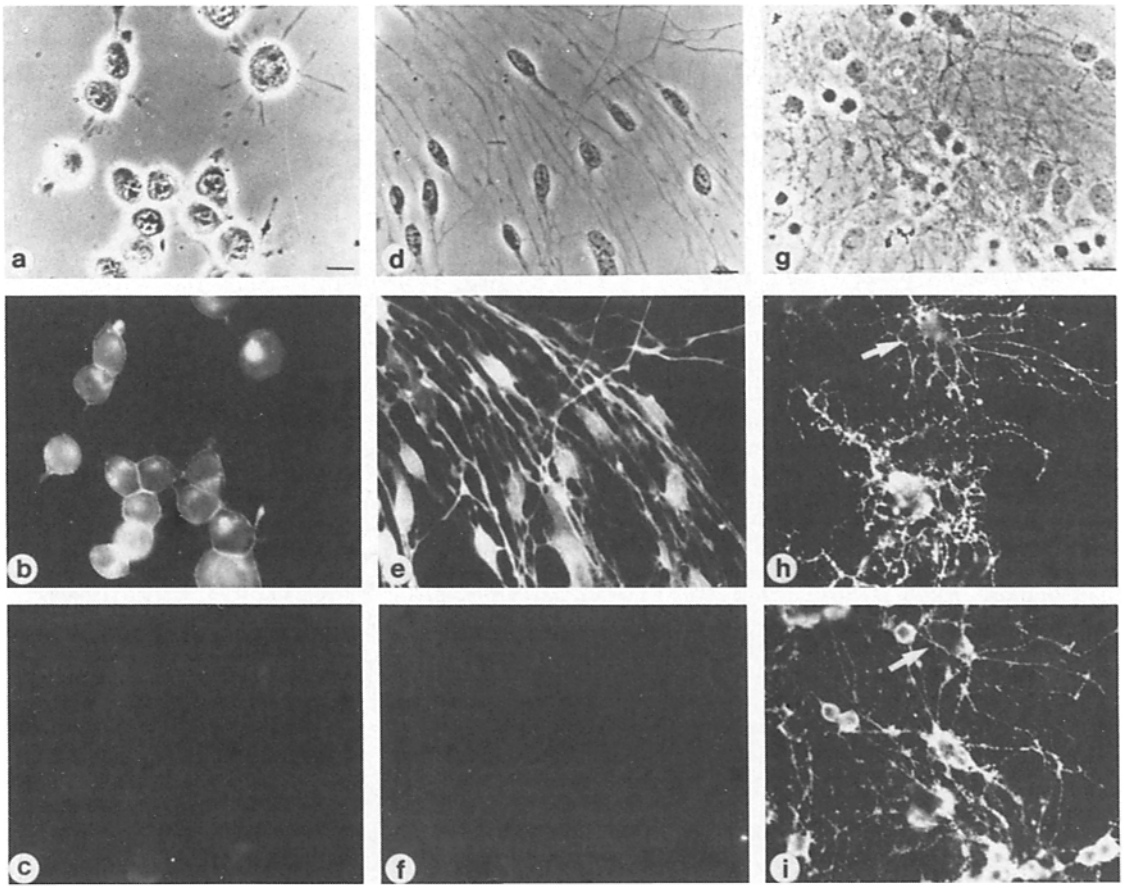

Figure 4. Immunodoublefluorescence labeling of cultured neuroblastoma N2A (*a–c*), Schwann (*d–f*), and cerebellar (*g–i*) cells with monoclonal L3 antibody (*c*, *f*, and *i*), polyclonal L1 antibodies (*b* and *e*), and monoclonal 04 antibody (*h*); *a*, *d* and *g* are the phase contrast micrographs to fluorescence images *b* and *c*, *e* and *f*, and *h* and *i*, respectively. Arrows in *h* and *i* show an 04 antigen-positive oligodendrocyte that is also labeled by L3 monoclonal antibody. Bars, 10 μm.

Identification of the L3 Epitope-expressing Neural Cell Types

To investigate whether all L1-, MAG-, and AMOG-expressing cell types also express the L3 epitope, immunodoublefluorescence-labeling experiments were performed on monolayer cultures from early postnatal mouse cerebellum and from the peripheral nervous system (Fig. 4).

Approximately 95% of all L1-positive neurons in the cerebellar cultures were L3-positive (not shown). Approximately 30% of all 04 antigen-positive oligodendrocytes and 20% of all MAG-positive oligodendrocytes expressed the L3 epitope (Fig. 4, *g–i*). The L3 epitope could not be detected on fibronectin-positive fibroblasts (not shown). It was also not detectable on glial fibrillary acidic protein–positive astrocytes, most of which are AMOG-positive (Antonicek et al., 1987) (not shown). Immunodoublefluorescence by indirect labeling procedures could not be performed with L3 and AMOG antibodies since both are derived from rat.

In cultures of neural cells from the peripheral nervous system, the L3 epitope could not be detected on L1-positive Schwann cells (Fig. 4, *d–f*), neuroblastoma clone C1300 derived from the peripheral nervous system (Fig. 4, *b* and *c*), or neurons from dorsal root ganglia (not shown).

These experiments indicated that L3 carbohydrate epitope expression was regulated independently of the protein backbone as had been previously observed for the L2 carbohydrate epitope and its glycoproteins (Wernecke et al., 1985).

Discussion

Monoclonal L3 antibody recognizes an *N*-glycosidically linked carbohydrate epitope that is shared by at least nine glycoprotein bands in the mouse nervous system. From these glycoproteins three could be identified as the neural cell adhesion molecules: L1, MAG, and the AMOG that is described in the accompanying paper (Antonicek et al., 1987). Based on the observation that out of three functionally characterized cell surface glycoproteins three are indeed adhesion molecules, we would like to suggest, as we have done previously for the L2/HNK-1 family (Kruse et al., 1984), that all other members of the L3 family are cell adhesion molecules. This reasoning derives also from further analogies between the two groups of adhesion molecules: the epitope-carrying carbohydrate moieties are *N*-glycosidically linked and regulated independently of the protein backbone, and only a subpopulation of a particular adhesion molecule expresses the epitope. Thus, the L3 epitope may define a novel family of cell adhesion molecules with members overlapping in part with the other identified family of neural adhesion molecules, the L2/HNK-1 family. This overlap is

only partial since two members of the L2/HNK-1 family, N-CAM and J1, do not belong to the L3 family. The overlapping members are L1 and MAG. AMOG belongs exclusively to the L3 family.

As has been previously observed for the L2/HNK-1 family (Kruse et al., 1984; Poltorak, M., R. Sadoul, G. Keilhauer, C. Landa, T. Fahrig, and M. Schachner, manuscript submitted for publication; our unpublished observations), not all glycoproteins of the L3 family express the epitope. For instance, of the carbohydrate variants of myelin-associated glycoprotein which form a broad band by SDS PAGE, only those in the lower apparent molecular mass region express the L3 epitope. Furthermore, when MAG is enriched by immunoaffinity chromatography on a monoclonal L2 antibody column, this carbohydrate variant fraction of MAG is L3-negative. Consistent with these findings is the observation that only 10–20% of all MAG-positive oligodendrocytes express the L3 epitope. Similarly, in cultures of early postnatal mouse cerebella, glial fibrillary acidic protein- and AMOG-positive astrocytes (see Antonicek et al., 1987) do not express the L3 epitope. However, AMOG isolated from adult mouse brain expresses the L3 epitope. It is interesting that the expression of the L3 epitope on L1-positive cells shows a striking difference between central and peripheral nervous system: while almost all L1-positive neurons from early postnatal mouse cerebellum express the L3 epitope, none of the L1-positive cells of peripheral nervous system origin tested so far express the L3 epitope. Further studies, however, are necessary to characterize the differences and similarities among L3-negative and -positive L1-expressing neural cells.

Unfortunately, the percentages of L1, MAG, or AMOG molecules that express the L3 epitope cannot be estimated by sequential immunoprecipitations, since the L3 carbohydrate epitope is no longer recognized by L3 antibody once the glycoproteins are solubilized in nonionic detergents such as NP-40 or Triton X-100 at concentrations compatible with the conditions for immunoprecipitation. The most straightforward explanation for this finding would be that the affinity of the antibody for its antigen is low. On the other hand, these experiments could suggest a masking of the L3 epitope by conformational changes in mild detergents. Also, it is presently not possible to investigate by sequential immunoprecipitation whether the L3 epitope is carried by the carbohydrate variant fractions of L2-negative or -positive glycoproteins. However, the present experiments indicate that the L3 carbohydrate epitope is not expressed on L2 epitope-carrying MAG molecules but that it is expressed on both the L2-negative and -positive L1 molecules. A simple relationship between the L2 and L3 carbohydrate epitope-carrying variants of adhesion molecules is therefore unlikely.

The present observations point to questions that need to be answered in the future: Is the next, functionally characterized member of the L3 family also an adhesion molecule? What is the structure and function of the L3 epitope? How many families of cell adhesion molecules exist that are combined by a common family feature in form of a distinct carbohydrate structure? And, is it possible that neural cell adhesion molecules are "presenters" of functionally important carbohydrate structures with immense combinatorial possibilities?

The authors are grateful to Horst Antonicek, Thomas Fahrig, Bernd Gehrig, Jan Kruse, and Heide Wernecke for antigens, Bernd Seilheimer for Schwann cell cultures, and Rémy Sadoul for comments on the manuscript.

The authors thank Deutsche Forschungsgemeinschaft (SFB 317) and Fonds der Chemischen Industrie for support.

Received for publication 16 December 1986, and in revised form 23 February 1987.

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