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Monoclonal antibodies raised against membrane glycoproteins from mouse brain recognize N-linked oligomannosidic glycans

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Monoclonal L3 and L4 antibodies have been shown to recognize carbohydrate epitopes on several neural cell adhesion molecules; these epitopes can be released by treatment with endoglycosidase H. In the present study, we have identified the oligosaccharides released by endoglycosidase H from the cell adhesion molecules AMOG and L1 by fast-atom bombardment mass spectrometry as being solely of the oligomannosidic type. Using neoglycolipids of oligomannosidic glycans, we also report that both antibodies show the highest reactivity with the α-manno-pentaose Manα1-3-[Manα1-6(Manα1-3)Manα1-6]-Man, but decreasing reactivity with the α-manno-hexaose, heptaose, octaose and nonaose glycans. Thus, to our knowledge, we describe here for the first time monoclonal antibodies recognizing *N*-glycosidically linked oligomannosidic glycans.

Key words: monoclonal antibodies/nervous system/oligomannosidic glycans

Introduction

Oligosaccharides *N*- or *O*-glycosidically linked to cell surface glycoproteins have only recently become recognized as having important functional roles not only in modulating, for example, the functional conformations of the protein moiety, but also as being directly involved in cell recognition and adhesion. Significant advances in understanding the functional roles for oligosaccharides have been made using carbohydrate-specific monoclonal antibodies. By means of this approach, the L2/HNK-1, L3, L4 and L5 carbohydrate epitopes have been detected and implicated in neural cell interactions. The L2/HNK-1 carbohydrate epitope is expressed by several neural cell adhesion molecules and by glycolipids of the peripheral and central nervous system (for a review, see Schachner, 1989; Jessell *et al.*, 1990; Chou *et al.*, 1991). This epitope has been identified as a 3′-sulphated glucuronic acid at the non-reducing end of glycolipids with a neolactosyl-type backbone (Chou *et al.*, 1986; Ariga *et al.*, 1987). It is involved in the migration of neural crest cells (Bronner-Fraser, 1987), the adhesion of astrocytes and neurons to laminin (Hall *et al.*, 1993), the outgrowth of astrocytic and neuritic processes (Künemund *et al.*, 1988), the preferential outgrowth of neurites from motor but not from sensory neurons (Martini *et al.*, 1992), the homophilic binding of the neural cell adhesion molecule P0 (Griffith *et al.*, 1992), and finally the binding to P- and L-selectin (Asa *et al.*, 1992) and to human serum amyloid P protein (Loveless *et al.*, 1992).

The L3 and L4 monoclonal antibodies recognize carbohydrate epitopes released from glycoproteins by treatment with endoglycosidase H (Fahrig *et al.*, 1990) . These epitopes have, so far, always been reported as being co-expressed by some cell adhesion molecules positive for the L2/HNK-1 carbohydrate, e.g. L1, MAG and P0, but not by other L2/HNK-1-positive molecules such as NCAM, J1/janusin (previously designated J1-160/180) and J1/tenascin. AMOG is the only adhesion molecule thus far found to carry the L3 and L4, but not the L2/HNK-1, epitope. Several other identified (e.g. Thy-1) or unidentified glycoproteins from different cell types, organs or species express the L3 and L4 carbohydrates (Kücherer *et al.*, 1987; Bajt *et al.*, 1990; Fahrig *et al.*, 1990). In short-term adhesion assays, the L3 antibody has been shown to inhibit neuron–neuron adhesion only, whereas the L4 antibodies also inhibited neuron–astrocyte adhesion, suggesting that the antibodies recognize different epitopes (Fahrig *et al.*, 1990).

Finally, the L5 carbohydrate, whose structure is currently not known, is expressed by L1, Thy-1 and astrochondrin, a chondroitin sulphate proteoglycan (Streit *et al.*, 1990, 1993). The monoclonal antibody against this carbohydrate inhibits the extension of astrocytic processes, as well as cerebellar granule cell migration *in vitro* in developing chicken embryos (Streit *et al.*, 1993). The L5 antibody also inhibits neural induction and closure of the neural tube (Roberts *et al.*, 1991).

In this paper, we present evidence that carbohydrates reacting with the L3 and L4 antibodies are N-linked oligomannosidic glycans. Studies of the specificity of antibody binding revealed that α1-3- and α1-6-, but not α1-2-linked mannose residues are important structural features for recognition by both antibodies. Binding of the antibodies to the peripheral myelin glycoprotein P0, whose *N*-glycosidically linked oligosaccharides contain a fucose residue at the innermost *N*-acetylglucosamine (GlcNAc) (Field *et al.*, 1992), is only weak and strongly increased after removal of the fucose with fucosidase. The fact that L3 and L4 antibodies have been shown to bind to neural cell surfaces, to inhibit cell adhesion and neurite outgrowth (Kücherer *et al.*, 1987; Fahrig *et al.*, 1990), and to interfere with the interaction between L1 and NCAM (Horstkorte *et al.*, 1993), implicates the involvement of oligomannosidic glycans in important functional roles.

Results

Monoclonal L3 and L4 antibodies recognize oligomannosidic glycans

For the structural analysis of the *N*-glycosidically linked glycans which are recognized by L3 and L4 antibodies, we used oligosaccharides of AMOG released by endoglycosidase H. Treatment of AMOG with this enzyme resulted in a complete removal of the epitopes recognized by both antibodies (Figure

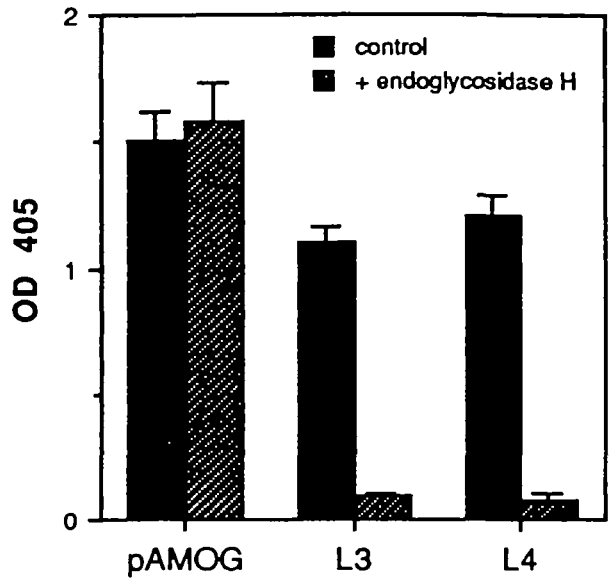

Fig. 1. Determination of the reactivity of polyclonal AMOG antibodies (pAMOG) and monoclonal L3 and L4 antibodies (L3, L4) with AMOG with (+ endoglycosidase H) or without endoglycosidase H treatment (control) as quantified by ELISA. The optical density at 405 nm (OD 405) of the reaction products with HRP-conjugated secondary antibodies is shown. Mean values ± SDs of one representative out of three experiments carried out in quintuplicate are shown.

1). The advantage of using AMOG as a source of oligosaccharides for the L3/L4 epitopes is that endoglycosidase H treatment of AMOG produces by far the largest shift in apparent molecular weightt, as compared to other glycoproteins (Bajt *et al.*, 1990; Fahrig *et al.*, 1990). From the reduction in apparent mol. wt from 50 to 35 kDa (Antonicek *et al.*, 1987) and the calculated mol. wt for the protein backbone of 32 kDa (Gloor *et al.*, 1990), it can be estimated that ~80% of N-linked oligosaccharides of AMOG are of the oligomannosidic and/or hybrid type. We could therefore expect to isolate reasonable quantities for the structural analysis of L3- and L4-positive oligosaccharides.

After the removal of protein, enzyme and salt on a Biogel P2 polyacrylamide column, released oligosaccharides were reduced with sodium borohydride, permethylated and submitted to fast-atom bombardment mass spectrometry (FAB-MS). Only three molecular $[M+H^+]$ ions at m/z = 1328, 1532 and 1736, with a relative abundance of 5:2:1, could be detected, which correspond to oligosaccharides containing one *N*-acetylhexosamine and 5, 6 or 7 hexoses (Figure 2). Hybrid-type glycans, which could also be released by endoglycosidase H, would contain at least one more *N*-acetylhexosamine instead of hexose (Hex) and thus give rise to characteristically different molecular ions; these were not observed. The same glycan composition and distribution was obtained after peracetylation of oligosaccharides released from AMOG by endoglycosidase H, and an almost identical profile of molecular ions was observed for oligosaccharides released from L1 by endoglycosidase H (not shown). From the known biosynthesis of *N*-glycans (Kornfeld and Kornfeld, 1985) and the mode of action of endoglycosidase H (Tarentino *et al.*, 1978), we conclude that the hexoses occurring in the identified oligosaccharides correspond to penta-, hexa- and heptamannosidic structures.

We further prepared neoglycolipids of oligomannosidic glycans, which in contrast to free oligosaccharides bind well to the plastic of microtitre wells so that the reactivity with antibodies can be measured by enzyme-linked immunosorbent assay (ELISA). Oligosaccharides released by endoglycosidase H from AMOG yielded neoglycolipids reacting with the L3 antibody (Figure 3) and, similarly, so did oligosaccharides from bovine RNase B, which is known to carry only oligomannosidic oligosaccharides (Liang *et al.*, 1980). No immunoreactivity

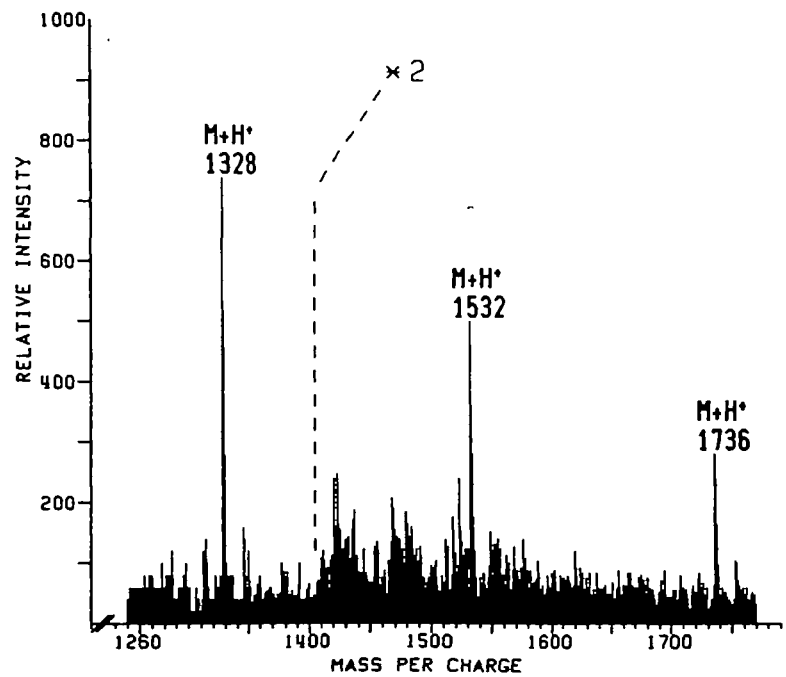

Fig. 2. Positive-ion FAB-MS of reduced and permethylated oligosaccharides released by endoglycosidase H from AMOG. Relative intensities of molecular $[M+H^+]$ ions corresponding to $Hex_{5-7}HexNAc$-ol are shown. No significant fragments were observed in the mass range < 1200 amu.

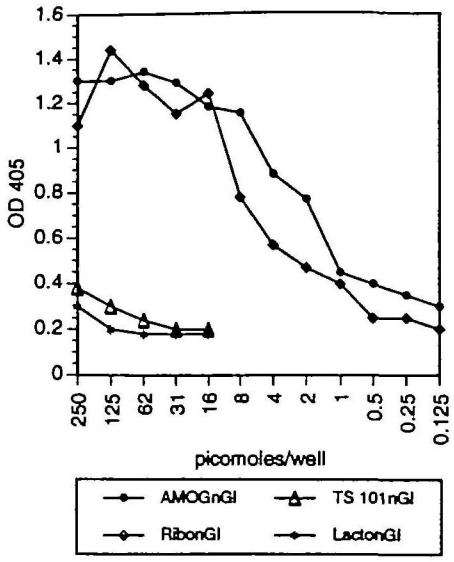

Fig. 3. Reactivity of L3 and L4 antibodies (L3, L4) with neoglycolipids as quantified by ELISA (AMOGnGl, RibonGl: neoglycolipids of oligosaccharides released from AMOG and RNase B by endoglycosidase H, respectively; TS101nGl, LactonGl: neoglycolipids containing the trisaccharide Gal-GlcNAc-Man and lactose, respectively). Wells were coated with neoglycolipids in serial 1:1 dilution from 250 pmol/well. Reactivity with the L3 antibody was determined by measuring the optical density at 405 nm (OD 405) of the reaction products with the HRP-conjugated secondary antibodies. One representative out of three experiments carried out in triplicate is shown, SDs were <5%.

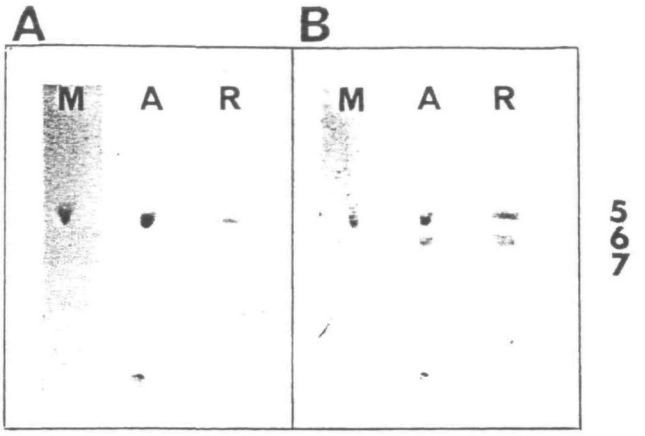

Fig. 4. (A) Binding of L3 antibody to $Man_5GlcNAc$-neoglycolipid (M, 3 μg) and neoglycolipids of oligosaccharides released by endoglycosidase H from AMOG (A, 5 μg) or RNase B (R, 3 μg), as detected by TLC immunostaining. After chromatography of neoglycolipids, TLC plates were incubated with L3 antibody and secondary HRP-conjugated antibodies. Binding of antibodies was visualized with chloronaphthol. (B) Detection of neoglycolipids by staining with orcinol; 5, 6, 7 at the right margin designate neoglycolipids containing 5, 6 and 7 mannoses.

was observed with lactosylneoglycolipid and a neoglycolipid containing the trisaccharide Gal-GlcNAc-Man (galactose-β1-4-*N*-acetylglucosamine-β1-4-mannose; TS101) (Figure 3). The same result was obtained with the L4 antibody (not shown).

On thin-layer chromatograms, neoglycolipids derived from AMOG or RNase B separated into three bands, of which the fastest co-migrated with the neoglycolipid derived from Man_5-GlcNAc-oligosaccharide (Figure 4). The two more slowly migrating bands should correspond to $Man_6GlcNAc$- and $Man_7GlcNAc$-neoglycolipids, in agreement with the structural evidence from the FAB mass spectrum. Immunostaining of TLC plates with the monoclonal L3 antibody gave a strong reaction with the $Man_5GlcNAc$-neoglycolipid and a weaker reaction with the $Man_6GlcNAc$-neoglycolipid. No immunoreactivity was observed with the $Man_7GlcNAc$-neoglycolipid (Figure 4). This is probably not only due to the lower abundance of this compound in the mixture, as seen in the orcinol-stained chromatogram (Figure 4B), but also to the lower reactivity of $Man_7GlcNAc$-neoglycolipid with the antibody (see the next paragraph). Similar results were obtained with the L4 antibody (not shown).

Manα1-3Man and Manα1-6Man, but not Manα1-2Man, are important carbohydrate units for the reactivity with the monoclonal L3 and L4 antibodies

In order to identify the structural features necessary for antibody recognition, neoglycolipids were prepared individually from oligosaccharides of known structures containing 5, 6, 7, 8 or 9 mannoses (Figure 5). Under the conditions used, Man_5-$GlcNAc_2$- to $Man_8GlcNAc_2$-oligosaccharides were converted quantitatively into their neoglycolipids, whereas ~50% of the $Man_9GlcNAc_2$-oligosaccharide remained underivatized, as estimated by orcinol staining of thin-layer chromatograms (not shown). This was taken into account when equimolar amounts of the neoglycolipids were coated in microtitre plate wells for the comparison of their reactivities with the antibodies. Interestingly, an increasing reactivity with the L3 antibody (Figure 6) and the L4 antibody (not shown) was observed with a decreasing number of mannoses. The $Man_9GlcNAc_2$-neoglycolipid gave only an extremely low reactivity. A significant increase in reactivity was observed from the $Man_9GlcNAc_2$- to the $Man_8GlcNAc_2$- and from the $Man_8GlcNAc_2$- to the $Man_7GlcNAc_2$-neoglycolipid, whereas only small further increases in antibody reactivity were seen for $Man_6GlcNAc_2$- and $Man_5GlcNAc_2$-neoglycolipids. From these observations, it is concluded that α1-3- and α1-6-linked mannose residues exposed at the non-reducing end of the oligosaccharides are recognized by the antibodies. α1-2-linked mannose at the non-reducing end of oligosaccharide structures was, if at all, only weakly recognized by the antibodies, and also appeared to block the recognition of internal α1-3- and α1-6-linked mannose residues.

After treatment of Man_5Glc-NAc-neoglycolipid with jack bean mannosidase, reactivity with the L3 antibody was similar to the reactivity of the $Man_8GlcNAc_2$-neoglycolipid, indicating that further removal of mannose units leads to a reduction in immunoreactivity (Figure 6). Similar results were obtained for the L4 antibody (not shown). Negative-ion mass spectrometric analysis of the product of the mannosidase-treated Man_5-GlcNAc-neoglycolipid gave only one detectable molecular $[M-H^+]$ ion at m/z = 1219, corresponding to $Man_2GlcNAc$-neoglycolipid (not shown).

Fucose linked to the innermost N-acetylglucosamine of N-linked oligosaccharides inhibits recognition by the L3 and L4 antibodies

The major glycoprotein from the peripheral nervous system, P0, has been shown to carry the L3 and L4 epitopes (Bollensen *et al.*, 1988; Fahrig *et al.*, 1990). Treatment of P0 with

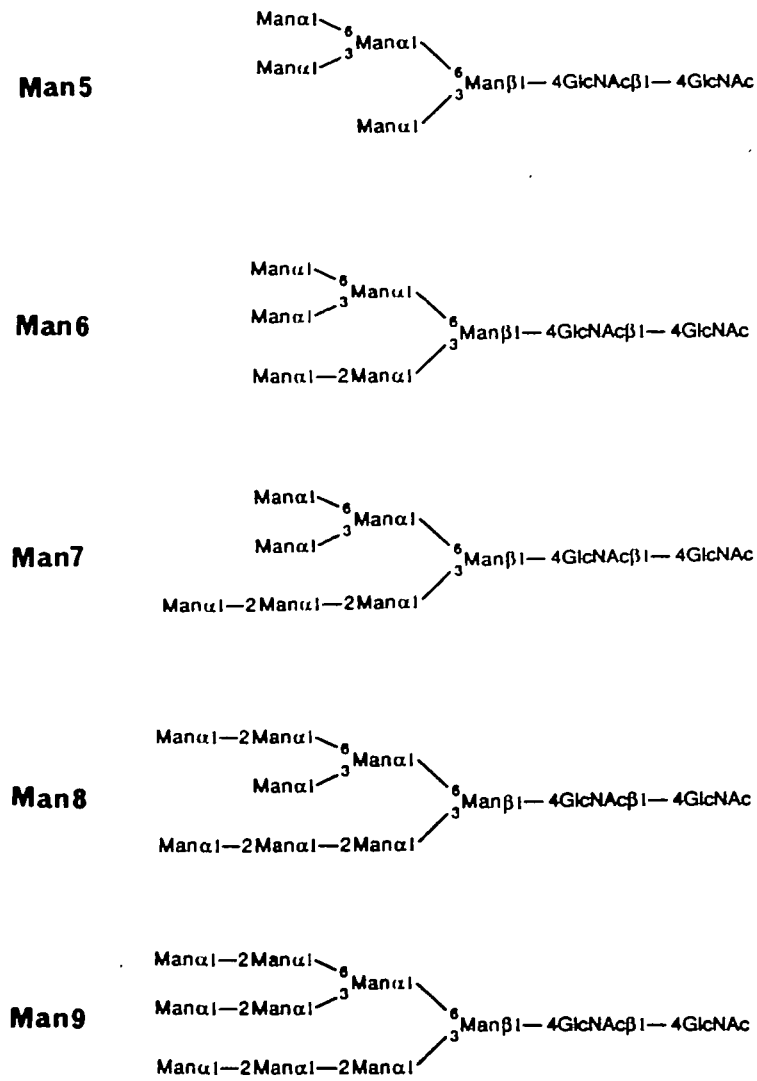

Fig. 5. Structures of oligomannosidic glycans carrying 5, 6, 7, 8 or 9 mannose residues (Man5–Man9) used for the preparation of defined neoglycolipids. According to the supplier's NMR analysis, the structures shown for Man7 and Man8 represent 70% and 80%, respectively, of the isomer mixtures.

α-fucosidase from *Charonias lampas* increased the binding of the L3 antibody ~12-fold (Figure 7). No significant increase in L3 immunoreactivity was observed for L1 and for AMOG after treatment with fucosidase. The same results were obtained with the L4 antibody (not shown). As P0 carries α-fucose only at the innermost GlcNAc (Field *et al.*, 1992; Uyemura and Kitamura, 1991), we believe that fucose in this position may prevent binding of L3 and L4 antibodies to mannosidic glycans by steric hindrance.

Maintenance of cerebellar neurons in culture in the presence of swainsonine increases L3 and L4 immunoreactivity

The oligomannosidic L3 and L4 epitopes have been detected by indirect immunofluorescence studies at the cell surface of cerebellar neurons (Kücherer *et al.*, 1987). The expression of the epitopes should therefore be increased by treatment of cells with the glycan processing inhibitor swainsonine, which blocks Golgi mannosidase II, thus causing an accumulation of oligomannosidic and hybrid-type glycans (Tulsiani *et al.*, 1990). When cells were maintained *in vitro* in the absence of inhibitors, expression of the L3 epitope could be observed on most cells (>90%) and on their processes. Maintenance of these cell cultures in the presence of swainsonine for 2 days led to a strong increase of L3 expression (Figure 8). Cell viability was unaffected by this treatment, as judged by the morphological appearance of the cultures, and seems therefore not to depend on the expression of complex-type carbohydrates, which is strongly reduced under the same culture conditions (Hall *et al.*, 1993). Similar results were obtained with the L4 antibody (not shown).

Discussion

In this study, we have identified the carbohydrate epitopes recognized by monoclonal L3 and L4 antibodies. Only three molecular ions could be detected by mass spectrometric analysis of the endoglycosidase H-released oligosaccharides from AMOG, which had previously been shown to contain the

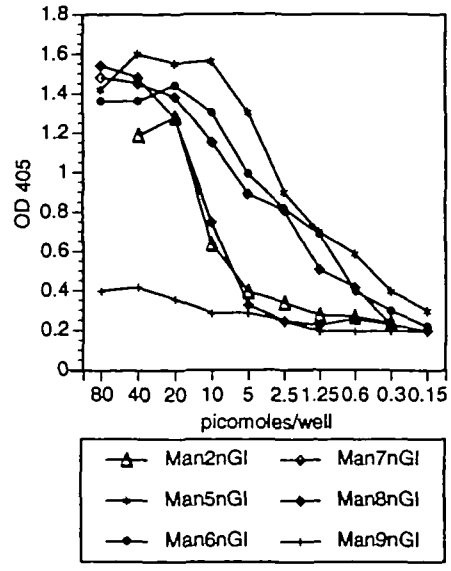

Fig. 6. Reactivity of L3 antibody (L3) with Man_2,- Man_5-, Man_6-, Man_7-, Man_8- and $Man_9GlcNAc_2$-neoglycolipids (Man2,5,6,7,8,9nGl), as quantified by ELISA. Man2nGl was obtained from Man5nGl after jack bean mannosidase treatment, as determined by FAB-MS (not shown). Neoglycolipids were coated in a 1:1 serial dilution from 80 pmol/well on. Reactivity with the L3 antibody was determined by measuring the optical density at 405 nm (OD 405) of the reaction products with the HRP-conjugated secondary antibodies. One representative out of three experiments carried out in triplicate is shown; SDs were <5%.

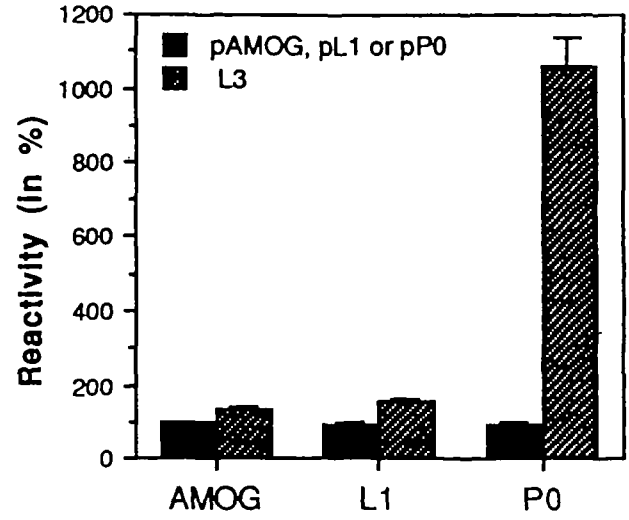

Fig. 7. Reactivity of L3 antibody (L3) after fucosidase treatment of AMOG, L1 or P0, as quantified by ELISA. The optical density at 405 nm of the reaction products with the HRP-conjugated secondary antibodies is shown. Reactivity of monoclonal L3 (L3) and polyclonal AMOG, L1 or P0 antibodies (pAMOG, pL1 or pP0) is expressed in percent, with reactivity for non-treated samples set to 100%. Mean values ± SDs from one representative out of three independent experiments carried out in triplicate are shown.

epitopes recognized by the L3 and L4 antibodies (Kücherer *et al.*, 1987; Fahrig *et al.*, 1990). These molecular ions correspond to oligosaccharides composed of one *N*-acetylglucosamine and 5, 6 or 7 mannoses. Thus, only oligomannosidic glycans are released from AMOG and, as a consequence, the L3 and L4 antibodies must recognize oligomannosidic structures.

The mass spectrometric results were corroborated by TLC immunostaining and ELISA, showing that the antibodies react with neoglycolipids derived from the glycans of AMOG, RNase B and from oligomannosidic glycans of defined structures, but not with neoglycolipids containing other neutral glycans. Obviously, both antibodies do not require the intact di-*N*-acetylglucosamine unit for recognition because the terminal *N*-acetylglucosamine residue is cleaved by endoglycosidase H treatment and the *N*-acetylglucosamine residue linked β1-4 to the mannotriose core is modified during the derivatization of oligosaccharides to neoglycolipids (Tang *et al.*, 1985). The binding of the L3 and L4 antibodies to neoglycolipid containing defined oligomannosidic glycans increased from Man_9- to Man_5-neoglycolipid, i.e. with decreasing numbers of mannoses paralleling the decreasing number of α1-2-linked and increasing number of α1-3- and α1-6-linked mannoses at the non-reducing end. The most straightforward interpretation of this result is that terminally exposed α1-3- and α1-6-linked mannose residues are required for antibody binding, whereas terminal α1-2-linked mannose seems not to be recognized and, in addition, to prevent the recognition of internally located α1-3- and α1-6-linked mannose residues. Whether the antibodies bind better to the α1-3- or α1-6-linked glycans cannot be distinguished in our assay. Interestingly, recombinant interleukin IL-2 binds with similar preference to oligomannosidic glycans as the L3 and L4 antibodies. The binding of IL-2 to uromodulin was inhibited by Man_5- and Man_6-glycopeptides at micromolar concentrations, whereas the Man_9-glycopeptide was ineffective as an inhibitor (Sherblom *et al.*, 1989).

Another interesting aspect of the specificity of the L3 and L4 antibodies is the strong increase in antibody reactivity which was only observed with P0, but not with AMOG or L1, after treatment with α-L-fucosidase. The glycan structures of P0 have been shown to be almost exclusively of the hybrid type carrying fucose at the innermost *N*-acetylglucosamine, but not in other positions (Uyemura and Kitamura, 1991; Field *et al.*, 1992). This observation suggests (i) that the mannosidic branch of hybrid-type structures may be sufficient for immunoreactivity and (ii) that fucose in this location inhibits the recognition by the L3 and L4 antibodies. The α1-6 glycosidic linkage, which seems to be part of the epitope recognized by the antibodies, is thought to confer high rotational freedom on branched oligosaccharides. It is, therefore, likely that the antibodies bind preferably to a specific conformation in which only non-fucosylated structures can exist. This could possibly be a conformation also described as the 'broken wing' conformation (Montreuil, 1984), in which the α1-6 branch folds back towards the *N*-glycosidic linkage and which is probably perturbed when a fucose is attached to the terminal *N*-acetylglucosamine.

In the present study, the L3 and L4 antibodies showed no difference in their reactivity towards all compounds tested. Earlier results, however, indicated that the antibodies recognize different epitopes on L1 because they did not compete with each other for binding to their epitopes (Fahrig *et al.*, 1990). It is, therefore, difficult to understand why we observed the same pattern of reactivity of both antibodies with different oligomannosidic neoglycolipids. One possible reason could be that, through interaction with the protein backbone, the oligomannosidic glycans exist as distinct conformers which are recognized by either the L3 or the L4 antibody.

Polyclonal antibodies raised against yeast mannans have been described previously to recognize Manα1-3Man structures

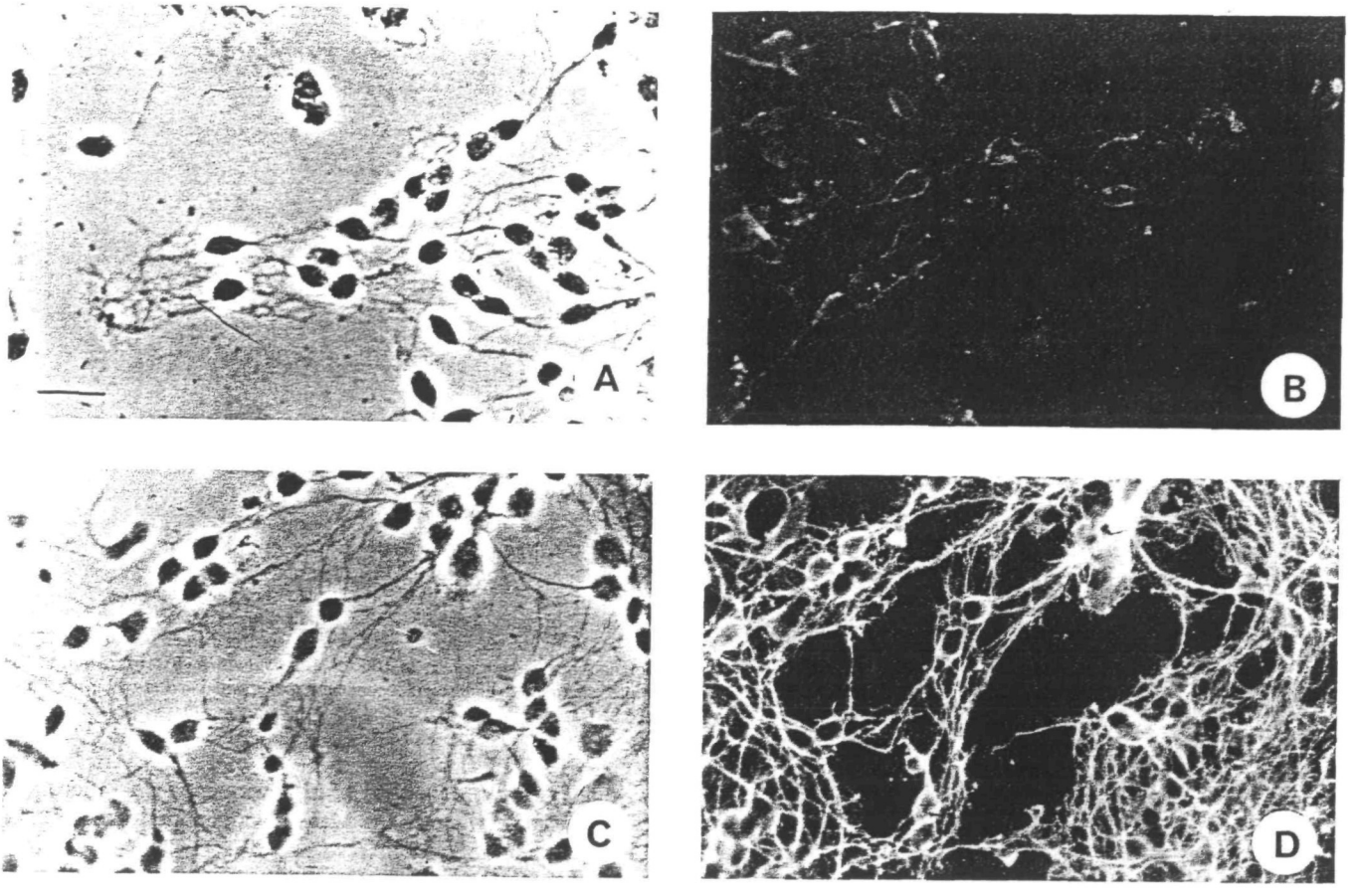

Fig. 8. Immunocytochemical detection of the L3 epitope in cultures of cerebellar neurons. Indirect immunofluorescence staining of live cells in monolayer culture from early postnatal mouse cerebella maintained for 2 days *in vitro* was carried out with monoclonal L3 antibody. Cultures were maintained in the presence (C, D) or in the absence (A, B) of the glycosylation processing inhibitor swainsonine. Note the increased L3 expression in (D) as compared to (B). The phase-contrast micrographs corresponding to fluorescence images (B) and (D) are shown in (A) and (C), respectively. The bar in (A) represents 10 μm for A–D.

(Nakajima *et al.*, 1978; Hamada *et al.*, 1981). To our knowledge, the L3 and L4 antibodies are the first monoclonal antibodies described to recognize oligomannosidic glycans. Lectins are yet other carbohydrate-binding proteins found in bacteria, plants and animals, and amongst them are several mannose-specific lectins (Lis and Sharon, 1991; Taylor *et al.*, 1992). The function of carbohydrate–lectin interactions in plants is largely unknown, but in mammals it has been shown that they play an important role in host defence mechanisms against infectious microorganisms. Bacteria, for example, interact through these glycans with mannose-binding proteins on phagocytic cells (Ofek and Sharon, 1988). Conversely, bacteria may bind through lectins they express to mannosidic glycans carried by the leucocyte adhesion molecule CD11/CD18, an integrin (Gbarah *et al.*, 1991). These processes, termed lectinophagocytosis, are antibody-independent responses relying on interactions between cell surface-exposed carbohydrates and lectins (Ofek and Sharon, 1988).

That oligomannosidic glycans can be expressed at cell surfaces and are not merely required as precursors of complex-type oligosaccharides during the biosynthesis of glycoproteins in the endoplasmic reticulum (ER; Kornfeld and Kornfeld, 1985), has been demonstrated by immunofluorescence studies with the L3 and L4 antibodies. Neurons, subpopulations of astrocytes and oligodendrocytes showed cell surface reactivity with both antibodies (Kücherer *et al.*, 1987; Fahrig *et al.*, 1990). The antibodies also inhibited cell adhesion and outgrowth of processes from neurons and astrocytes, suggesting that the expression of oligomannosidic glycans at the cell surface is of functional importance (Fahrig *et al.*, 1990). This assumption is strongly supported by the observation that the oligomannosidic glycans themselves, when added to the culture medium of cerebellar neurons, drastically inhibited neurite outgrowth (Horstkorte *et al.*, 1993). Several findings provide evidence that the inhibition of neurite outgrowth is the result of a disturbed association of L1 with NCAM via oligomannosidic glycans carried only by L1, but not by NCAM, implicating oligomannosidic glycans in important cell recognition and adhesion processes (Horstkorte *et al.*, 1993).

Apart from L1 and AMOG, a large number of glycoproteins have been shown to carry oligomannosidic glycans, either by reactivity with lectins, the L3 and L4 antibodies or by structural analysis, e.g. chorionogonadotrophin (Blithe, 1990), integrins (Pesheva *et al.*, 1987; Asada *et al.*, 1991), myelin-associated glycoprotein (MAG; Fahrig *et al.*, 1990), ovalbumin (Tai *et al.*, 1977), P0 (Bollensen *et al.*, 1988), RNase B (Liang *et al.*, 1980), Thy-1 (Parekh *et al.*, 1987; Fahrig *et al.*, 1990), thyroglobulin (Arima *et al.*, 1972) and uromodulin (Sherblom *et al.*, 1987). Interestingly, some of these glycoproteins have been shown to exist in association with other proteins. Functionally important associations mediated by oligomannosidic glycans are so far only known for L1 and NCAM and for the

subunits of human chorionogonadotrophin (Blithe, 1990; Horstkorte *et al.*, 1993). It remains to be seen whether these glycans play a role in the non-covalent linkage between other cell surface glycoproteins, as for example the subunits of thyroglobulin, the α- and β-subunits of integrins or AMOG, the β2-subunit of the Na/K-ATPase and the catalytically active α-subunit (Gloor *et al.*, 1990; Schmalzing *et al.*, 1992).

Association of proteins can take place during their biosynthesis in the ER (Hurtley and Helenius, 1989), where only oligomannosidic glycans exist. The possibility of subunit association in the ER, together with the locally restricted synthesis of different types of oligosaccharides in the compartments of the ER and Golgi apparatus, provides a basis for the hypothesis that the interaction between molecules like L1 and NCAM may take place in the ER. This association may prevent trimming and processing of oligomannosidic glycans by glycosidases and glycosyltransferases by steric hindrance. As a result, the oligomannosidic glycans would pass through the Golgi apparatus unmodified and reach the cell surface. It is possible, therefore, that the oligomannosidic glycan-mediated association of proteins represents a more general mechanism for the assembly of cell surface expressed and secreted proteins.

Materials and methods

Antibodies

Monoclonal L3 and L4 antibodies (Kücherer *et al.*, 1987; Fahrig *et al.*, 1990), both of the IgM subclass, were either used as ascites or as immunoaffinity-purified antibodies using a monoclonal MARK-1 antibody column reacting with rat Ig kappa chain (Bazin, 1982). Polyclonal antibodies were prepared in rabbits against AMOG (Antonicek *et al.*, 1987), P0 (Schneider-Schaulies *et al.*, 1990) and L1 (Martini and Schachner, 1986). Horseradish peroxidase (HRP)-conjugated secondary antibodies directed against rat IgG and IgM or rabbit IgG, as well as fluorescein isothiocyanate-conjugated secondary antibodies, were purchased from Jackson Immunochemicals (Hamburg, FRG).

Glycoproteins

AMOG and L1 were immunoaffinity purified from detergent lysates of crude membrane fractions from adult mouse brain using monoclonal antibody columns (Rathjen and Schachner, 1984; Antonicek *et al.*, 1987). PO was obtained from human sciatic nerve myelin according to Kitamura *et al.* (1976). RNase B and pronase were purchased from Sigma.

Isolation of oligosaccharides

Oligosaccharides were released from AMOG or RNase B either by hydrazinolysis (Takasaki *et al.*, 1982) or by treatment with endoglycosidase H (Boehringer Mannheim). For the enzymatic release, 500 μg of immunoaffinity-purified AMOG were incubated for 24 h at 37°C with 50 mU endoglycosidase H in 1 ml of 50 mM sodium acetate buffer (pH 5.5). The completeness of the reaction was monitored by ELISA (see below). The reaction products were purified by Biogel P2 (Biorad) column chromatography with 1% acetic acid in water as eluent. Oligosaccharide-containing fractions were detected by silica gel TLC (Merck, catalog no. 5721) with butanol/ethanol/water/acetic acid/pyridine (10/100/30/3/10, by vol.) as solvent. Orcinol-positive fractions beyond the void volume of the column were collected, neutralized with pyridine and the solvents removed using a rotary evaporator.

Mass spectrometry

FAB-MS was performed on a ZAB HF mass spectrometer (VG Analytical, Manchester, UK) in the positive-ion mode on glycans released by endoglycosidase H treatment, followed by peracetylation or permethylation (Egge and Peter-Katalinic, 1987) and in the negative-ion mode on neoglycolipids derived from native glycans using thioglycerol as a matrix. Spectra were acquired as single scans in the upscan mode using an AMD DP10 data system equipped with SAMII (KWS) hardware and SUSY software (AMD Intectra, Beckeln, FRG).

Synthesis of neoglycolipids

Neoglycolipid derivatives of the following carbohydrates were prepared: oligosaccharides from AMOG released by endoglycosidase H or from RNase B released either by hydrazine or endoglycosidase H as described above, lactose and TS101 (the trisaccharide galactose-*N*-acetylglucosamine-mannose). Furthermore, oligomannosidic glycans of defined structures containing 5, 6, 7, 8 or 9 mannose residues ($Man_{5-9}GlcNAc_2$ released from thyroglobulin, Oxford GlycoSystems; see Figure 5 for structures) and $Man_5GlcNAc$ (a gift from A.Haselbeck, Boehringer Mannheim) were derivatized to neoglycolipids. Neoglycolipid synthesis was performed mainly according to the procedure described by Stoll *et al.* (1988). Briefly, 500 μg of oligosaccharides in 20 μl methanol/water (1/1, v/v) were mixed with 4 mg dipalmitoyl-phosphatidyl-ethanolamine (Sigma) dissolved in 800 μl chloroform/methanol (1/1, v/v). After incubation at 60°C for 2 h, 1 mg of sodium cyanoborohydride in 100 μl methanol was added and the reaction continued for up to 5 days. The progress of the reaction was monitored by TLC. The chromatogram was first developed with chloroform/methanol/water (105/100/28, by vol.) until the front had reached about 2/3 across the plate and after drying, with chloroform/methanol/water (75/125/4, by vol.) until the top of the thin-layer plate. Glycolipids were visualized by staining with orcinol reagent.

The neoglycolipids were purified on Sephadex LH 20 (column size 140 × 18 mm; Pharmacia) with chloroform/methanol/water (5/5/1, by vol.) as eluent and then on Iatrobeads (column size 80 × 18 mm; Macherey & Nagel, Düren, FRG), first with chloroform/methanol/water (75/25/4, by vol.) until the underivatized phosphatidylethanolamine eluted, and finally with chloroform/methanol/water (105/100/28, by vol.). The fractions from each column were analysed by TLC and the neoglycolipid-containing fractions pooled.

Synthesis of neoglycolipids with mannosidic glycans of defined structures was carried out with 10 nmol of each compound. All solvents and reagents were scaled down to 1/20 of the level described in the procedure above.

Determination of antibody binding to neoglycolipids by TLC immunostaining

Neoglycolipids derived from $Man_5GlcNAc_2$ (~3 μg), from oligosaccharides of AMOG (~5 μg) and oligosaccharides of RNase B (~3 μg) were chromatographed on $HPTLC_{60}$ silica gel plates (Merck) in chloroform/methanol/water (105/100/28, by vol.). Incubation of TLC plates with antibodies was essentially carried out as described by Felding-Haberman *et al.* (1986) with the modification that the plates were incubated with the first antibody (50 μg/ml) at 4°C overnight and with the secondary HRP-conjugated antibody for 3 h at room temperature. After the development of the HRP reaction with 4-chloronaphthol, the TLC plate was washed three times with water, air dried and treated with acetone to remove the polyisobutyl-methacrylate. After drying, the plate was sprayed with orcinol and heated at 100°C for colour development.

ELISA

(i) Determination of antibody binding to AMOG before and after treatment with endoglycosidase H. AMOG was treated with endoglycosidase H as described above for the isolation of the oligosaccharides. As a control, the incubation of AMOG was carried out under identical conditions as for the enzymatic digestion, but in the absence of the enzyme. The incubation solutions were then diluted in 0.1 M sodium bicarbonate to a concentration of 1 μg AMOG/ml and incubated in 96 well microtitre plates (Falcon, catalogue no. 3912) at 4°C overnight. After blocking for 1 h with 1% bovine serum albumin (BSA) in phosphate-buffered saline, pH 7.2 (PBS), wells were incubated for 3 h with appropriate dilutions of monoclonal L3, L4 (10 μg/ml) or polyclonal AMOG antibodies (100 μg/ml). After washing of plates with PBS, incubation with HRP-conjugated antibodies to rabbit Ig or rat IgG and IgM for 2 h and another washing step, the HRP reaction was carried out as described by Bollensen *et al.* (1988). The optical density of the HRP reaction product was measured at 405 nm using a Titertek Multiscan Plus (Flow). All incubations were carried out at room temperature.

(ii) Determination of the binding of the L3 and L4 antibodies to AMOG, L1 and P0 after fucosidase treatment. AMOG, L1 or P0 (10 μg) were dissolved in 200 μl of 0.1 M citrate/phosphate buffer (pH 4.0), and incubated for 48 h at 37°C with 15 mU α-fucosidase from *C.lampas* (Seikagaku Kogyo, Japan; the enzyme is no longer available). For a control, the antigens or enzymes were incubated in the absence of enzyme or antigens, respectively, under otherwise identical conditions. The incubation solutions were diluted 1:10 in 0.1 M sodium bicarbonate and the ELISA performed as described in (i) using monoclonal L3 and L4, and polyclonal AMOG, L1 or P0 antibodies.

(iii) Detection of neoglycolipids. Solutions of purified neoglyocolipids in ethanol were applied onto microtitre plates either at constant concentrations or serially diluted (see legends to Figures 3 and 6 for concentrations) and the ethanol

evaporated at room temperature. After incubation of wells with 1% defatted BSA (Sigma, catalogue no. A 7030) in 0.1 M sodium bicarbonate for 1 h, incubation with L3 or L4 antibody for 3 h was followed by incubation with HRP-conjugated secondary antibodies to rat IgG and IgM for 2 h, and the ELISA developed as described in (i). All steps were carried out at room temperature. For the detection of L3 and L4 immunoreactivity after α-mannosidase treatment, an aliquot of a $Man_3GlcNAc_2$-neoglycolipid preparation (~10 μg) was incubated for 48 h at 37°C with jack bean mannosidase (2 U; Sigma) in 10 mM sodium acetate buffer (pH 4.2) containing 0.1 mM zinc acetate. A control aliquot was incubated in parallel without enzyme. After freeze–drying, the incubation mixtures were dissolved in 100 μl of chloroform/methanol/water (100/105/28, by vol.) and diluted 1:100 in ethanol. The ELISA was then performed as described above. In order to ensure that glycolipids remained bound to the plastic of the microtitre plates during the ELISA procedure, substrate-coated glycolipids (1 μg/ml) were incubated for 6 h with 1% defatted BSA in PBS. After washing of the microtitre plates with distilled water, the coated glycolipids were redissolved in chloroform/methanol/water (1/1/0.15, by vol.) and the combined solutions from 20 wells evaporated under nitrogen. TLC was carried out as described for immunostaining. Similar amounts of the different glycolipids were recovered, as judged by eye from the orcinol staining of chromatograms.

Cell culture

Freshly dissociated cerebellar cells (~90% of which are small cerebellar neurons) from 5- to 7-day-old NMRI mice were plated at a concentration of 3–4 × 10^6 cells/ml on poly-L-lysine-coated glass coverslips (Schnitzer and Schachner, 1981; Keilhauer *et al.*, 1985). For treatment of cells with swainsonine, cerebellar cells were cultured for 2 days in the presence of swainsonine (2 μg/ml, from Sigma) in serum-free, hormone-supplemented medium (Fischer, 1982). The inhibitor was added to the single-cell suspensions prior to plating them on glass coverslips.

Immunocytological procedures

Indirect immunofluorescence staining of monolayer cultures with the monoclonal L3 antibody was carried out according to Fischer *et al.* (1986) using fluorescein isothiocyanate-conjugated secondary antibodies.

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Abbreviations

BSA, bovine sorum albumin; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FAB-MS, fast-atom bombardment mass spectrometry; Gal, galactose; GlcNAc, *N*-acetylglucosamine; Hex, hexose; HexNAcol, *N*-acetylhexosaminitol; HRP, horseradish peroxidase; Man, mannose; PBS, phosphate-buffered saline; TS101, galactose-β1-4-*N*-acetyl-glucosamine-β1-4-mannose.

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