Myelin-associated Glycoprotein, a Member of the L2/HNK-1 Family of Neural Cell Adhesion Molecules, Is Involved in Neuron–Oligodendrocyte and Oligodendrocyte–Oligodendrocyte Interaction

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Abstract. A monoclonal antibody to the myelin-associated glycoprotein (MAG) was prepared and characterized to probe for the involvement of MAG in cell surface interactions among neural cells in vitro. The antibody reacts specifically with oligodendrocyte cell surfaces and myelin-rich brain regions as expected from previous investigations. Not all O4 antigen-positive oligodendrocytes express MAG in vitro. Fab fragments of the antibody interfere with neuron to oligodendrocyte and oligodendrocyte to oligodendrocyte adhesion, but not with oligodendrocyte to astrocyte adhesion. MAG-containing liposomes bind to the cell surfaces of the appropriate target cells by a mechanism that is specifically inhibitable by Fab fragments of monoclonal MAG antibodies, demonstrating that MAG is a neural cell adhesion molecule.

THE myelin-associated glycoprotein (MAG)[1] is a constituent of central and peripheral nervous system myelin sheaths (for review, see Quarles, 1984). Because of its particular localization in periaxonal membranes it has been implicated in neuron-myelinating cell interactions (Trapp and Quarles, 1984; Sternberger et al., 1979). Furthermore, during myelination MAG has been localized on the turning loops of Schwann cells around the axon and has been thought to play a role in glia–glia interaction (Martini and Schachner, 1986). Like the neural cell adhesion molecule (N-CAM), MAG shares immunoglobulin-like domains with other members of the immunoglobulin supergene family (Arquint et al., 1987; Williams, 1982). However, there is no direct experimental evidence proving that MAG is indeed involved in cell surface interactions. On the basis of recent evidence that MAG belongs to a family of glycoproteins that shares a common carbohydrate epitope designated L2/HNK-1 (Kruse et al., 1984, 1985) and includes the cell adhesion molecules L1, J1, and N-CAM, we have suggested that MAG is involved in cell surface recognition (Kruse et al., 1984). Here we show that in the central nervous system MAG antibodies interfere with neuron–oligodendrocyte and oligodendrocyte–oligodendrocyte, but not astrocyte–oligodendrocyte adhesion. Furthermore, we show by use of the purified molecule that MAG itself is the ligand involved in adhesion.

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1. *Abbreviations used in this paper*: MAG, myelin-associated glycoprotein; N-CAM, neural cell adhesion molecule.

Materials and Methods

Production and Analysis of Mono- and Polyclonal Antibodies to MAG

Monoclonal antibody to MAG was prepared in mice by immunization with L2 epitope-carrying glycoproteins from 1- to 2-d-old chicken brains. These glycoproteins were isolated by immunoaffinity chromatography using a monoclonal L2 antibody column (Kruse et al., 1984). The monoclonal antibody to MAG, an IgG by molecular weight determination, was obtained by fusion of mouse myeloma clone P3X63Ag8.653 with spleen cells from immunized mice as described (Lagenaur et al., 1980). For immunization, 4- to 6-wk-old BALB/c females were injected subcutaneously with 50 μg glycoproteins in PBS, pH 7.3, in complete Freund's adjuvant. Two consecutive immunizations were carried out in incomplete Freund's adjuvant at 3-wk intervals. The serum of immunized animals was examined by the immunospot-binding test (Hawkes et al., 1982) using a crude membrane fraction from 1- to 2-d-old chicken brains as described (Rathjen and Schachner, 1984). The monoclonal antibody was identified as MAG-reactive by the immunospot-binding test with purified MAG from bovine brain (Quarles et al., 1983).

Polyclonal MAG antibodies were prepared in rabbits against MAG isolated by the lithium diiodosalicylate-phenol method (Quarles et al., 1981, 1983) and reacted only with the intracellularly exposed domains of MAG (see legend to Fig. 2). Polyclonal antibodies from rabbits immunized with MAG obtained by immunoaffinity purification from nonionic detergent lysates of a crude membrane fraction from adult mouse brain (Rathjen and Schachner, 1984) reacted with the cell surface-exposed domains of MAG. Both antibodies reacted identically in Western blots.

Western blot analysis, radioiodination of the L2 epitope carrying glycoproteins, and immunoprecipitation were carried out as described previously (Kruse et al., 1984; Faissner et al., 1985).

Cell Culture and Immunocytology

Cell culture, double immunofluorescence, and indirect immunohistological procedures were carried out as described (Goridis et al., 1978; Schnitzer and Schachner, 1981*a*). Double immunofluorescence labeling with antibodies to the cell adhesion molecule L1 (Rathjen and Schachner, 1984), glial

fibrillary acidic protein and vimentin (Schnitzer et al., 1981), O4 antigen (Sommer and Schachner, 1981), and fibronectin (Schnitzer and Schachner, 1981a) show that MAG expression is confined to oligodendrocytes in the central nervous system.

Immunoaffinity Purification of MAG, Preparation of Liposomes, and Liposome Binding Test

MAG was immunoaffinity-purified from adult mouse brain membranes solubilized with 0.5% NP-40 (as described by Rathjen and Schachner, 1984) by the monoclonal MAG antibody column with the following modifications. After washing the antibody column with NP-40–containing buffers (Rathjen and Schachner, 1984) the detergent was changed to 34 mM octylglucoside in 20 mM Tris buffer, pH 7.3. The bound antigen was eluted from the column with 50 mM diethylamine, 1 mM EDTA, 1 mM EGTA, containing 34 mM octylglucoside, pH 11.5. The eluate was quickly neutralized and dialyzed against Tris-buffered saline containing 34 mM octylglucoside.

For incorporation of MAG into liposomes the method by Sadoul et al. (1983) was used. In brief, 50 μg purified MAG was added to 1 μmol egg yolk phosphatidylcholine and 0.58 μmol cholesterol in half-strength Tris-buffered saline containing 50 mM octylglucoside and 20 mM carboxyfluorescein. The mixture was maintained for 2 h at 25°C and then passed over a Sephadex G25 column (30 ml) that had been prefilled with 10 ml of 20-mM carboxyfluorescein. Liposomes were separated from unbound protein by sucrose gradient centrifugation and dialyzed against 0.15 M NaCl, 20 mM Hepes-buffer, pH 7.2. Dorsal root ganglion cells were dissected from 1-d-old NMRI mice, dissociated, and maintained in vitro with 100 μg/ml nerve growth factor for 4 d as described (Ranscht et al., 1987). Spinal cord cells were obtained from 13-d-old NMRI mouse embryos, dissociated, and maintained in vitro for 12 d as described for neonatal mouse cerebellum (Schnitzer and Schachner, 1981*b*).

For the liposome binding test, 50 μl of the liposome solution was diluted in 50 μl 0.15 M NaCl, 20 mM Hepes-buffer, pH 7.2, containing 5% horse serum, and incubated for 30 min with the monolayer cultures at 25°C. Cultures were washed and mounted in Mowiol (Hoechst, Frankfurt, Federal Republic of Germany). When Fab fragments of poly- and monoclonal MAG antibodies were included in the test, liposomes were incubated with cells in the presence of 100 μg/ml Fab fragments of monoclonal or polyclonal MAG antibodies. Polyclonal MAG antibodies used in this test were obtained by immunization with MAG obtained from nonionic detergent lysates of adult mouse brain membranes by immunoaffinity chromatography and reacted with the cell surfaces of live cultured oligodendrocytes. Fab fragments of polyclonal L1 (Rathjen and Schachner, 1984) were used at 200 μg/ml.

Adhesion Test

The adhesion test was carried out as described (Keilhauer et al., 1985*a*) using monolayer cultures as target cells and fluorescein diacetate–labeled single cell suspensions as probe cells, except that 96-well microtiter plates were used to culture the target cells. Pure populations of small cerebellar neurons were obtained by buoyant density centrifugation of single cell suspensions through Percoll and immunocytolysis (Keilhauer et al., 1985*a*). Oligodendrocytes and astrocytes were prepared from cerebral hemispheres of 0–2-d-old Sprague-Dawley rats according to the method of McCarthy and de Vellis (1980) as modified by Keilhauer et al. (1985*b*). Neurons were plated at 200,000 cells per well and used as target cells after 3–4 d in vitro. Oligodendrocytes were plated at 25,000 cells per well and used as target cells after 5–7 d in vitro. Neurons, astrocytes, and oligodendrocytes were used as probe cells at 10,000, 25,000, and 50,000 cells per well and taken after 1, 1, and 5–7 d in vitro, respectively. The purity of neurons, astrocytes, and oligodendrocytes was >99, >99, and 70–80%, respectively, using tetanus toxin receptor, glial fibrillary acidic protein, and O4 antigen as cell type–specific markers (Schnitzer and Schachner, 1981*a*; Sommer and Schachner, 1981). The predominant contaminating cell type in the enriched oligodendrocyte preparation is glial fibrillary acidic protein-positive astrocyte. Only ~50% of all O4 antigen–positive oligodendrocytes express MAG. Of the probe cells the percentage of input cells that bound to their target cells (oligodendrocytes) was 65 ± 12 for neurons, 40 ± 7 for astrocytes, and 73 ± 9 for oligodendrocytes. Neurons bound to neurons with ~90% of all input cells. Probe and target cells were treated with Fab fragments (1 mg/ml) of monoclonal MAG antibody for 20 min on ice before the adhesion test. Probe and target cells were then incubated for 30 min at room temperature in a reciprocal shaker at 40 cycles per min. Aggregation among probe cells was negligible in the presence or absence of Fab fragments. Adhering cells were scored by eye in a fluorescence microscope.

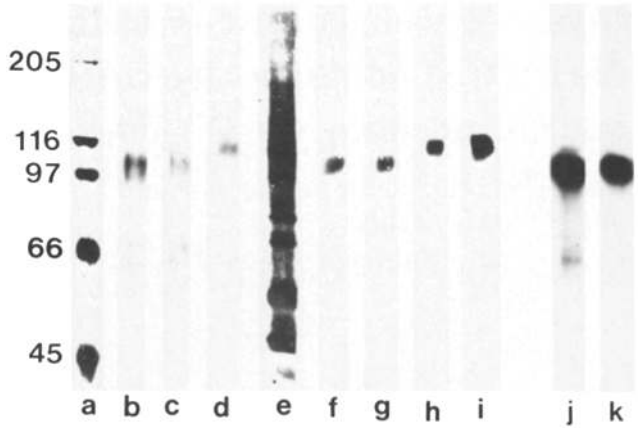


Figure 1. Purified MAG, visualized after SDS-PAGE (8% slab gels) by the reducing silver method (Merril et al., 1982) (lanes *b–d*) and by Western blot analysis using polyclonal MAG antibodies (lanes *f–h*). MAG was isolated by immunoaffinity chromatography on the monoclonal MAG antibody column from adult human (lanes *b* and *f*) and mouse (lanes *c* and *g*) brains, and from adult bovine brain by the lithium diiodosalicylate–phenol method (Quarles et al., 1983) (lanes *d* and *h*). Myelin purified from adult bovine brain (Norton and Poduslo, 1973) visualized by the reducing silver method (lane *e*) and developed by Western blot using polyclonal MAG antibodies (lane *i*). Numerals on the left represent apparent M_r ($\times 10^{-3}$) of molecular mass markers (lane *a*). Note the differences in M_r of MAG isolated from human and mouse vs. bovine (see also Quarles, 1984). Autoradiography of immunoprecipitates of ^{125}I-labeled glycoproteins from adult mouse brain isolated by immunoaffinity chromatography on a monoclonal L2 antibody column (Kruse et al., 1984), incubated with polyclonal (lane *j*) and monoclonal (lane *k*) antibodies to MAG and separated by SDS-PAGE on 8% slab gel.

Results

Monoclonal antibodies were prepared from mice immunized with the fraction of L2 epitope–carrying glycoproteins from chicken brain. One of the antibodies recognized MAG from mouse, bovine, human (Fig. 1), rat, chicken, and frog. The antibody reacted with the cell surface of oligodendrocytes in monolayer cultures of early postnatal mouse cerebellum, but not with neurons, astrocytes, or fibroblasts as seen by double-immunolabeling with antibodies to established cell type–specific markers (not shown). Approximately 50% of all O4 antigen–positive oligodendrocytes (Sommer and Schachner, 1981) were stained by the monoclonal antibody to MAG in 3-d-old cultures of 7-d-old mouse cerebellum. All oligodendrocytes stained by the monoclonal antibody were also stained by polyclonal antibodies to MAG and vice versa (Fig. 2). In histological sections of mouse cerebellum MAG was first detectable at postnatal day 6 in prospective white matter. In the adult cerebellum only white matter tracts were labeled by MAG antibodies (Fig. 2). Approximately 30% of all MAG-positive oligodendrocytes express the L2/HNK-1 epitope in cultures of early postnatal cerebellum, suggesting a heterogeneity in expression of this carbohydrate moiety among MAG-expressing cells. This heterogeneity in L2/HNK-1 expression has also been previously observed for two other members of the L2/HNK-1 family, N-CAM and L1 (Kruse et al., 1984; Wernecke et al., 1985).

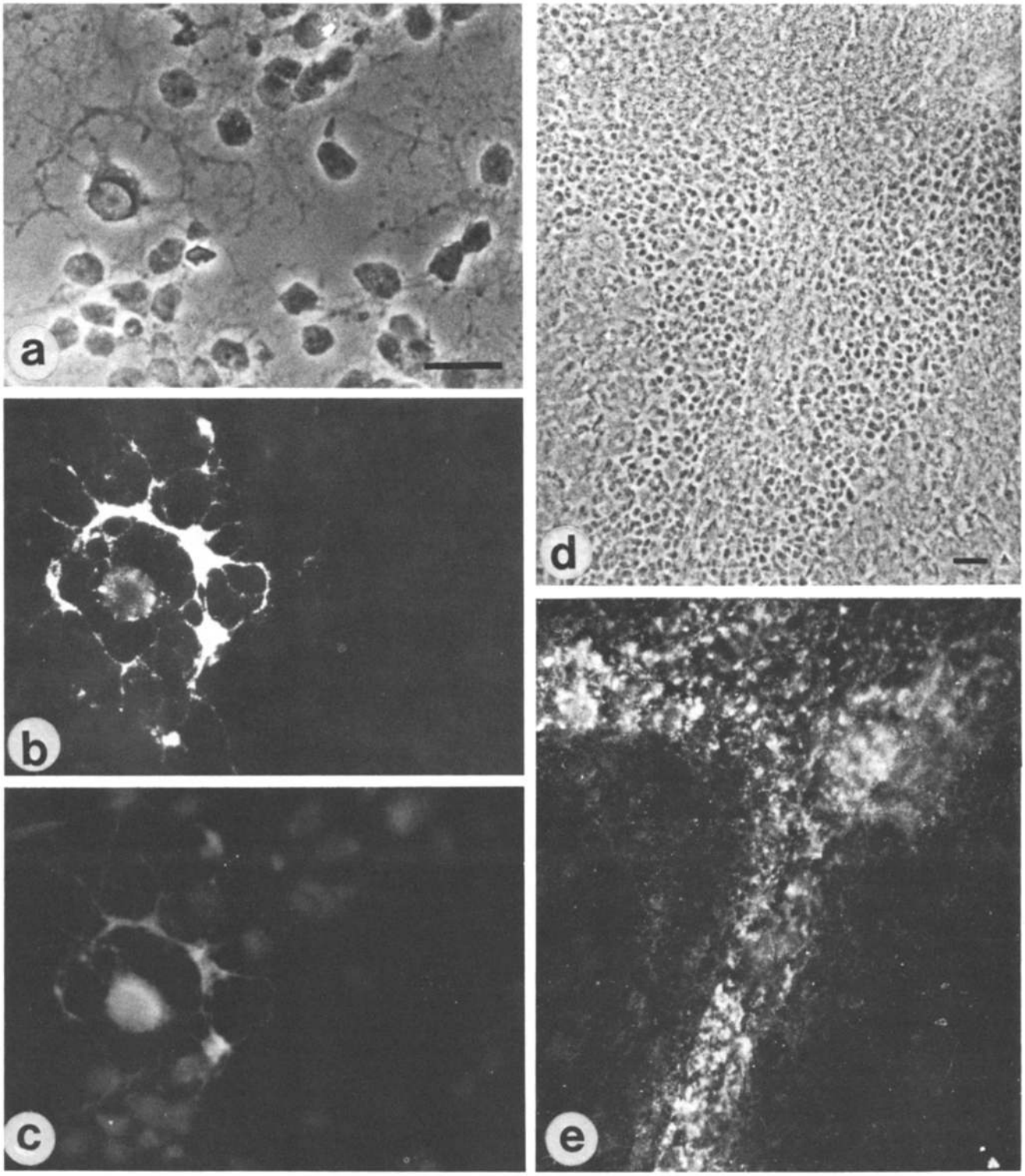


Figure 2. Double immunofluorescence labeling of monolayer cultures of 7-d-old C57BL/6J mouse cerebellum maintained in vitro for 3 d using monoclonal (*b*) and polyclonal MAG (*c*) antibodies. Cells had to be permeabilized by fixation (Schnitzer and Schachner, 1981*a*) for reactivity with polyclonal antibodies prepared against MAG obtained by the lithium diiodosalicylate method. Generally, these antibodies were weakly reactive in indirect immunofluorescence labeling procedures. (*a*) Corresponding phase-contrast micrograph to fluorescence images (*b* and *c*). Indirect immunohistology using monoclonal MAG antibody on a fresh frozen section of adult C57BL/6J mouse cerebellum (*e*). (*d*) Corresponding phase-contrast micrograph of fluorescence image (*e*). Bars, 20 μm.

Table I. Inhibition of Adhesion between Oligodendrocytes and Neurons, Astrocytes, or Oligodendrocytes in the Presence of Fab Fragments from Monoclonal MAG Antibodies

Antibody	Neuron* to oligodendrocyte‡	Neuron* to neuron‡	Astrocyte* to oligodendrocyte‡	Oligodendrocyte* to oligodendrocyte‡
None	0 ± 2	0 ± 2	0 ± 4	0 ± 2
Mono-MAG	25 ± 6	2 ± 4	−2 ± 1	16 ± 4
Poly-liver	−1 ± 3	0 ± 3	4 ± 2	−1 ± 2

Percent inhibition of adhesion in the presence of Fab fragments was calculated: % inhibition = (adhesion [control] − adhesion [+Fab])/(adhesion [control]) × 100. Numbers are mean values from several experiments (± SD). Seven experiments were performed for neuron–oligodendrocyte, five for neuron–neuron, three for astrocyte–oligodendrocyte, and five for oligodendrocyte–oligodendrocyte adhesion. The difference in inhibition of adhesion given by liver membrane (Lindner et al., 1983; Pollerberg et al., 1986) and monoclonal MAG antibodies is significant for oligodendrocyte–neuron and oligodendrocyte–oligodendrocyte adhesion ($P < 0.001$ according to Student's t test).
*, probe cells; ‡, target cells. mono, Fab fragments of monoclonal antibody; poly, Fab fragments of polyclonal antibody.

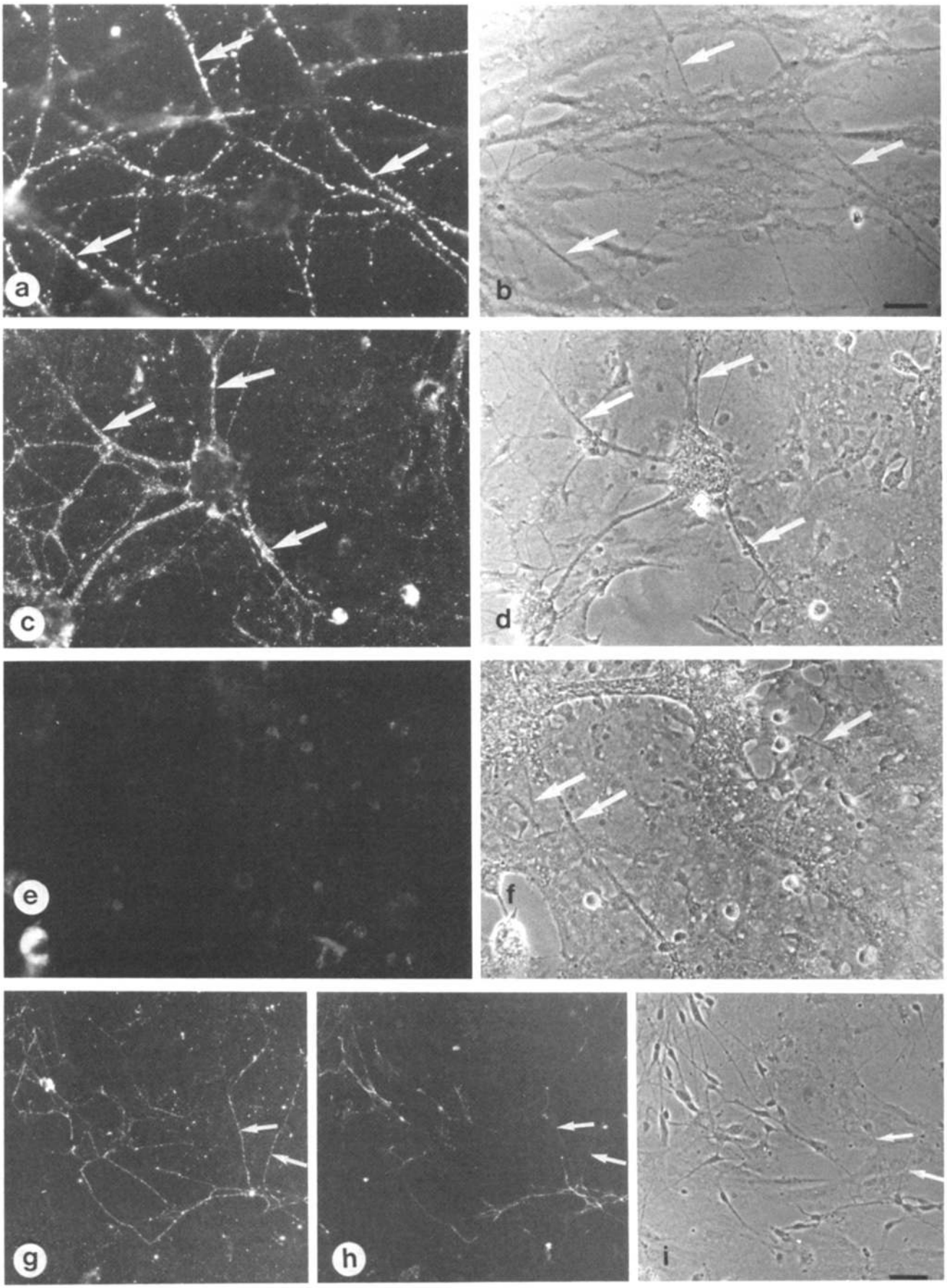
The epitope recognized by monoclonal MAG antibody is most likely localized in the protein part of the molecule, since treatment of purified MAG with protease-free endoglycosidase F does not destroy immunoreactivity. Furthermore, MAG is not recognized any more by the antibody after treatment with chloroform-methanol or SDS for Western blot analysis.

To investigate whether all molecules of a population of MAG molecules isolated from adult mouse brain express the L2/HNK-1 epitope, sequential immunoprecipitations were performed as described previously for N-CAM (Kruse et al., 1984). For these experiments MAG was isolated from adult mouse brain by immunoaffinity chromatography using the monoclonal MAG antibody column, radioiodinated, and exhaustively immunoprecipitated, first with monoclonal L2 antibodies and then with mono- or polyclonal MAG antibodies. Only ~30% of the total radioactivity that could be precipitated with mono- or polyclonal MAG antibodies was precipitated by L2 antibodies. When immunoprecipitations were carried out first with mono- or polyclonal MAG antibodies, no more counts could be recovered by subsequent immunoprecipitation with L2 antibodies. These experiments show that only a subpopulation of ~30% of all MAG molecules isolated from adult mouse brain carry the L2/HNK-1 epitope. Similarly, only ~20% of all N-CAM molecules (Kruse et al., 1984) and 50% of all L1 molecules (A. Faissner, unpublished observations) isolated from adult mouse brain express the L2/HNK-1 epitope.

To investigate whether MAG is a cell adhesion molecule adhesion of single cell suspensions of small neurons from early postnatal mouse cerebellum and astrocytes and oligodendrocytes from rat cerebral hemispheres to monolayers of oligodendrocytes was measured under Ca^{++}-free conditions (Keilhauer et al., 1985*a*) in the absence and presence of Fab fragments of monoclonal MAG antibodies. These antibodies inhibited oligodendrocyte–neuron and oligodendrocyte–oligodendrocyte, but not oligodendrocyte–astrocyte interaction (Table I). Inhibition values of 25 ± 6 for neuron-oligodendrocyte adhesion and 16 ± 4 for oligodendrocyte–oligodendrocyte adhesion were significantly different from control values (see legend to Table I). Partial and often small antibody-mediated inhibition is not unusual in adhesion tests (e.g., Keilhauer et al., 1985*a*) and suggests a heterogeneity in adhesion mechanisms. Furthermore, it has to be kept in mind that only ~50% of all O4 antigen-positive oligodendrocytes in the enriched population of oligodendrocytes expressed MAG, 70–80% of which are O4 antigen-positive oligodendrocytes, the rest being predominantly glial fibrillary acidic protein-positive astrocytes that do not engage in MAG-dependent adhesion of oligodendrocytes (Table I). MAG antibodies were generally observed in these tests to inhibit oligodendrocyte–oligodendrocyte adhesion, but not to interfere with oligodendrocyte–astrocyte adhesion, when the cells were viewed by phase-contrast and fluorescence microscopy. Polyclonal antibodies to mouse liver membranes that react strongly with all three cell types by indirect immunofluorescence did not interfere with adhesion. These experiments suggest that MAG is involved in cell adhesion among certain types of neural cells. However, antibodies that bind to MAG may not only conceal MAG on the cell surface, but could also sterically block closely associated molecules that may be responsible for adhesion.

To investigate whether MAG itself is the binding ligand, MAG was isolated from nonionic detergent lysates of a crude membrane fraction of adult mouse brain by immunoaffinity purification. MAG was then incorporated in phosphatidylcholine and cholesterol-containing liposomes labeled with fluorescein (Sadoul et al., 1983). Incorporation of MAG into liposomes was shown by subjecting the liposome preparation to SDS-PAGE. MAG isolated by the lithium diiodosalicylate–phenol method (Quarles et al., 1983) did not incorporate into liposomes under the conditions of this study. Liposomes containing MAG from nonionic detergent lysates were added to monolayer cultures prepared from dorsal root ganglia, spinal cord, and cerebellum (Fig. 3). MAG liposomes specifically attached to neurites and neurite bundles

Figure 3. Binding of fluorescein-labeled MAG-containing liposomes to cultures of dorsal root ganglion (*a* and *b* and *g–i*) and spinal cord cells (*c–f*) in the absence (*a–d* and *g–i*) and presence (*e* and *f*) of Fab fragments of monoclonal MAG antibodies. *g* and *h* are double immunofluorescence micrographs showing binding of MAG-containing liposomes (*g*) and monoclonal L1 antibody to label neurites (*h*). *b*, *d*, *f*, and *i* are phase-contrast micrographs corresponding to fluorescence images *a*, *c*, and *e*, and *g* and *h*, respectively. Arrows point to neurites or neurite bundles (*a–d* and *f–i*). Fluorescent cell bodies in *e* are macrophages that autofluoresce with a reddish color different from the yellow-green of fluorescein. Bars: (*a* and *b*) 15 μm; (*c–i*) 80 μm.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(Fig. 3, *a*, *c*, and *g*), but not to the underlying Schwann cells of cultures of dorsal root ganglia (Fig. 3, *a* and *b* and *g*, *h*, and *i*). It should be noted that Schwann cells did not express MAG in these cultures. MAG liposomes also did not attach to astrocytes, fibroblasts, or macrophages of spinal cord (cf. Fig. 3, *c* and *d*). Binding of MAG liposomes to neurites was also observed in cultures of early postnatal cerebellum, up to 90% of which consists of small cerebellar neurons, the granule, stellate and basket cells (not shown) that are never contacted by myelinating oligodendrocytes in situ. Binding was not detectable after 4 d in culture, but was seen after 12 d. However, binding of MAG liposomes to cerebellar neurons was always less pronounced than to spinal cord or dorsal root ganglion neurons. Liposomes without MAG did not bind to neurites or cells (not shown). Binding of MAG-containing liposomes to neurites was inhibited by Fab fragments of mono- and polyclonal MAG antibodies (Fig. 3, *e* and *f*). Polyclonal antibodies used in this study reacted with the extracellularly exposed domains of MAG. Fab fragments of polyclonal L1 antibodies did not interfere with the adhesion of MAG liposomes to neurites. These experiments show that MAG is itself the binding ligand and therefore a true adhesion molecule.

Discussion

The present study has demonstrated that the myelin-associated glycoprotein is expressed on the cell surface of a subpopulation of O4 antigen–positive oligodendrocytes and acts as a Ca^{++}-independent cell adhesion molecule. After N-CAM (Sadoul et al., 1983; Hoffman and Edelman, 1983) MAG is the second and only neural cell adhesion molecule for which direct ligand binding could be shown. Not all MAG-positive oligodendrocytes and not all MAG molecules express the L2/HNK-1 carbohydrate epitope as it has been previously observed for N-CAM (Kruse et al., 1984) and L1 (A. Faissner, unpublished observations). These findings are noteworthy, since the epitope has been implicated in cell adhesion (Keilhauer et al., 1985*a*) and is developmentally regulated (Wernecke et al., 1985). It is therefore possible that other molecular features of MAG are also implicated in cell adhesion.

It appears worth mentioning that MAG is involved in adhesion of oligodendrocytes to neurons that are normally not myelinated in vivo, such as the small cerebellar neurons. These observations point to the involvement of MAG in neuron–oligodendrocyte interactions other than myelination and to a more general role of MAG in these interactions. However, the small cerebellar neurons appear to be less adhesive partners for MAG-containing liposomes than the normal targets for myelination, the dorsal root ganglion and spinal cord neurons. The small but very significant inhibitory effect of monoclonal MAG antibodies on adhesion between cerebellar neurons and oligodendrocytes may thus be explained by the prevalence of other adhesive mechanisms that are operant between these neurons and oligodendrocytes. It is, however, also conceivable that the monoclonal antibody recognizes only the periphery and not the center of the cell-binding site of MAG.

In addition to neuron–oligodendrocyte adhesion MAG is also involved in oligodendrocyte–oligodendrocyte adhesion. This observation points to the role of MAG in self-recognition of oligodendrocyte cell surface membranes, which is a striking feature of the myelination process involving the apposition of spiralling loops of oligodendrocyte processes. It is interesting in this respect that MAG has been found on the outer mesaxon, paranodal loops of myelin, and Schmidt–Lanterman incisures, but is absent from compact myelin (Trapp and Quarles, 1984; Martini and Schachner, 1986) at adult stages. MAG was also detectable during development on the cell surface of immature myelin-forming and uncompacted glial processes in the sciatic nerve (Martini and Schachner, 1986). The contention that MAG is also present in compacted myelin lamellae (Webster et al., 1983) has been disputed (Trapp and Quarles, 1984) and can be ruled out on the basis of recent experiments that used postembedding staining procedures to avoid antibody penetration problems (Martini and Schachner, 1986). It remains to be investigated whether MAG binds to itself by a self-binding binding mechanism, as has been suggested for N-CAM (Hoffman and Edelman, 1983) and whether it can also serve as a ligand in binding to other molecules at the cell surface or in the extracellular matrix (see Martini and Schachner, 1986). A binding mechanism to a different receptor than MAG itself has to be postulated, since axons do not express MAG (Martini and Schachner, 1986), but bind MAG liposomes. Whether an altered form of MAG in Trembler (Inuzuka et al., 1985) and Quaking (Matthieu et al., 1974) mice is related to a defect in cell adhesion between axon and glia or between myelinating processes remains to be seen. Elucidation of the cellular and molecular signals that regulate MAG expression by oligodendrocytes and Schwann cells will yield important insights into the mechanisms underlying the complex process of myelination.

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