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Recognition of Human Recombinant Myelin Associated Glycoprotein by Anti-carbohydrate Antibodies of the L2/HNK-1 Family

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Summary: The L2 and HNK-1 monoclonal antibodies recognize carbohydrate determinants containing sulfate-3-glucuronate that are prominent on cells of neural crest lineages. In humans these epitopes are most abundant on the Myelin Associated Glycoprotein and it was assumed that they co-localize on the same molecules. Recently, in vitro synthesized carbohydrates have provided a basis for the different recognition requirements of these two antibodies. We now provide in vivo evidence that a human melanoma cell line can produce glycoproteins such as fibronectin, which is recognized by both the L2 and HNK-1 antibodies, and simultaneously a transfected Myelin Associated Glycoprotein carrying only L2-type carbohydrates. Conceivably, the differential expression of L2-and HNK-1 type glycans could have a role in development.

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The L2/HNK-1 epitope is common to a variety of cell adhesion molecules including the myelin proteins Myelin Associated Glycoprotein (MAG), P_0 and PMP-22 (1-3). These glycoproteins bear glycans that are recognized by the HNK-1 murine monoclonal antibody (4). These carbohydrates are thought to be involved in various neuronal recognition and adhesion functions. Recently MAG has been shown to inhibit neurite outgrowth from both developing cerebellar and adult dorsal root ganglia (5). Cooperative binding has been shown previously to be important for carbohydrate recognition by antibodies (6) and of all HNK-1 displaying proteins MAG has the highest density of such residues (7). Eight of the nine possible glycosylation sites on MAG can carry HNK-1 epitopes (7). The expression of the HNK-1 epitope has been shown to vary considerably during development and regeneration in the peripheral nervous system of the mouse (8, 9). In this context it is interesting to note that a subset of patients with monoclonal IgM gammopathies and neuropathies have

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Abbreviations: MAG, Myelin Associated Glycoprotein; fluorescein isothiocyanate, FITC; phycoerythrin, PE.

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autoantibodies that are directed against the HNK-1 epitope present on myelin proteins and glycolipids (10). The main pathological findings are a demyelinating neuropathy with deposits of the IgM paraprotein and a characteristic widening of the myelin lamellae at the intraperiod line (11). It has been shown by adoptive transfer that these features can be produced by anti-MAG antibodies (12) suggesting that the demyelination may be due to a perturbed adhesive function of HNK-1 bearing glycoproteins in peripheral nerve.

With the aim to produce a recombinant MAG recognized by the monoclonal IgM autoantibodies of patients with neuropathy, we have selected a human malignant melanoma cell line, Melur, which has been reported to produce HNK-1 positive fibronectin (13). The recombinant MAG immunoprecipitated from transfected Melur cells can be recognized by the L2 but not the HNK-1 antibody.

Materials and Methods

Cell culture and staining: Melur, D3A2G5 anti-MAG hybridoma (14) and HNK-1 hybridoma (4) cells were grown in Optimem I (Gibco BRL) medium supplemented with 5% fetal calf serum (Seromed). Melur cells were harvested with EDTA (0.2 g EDTA and 0.85 g NaCl/L). After two washes in PBS with 1% fetal calf serum they were incubated with D3A2G5 (anti-MAG polypeptide mouse IgG1) and HNK-1 (mouse IgM) hybridoma culture supernatants on ice for 30 min. After three washes with PBS/1% fetal calf serum they were incubated with a second layer of fluorescence labeled antibodies, goat anti-mouse IgG-Cy3 and goat anti-mouse IgM-Cy3 (both from Jackson ImmunoResearch Laboratories, Inc.) in PBS/1% fetal calf serum on ice for 30 min. The surface labeled cells were resuspended in PBS/1% fetal calf serum at $1x10^6$ cells /ml and either sorted by two color fluorescence using a FACS Star sorter (Becton Dickinson, Sunnyvale, CA) or mounted on coverslips for fluorescence microscope analysis.

Transfection: A full length human L-MAG cDNA (15) was introduced into the pRc/CMV and pRc/RSV expression vectors (Invitrogen) in front of the respective promoter and enhancer elements. The recombinant plasmids were verified by mapping with restriction enzymes and were transfected into Melur cells using the Lipofectin reagent obtained from GIBCO BRL. Stably transfected populations were selected using G418 at 0.4 mg/ml and sorted to obtain a population expressing high levels of HNK-1 and MAG. The selected cells were further expanded as required and checked regularly by immunostaining to verify the high expression of MAG and HNK-1. Petri dishes with confluent cells were harvested at 4°C in lysis buffer (50 mM Tris pH=8, 150 mM NaCl, 10 mM EDTA, 1% NP 40, 100 µM PMSF, 1.25 µg/ml of a protease inhibitor mixture with aprotinin, antipain, amastatin, bestatin). The lysates were sonicated for 30 sec and spun at 15'000 x g for 15 min. The protein concentration of supernatants was measured by Bradford assay. The non-transfected cell lysate was prepared the same way.

Immunoprecipitation: Transfected and nontransfected cell lysates and human brain myelin were immunoprecipitated using the D3A2G5 monoclonal antibody which recognizes the polypeptide chain of MAG. Protein A Sepharose beads (Pharmacia) were incubated with rabbit anti-mouse IgG (DACO) for 1 hour at room temperature. The coated beads were collected by centrifugation and washed with lysis buffer three times. The cell lysates (200 µg protein) were precleared with Sepharose 6B (Pharmacia) at 4°C for 1 h and then incubated with monoclonal anti-MAG antibody (D3A2G5) for 1 hour at room temperature. The rabbit anti-mouse IgG coated beads and the lysates with D3A2G5 monoclonal antibody were incubated together for 4 hours and washed 5 times in lysis buffer before boiling in SDS loading buffer with 5% ß-mercaptoethanol and electrophoresis in 7.5% SDS polyacrylamide gels (16).

Western blot analysis: Following electrophoresis, samples were electro-transferred onto nitrocellulose membranes (Schleicher and Schuell). The blots were blocked with PBS/0.15% casein for overnight at 4°C. They were incubated at room temperature for one hour with the following antibodies: D3A2G5, HNK-1, L2 (rat IgG) (2), anti-fibronectin antiserum (Accurate Chemical & Scientific Co.) and sera from patients with paraproteinemic peripheral neuropathy and monoclonal IgM anti-MAG antibodies. The primary antibodies were detected by goat anti-mouse IgG, goat anti-mouse IgM, goat anti-rat IgG or goat anti-human IgM horseradish peroxidase labeled secondary antibodies as required. The bands were visualized by ECL western blotting system (Amersham Life Science). The ECL films were scanned at 400 dpi with a Cannon CLC 10 and processed using the Adobe Photoshop Version 2.5.1 software.

Results

Transfection and immunostaining of transfected Melur cells: Untransfected Melur cells treated with 0.4 mg/ml of G418 were killed to over 95% within three weeks and no G418 resistant cells were observed in long term cultures. Three weeks following transfection with expression vectors for L-MAG only a minority (12%) of the Melur cells under G418 selection expressed readily detectable MAG cell surface fluorescence (Fig. 1). Similar results were obtained with expression vectors under the control of either RSV or CMV transcriptional enhancers and in the following only the results obtained with RSV constructs are shown. The cell surface fluorescence observed with the HNK-1 antibody also represented a considerable spectrum from weakly stained to very bright cells (Fig. 1). The transfected population was therefore subjected to 2 color FACS sorting to select for cells expressing high levels of both MAG and HNK-1 (Fig. 1). The phenotype of this double positive population was maintained following expansion of the original $5x10^5$ cells even after repeated

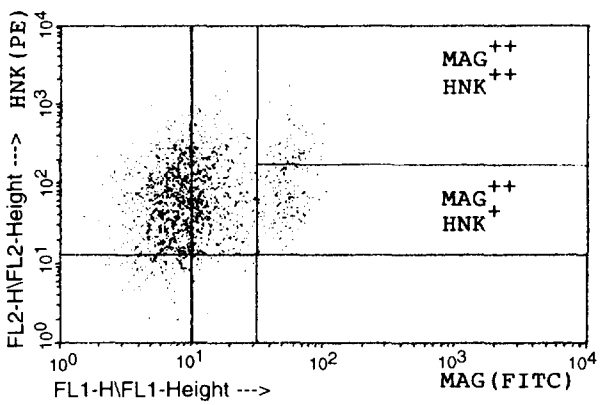


Figure 1. Fluorescence profiles of Melur cells transfected with MAG. Two color fluorescence sorting was used to select for transfectants expressing high levels of both HNK-1 and MAG. The fluorescein isothiocyanate (FITC)/MAG fluorescence and the phycoerythrin (PE)/HNK-1 fluorescence were gated as indicated to select $5x10^5$ MAG^{++} HNK^{++} cells. Background staining with second layer antibodies alone was set to less than 10^1 relative units.

subculturing (data not shown). The MAG transfected cells were also characterized by immunostaining using D3A2G5 (Fig. 2a) and HNK-1 (Fig. 2c) monoclonal antibodies.

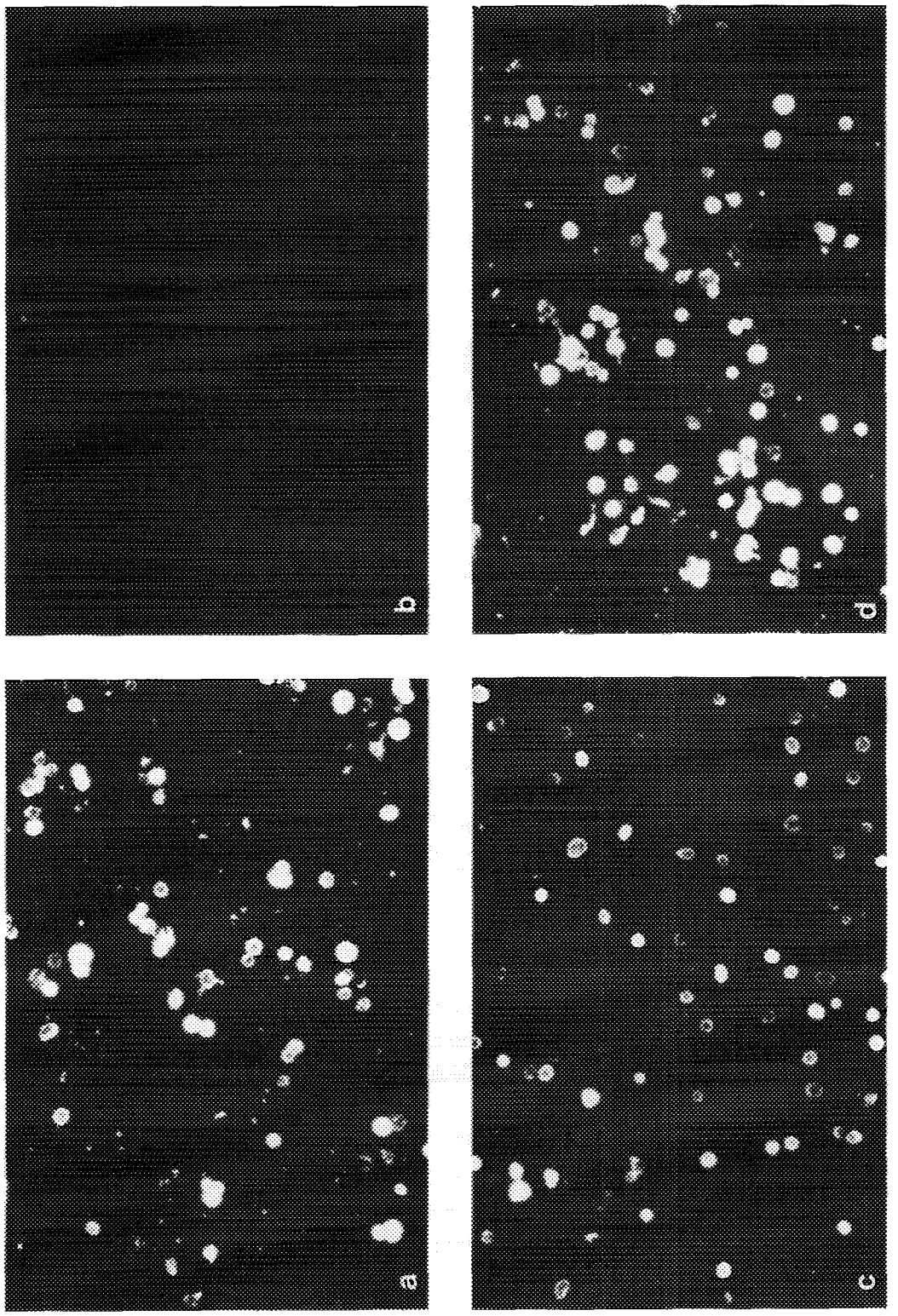
Immunoprecipitation and Western blot analysis: The stably transfected cell population selected for high levels of MAG and HNK-1 expression was used for immunoprecipitation and Western blot analysis. Human brain myelin and nontransfected cell lysates were used as positive and negative controls. The recombinant MAG immunoprecipitated with the D3A2G5 antibody (directed against the MAG polypeptide) is resolved on the blots by the D3A2G5 monoclonal antibody as two bands of approximately 90 kD and 100 kD mass as well as a faint band of approximately 80 kD (Fig. 3a). Predominantly the top band is also recognized by the L2 antibody and no signal was detectable with the HNK-1 monoclonal antibody or by human anti-MAG autoantibodies (Fig. 3b-d). Melur cells have been reported to produce HNK-1 positive fibronectin in particular under serum-free conditions. To characterize the HNK-1 type carbohydrates produced by Melur cells, lysates of cells grown in medium without serum were immunoprecipitated by a rabbit anti-fibronectin antibody and revealed with either HNK-1 or L2 antibodies. Under serum free conditions, the fibronectin in the Melur cells are both L2 positive (Fig. 3b) and HNK-1 positive (Fig. 3c), while recombinant MAG still reacted with the L2 and not with the HNK-1 antibody (data not shown).

Discussion

HNK-1/L2 determinants are found on numerous neural recognition and cell adhesion molecules. They are subject to considerable developmental and cell-type specific regulation (9). While antibody recognition of these carbohydrates in different species is variable it has generally been assumed that the determinants detected by both antibodies are co-localized on the same molecules (17, 18). The Melur cell line which has been used in this study produces different HNK-1/L2 positive proteins including fibronectin (13, 19). We now demonstrate that MAG produced by transfected Melur cells is reactive with the L2 monoclonal antibody. In murine Schwann cells L2 positive MAG may represent a functionally distinct form of MAG. Recently MAG carrying L2 determinants was found to be selectively expressed by Schwann cells ensheathing motor axons and suggested to be a selective guidance molecule for regenerating motor axons (20).

Surprisingly MAG expressed by transfected Melur cells was not reactive with the HNK-1 monoclonal antibody in spite of the production by these cells of HNK-1

Figure 2. Immunostaining of untransfected and transfected Melur cells using D3A2G5 (a and b) and HNK-1 (c and d) monoclonal antibodies. FACS sorted double positive MAG and HNK-1 expressing Melur cells were analyzed following five passages of continuous subculturing. Only the transfected cells (a) but not the untransfected controls (b) show cell surface fluorescence when stained with the D3A2G5 anti-MAG polypeptide antibody. The staining with the HNK-1 antibody produces a similar signal on transfected (c) and untransfected cells (d).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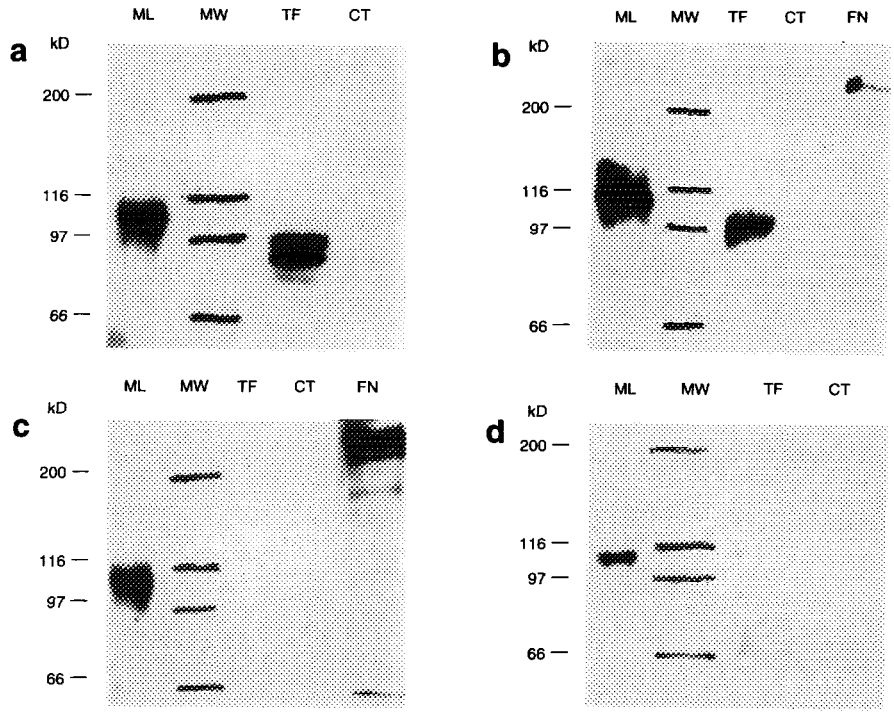

Figure 3. Western blot analysis of proteins immunoprecipitated by D3A2G5 anti-MAG antibody. Lane ML: human myelin prepared from human brain, Lane TF: cell lysate from MAG transfectants, Lane CT: cell lysate from non-transfected controls, Lane FN: cell lysate from MAG transfectants immunoprecipitated with a rabbit anti-fibronectin antiserum. a) The immunoprecipitated MAG protein was detected by Western blot analysis using the anti-MAG polypeptide antibody D3A2G5. b) The immunoprecipitated MAG protein was reacted with the L2 anti-carbohydrate monoclonal antibody. The immunoprecipitated fibronectin is L2 positive. c) The L2 positive MAG from myelin but not from MAG transfected Melur cells is shown to react with the HNK-1 monoclonal antibody. The immunoprecipitated fibronectin is HNK-1 positive. d) Detection of MAG with a representative serum of a patient with paraproteinemic neuropathy and IgM anti-MAG antibodies. All four sera tested reacted with the MAG from human myelin but not from transfected Melur cells.

reactive proteins. It has been assumed previously that the determinants recognized by the L2 and HNK-1 antibodies were co-localized on the same molecules in humans and other HNK-1 positive species. Now it will be necessary to establish if the differential glycosylation in terms of L2 and HNK-1 determinants as seen in Melur cells might be of functional significance, for example in regenerating tissues.

The identification of a MAG molecule binding to the L2 but not the HNK-1 antibody can be accommodated with recent information on the structural requirements for L2 and HNK-1 binding. Using synthetic carbohydrates it was shown that the tetrasaccharide to which the glucuronic acid is attached to is required for L2 binding while it is not critical for HNK-1 recognition (21). The latter requires sulfation of the glucuronic acid for optimal binding. The absence of sulfation could therefore be

responsible for the failure of the recombinant MAG to react with the HNK-1 antibody. Conceivably, the sulfotransferase activity could be rate limiting in Melur cells. Another possibility is that differential intracellular routing and processing could result in the secretion of HNK-1 positive fibronectin while only L2 positive MAG is produced.

The binding specificities of the HNK-1 and the human anti-MAG antibodies also differ somewhat as evidenced by the preferential recognition of MAG by antibodies from paraproteinemic peripheral neuropathy while the HNK-1 antibody gives a selectively stronger reaction with P_0 (14). In fact some human anti-MAG antibodies bind independently of the presence of sulfate but require a free carboxylic group instead (22, 23). The lack of binding of human anti-MAG autoantibodies to the recombinant MAG implies that there may be other differences than sulfate between the carbohydrate structures of the MAG expressed by Melur cells and the MAG in human myelin.

After attachment to protein, the N-linked oligosaccharide undergoes a complex series of trimming and elongation reactions leading to the final elaborate structures present on the mature molecule (24). The glycosylation pattern could be cell-type specific and may also be dependent on the stage of differentiation of the cells. The post-translational modifications on the MAG produced in the transfected Melur cells result in two predominant glycoprotein species of approximately 90 kD and 100 kD, i.e. with a distinctly higher banding than the 70 kD predicted for the unglycosylated peptide but lower than the MAG in myelin. Interestingly, most of the L2 labelling was on the 100 kD and not on the 90 kD MAG in Melur cells. The enzymatic specificity and catalytic kinetics of glucuronyl transferase and sulfotransferase, intracellular routing and cellular environment contribute to the regulation of glycosylation. There is, moreover, little information about the mechanisms controlling site-specific glycosylation and oligosaccharide transferase action.

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