Developmental Alterations in Molecular Weights of Proteins in the Human Central Nervous System That React With Antibodies Against Myelin-Associated Glycoprotein

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ABSTRACT By the use of a rat IgG monoclonal antibody (mab), a mouse mab and human serum containing an IgM mab, all of which react with isolated human myelin-associated glycoprotein (MAG) on immunoblots and bind only to proteins with relative mobilities identical to MAG and dMAG on immunoblots of homogenates of adult human spinal cord, we demonstrated the following: in homogenates of central nervous system tissue from human fetuses of gestational ages that antedate myelination, the anti-MAG antibodies react only with proteins with molecular weights of 250,000 or larger. During myelination the molecular weights of proteins with which the anti-MAG antibodies react shift towards the lower molecular weights found in adult myelin. Amongst those central nervous system regions examined, the shift towards the low molecular weights occurred earliest in the region that is first to become myelinated and latest in the one that is the last to myelinate. Once myelination is completed, the antibodies react only with proteins with relative mobilities identical to those of MAG and dMAG. These developmental changes in molecular weights of "MAG-related proteins" may prove useful as an index of chemical processes on the basis of which myelination occurs.

Myelin-associated glycoprotein (MAG)[1] is an acidic, concanavalin A binding glycoprotein that has been shown to be a minor component of both central (1) and peripheral myelin (2). Immunohistochemically it has been demonstrated in the inner and outer mesaxons and the Schmidt-Lanterman incisure of peripheral myelin sheaths (3) and in the periaxonal region of central myelin sheaths. MAG has also been found in the cytoplasm of oligodendrocytes prior to and during myelination (4). However, after myelination has been completed MAG is no longer detectable in oligodendrocytes. Interest in MAG was recently sparked when it was demonstrated that a group of patients with IgM monoclonal gammopathy and demyelinating neuropathy have their monoclonal IgMs directed against MAG (5, 6), and that when these IgMs are injected into the peripheral nerves of cats they cause demyelination (7). It is also of medical interest that in acute lesions of multiple sclerosis a decrease in immunohistochemically detectable MAG reaches well beyond the area of demyelination (8). Immunohistochemical staining for MAG therefore appears to provide a sensitive marker of myelin damage in multiple sclerosis.

MAG isolated from the central nervous system (CNS) myelin of rats or humans has a molecular weight of ~100,000 (9). Peripheral nervous system (PNS) MAG has a slightly greater molecular weight than does CNS MAG but peptide maps of PNS and CNS MAG are identical (2). In human myelin there is a neutral protease that breaks down MAG into dMAG which has a molecular weight of ~90,000 (10). During myelination in the rat CNS it has been shown that the molecular weight of MAG is greater than it is in adult myelin but the difference is small (11–13).

In this paper we describe a developmental alteration in molecular weights of human CNS proteins that reacts with antibodies to MAG. At early stages the antibodies react only with proteins with molecular weights over 250,000; in adult myelin they react only with proteins with molecular weights of 100,000 and 90,000. There are also intermediate forms that are present during various stages of myelination. The

[1] *Abbreviations used in this paper:* CNS, central nervous system; mab, monoclonal antibody; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; PNS, peripheral nervous system.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 NOVEMBER 1984 1642–1646

transition from high to low molecular weights occurs first in the parts of the CNS that become myelinated earliest.

MATERIALS AND METHODS

Monoclonal Antibodies to MAG: In attempts to obtain mabs specific for membrane-bound nicotinic acetylcholine receptor, Lewis rats were immunized with receptor isolated from the electric organ of *Torpedo californica* (14). Splenic lymphocytes from immune rats were fused with P2/0-Ag14 mouse cells using the method of Köhler and Milstein (15). Hybridoma supernatants were subsequently screened in an enzyme-linked immunosorbent assay (16) against a membrane preparation from the electric organ that contained acetylcholine receptor and was later shown to be contaminated by myelin. The electric organ of *Torpedo californica* is heavily innervated by myelinated axons. The hybridomas yielding positive supernatants were cloned by limiting dilutions and then recloned several times. Certain clones secreted mabs that on immunoblots of the receptor containing membrane preparation failed to bind to any component of nicotinic acetylcholine receptor but did bind to a protein with higher molecular weight. This protein was later shown to be a myelin protein. One of these mabs, an IgG termed $BRIC_{37}$, was selected for this study. $BRIC_{37}$ binds to isolated human MAG on immunoblots, and on immunoblots of homogenates from adult spinal cord $BRIC_{37}$ binds only to proteins with relative mobilities identical to those of MAG and dMAG (Fig. 1). $BRIC_{37}$ does not bind to anything on immunoblots of homogenates from liver, kidney, or striated muscle. The immunohistochemical distribution of the antigenic determinant with which $BRIC_{37}$ reacts is identical to that of MAG as defined with polyclonal antisera (3, 4).

A mouse mab (HNK_1, Becton-Dickinson, Mountain View, CA) that was originally raised against human natural killer cells but has subsequently been shown to react with MAG (17, 18) was also used. As control mabs we used several rat IgG mabs against the nicotinic acetylcholine receptor (generously provided by D. P. Richman, Department of Neurology, University of Chicago) and the mouse mabs anti-Leu 2a and anti-Leu 3a (Becton-Dickinson).

Human Serum Containing IgM Against MAG: We also used serum from a patient (M) with IgMλ monoclonal gammopathy and demyelinating neuropathy. IgM in this serum binds to isolated human MAG on immunoblots and on immunoblots of homogenates of adult human spinal cord binds only to proteins with relative mobilities identical to those of MAG and dMAG (Fig. 1) (6). Antibodies from the serum of patient M do not bind to anything on immunoblots of homogenates from liver, kidney, or striated muscle. As controls we used sera from two patients with IgM monoclonal gammopathy unaccompanied by neuropathy.

Attempts to Demonstrate Binding of $BRIC_{37}$ and IgM from the Serum of Patient M to Gangliosides Isolated from Human Peripheral Nerves and Human CNS: Gangliosides were isolated from fresh human sciatic nerves and human brains obtained at autopsy using a previously described method (19). The gangliosides were then separated by thin-layer chromatography (TLC) using aluminum-backed TLC plates (Silicagel 60: Merck, Darmstadt, Federal Republic of Germany). The plates were first developed in chloroform-methanol-0.2% $CaCl_2$ in H_2O (55-45-10, by volume), then dried and redeveloped, this time in chloroform-methanol-0.2% KCl in 2.5 M NH_4OH (50-40-10, by volume). Subsequently the plates were dried and cut into 1-cm wide strips that were placed individually into test tubes. The demonstration of binding of antibodies to the gangliosides on the plates was performed in the same manner as is described for binding of antibodies to proteins on nitrocellulose strips (see under Electrophoresis and Immunoblotting). The IgM from the serum of patient M bound to a ganglioside from peripheral nerve (Fig. 2); it did not bind to anything in the CNS-ganglioside preparation. This is in keeping with what Ilyas et al. (20) have recently described using sera from three patients with paraproteinemia and peripheral neuropathy. $BRIC_{37}$ did neither bind to the peripheral nerve gangliosides (Fig. 2) nor the CNS gangliosides.

Peptide Mapping to Demonstrate that $BRIC_{37}$ and IgM from the Serum of Patient M Probably Bind to Different Determinants: The method used is a variation of a method described by Cleveland et al. (21). In short, a piece of human white matter was homogenized, lyophilized, and delipidated by three exposures to chloroform:methanol (2:1, by volume). Subsequently the material was solubilized in 1% SDS-0.125 M Tris HCl at pH 6.8 and then boiled to inhibit endogenous enzymes. The following enzymes were used for peptide mapping: (*a*) trypsin; (*b*) protease 18; (*c*) chymotrypsin; and (*d*) ficin (all from Sigma Chemical Co., St. Louis, MO). The digestions were carried out at 37°C and various ratios of enzyme to white matter were used. The reactions were stopped by adding SDS and 2-mercaptoethanol and by boiling. The reaction products were separated by SDS polyacrylamide (10%) slab gel electrophoresis using the system of Laemmli (22) and then transferred onto nitrocellulose. The nitrocellulose was then stained

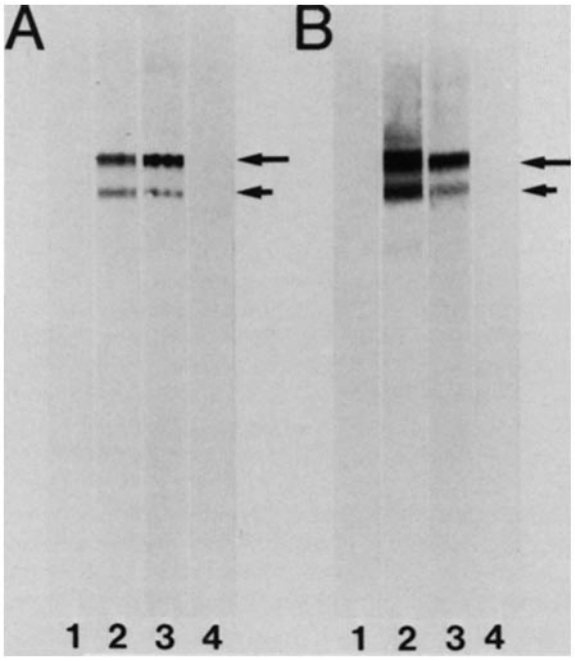

FIGURE 1 Binding of $BRIC_{37}$ and IgM from patient's serum to MAG on Western blots. Western blots of isolated human MAG (*A*) and of homogenates of adult human spinal cord (*B*) were stained using: (*1*) a control rat mab; (*2*) $BRIC_{37}$; (*3*) serum from patient M; and (*4*) a control human serum. Both $BRIC_{37}$ and IgM from patient M bind to isolated MAG (long arrow) and dMAG (short arrow), which is a breakdown product of MAG. On the spinal cord blots both sera identified MAG and dMAG and no other proteins.

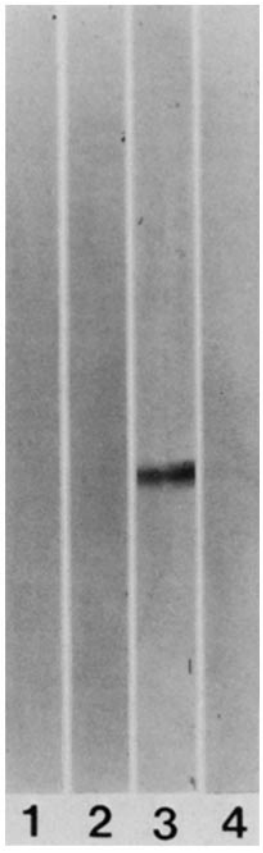

FIGURE 2 The figure shows strips of aluminum-backed TLC plates containing gangliosides from human peripheral nerve. The strips were stained with (*1*) $BRIC_{37}$; (*2*) control rat mab; (*3*) IgM from the serum of patient M; and (*4*) with IgM from control serum. IgM from the serum of patient M binds to a ganglioside that corresponds to the description of Ilyas et al. (20). The other antibodies bind to nothing in the ganglioside preparation.

either with $BRIC_{37}$ or IgM from the serum of patient M (see under Electrophoresis and Immunoblotting). Fig. 3 contains the results from one of these experiments and shows that there are substantial differences between the fragments recognized by $BRIC_{37}$ on one hand and IgM from the serum of patient M on the other.

Tissue Samples and Antigens: Fresh brains and spinal cords were obtained at autopsy from human fetuses at 31 and 37 wk of gestation and from postnatal humans at 7 mo and 30 y of age. Tissue was sampled from thoracic spinal cord, cerebellum, and frontal lobe. The tissue was either homogenized and solubilized directly or homogenized, lyophilized, and delipidated by three exposures to chloroform/methanol 2:1 (vol/vol), and subsequently solubilized. There were no differences between the results obtained by these two methods. Isolated human MAG was a generous gift from Dr. R. H. Quarles, (National Institutes of Health). Samples were solubilized in a solution containing 5.7 M urea, 1% wt/vol SDS, and 1% vol/vol 2-mercaptoethanol. Solubilized samples were heated at 100°C for 3 min.

Electrophoresis and Immunoblotting: The solubilized tissue samples and isolated MAG were separated with SDS polyacrylamide (5.6%) gel electrophoresis using the system of Fairbanks et al. (23). The gels were either stained with Coomassie Blue (23), or unstained gels were overlaid with nitrocellulose sheets and the protein bands were transferred electrically onto the nitrocellulose by use of a Bio-Rad Transblot TM cell (Bio-Rad Laboratories, Richmond, CA) at 60 V for 3 h in 20 mM Tris HCl buffer at pH 7.5 with 20% methanol. The nitrocellulose sheets were cut into strips, and the strips were placed individually into capped test tubes. The protein binding capacity of the nitrocellulose was saturated by overnight incubation with 5% BSA in 0.145 M NaCl-0.05 M Tris HCl buffer at pH 7.6 at room temperature. This was followed by a 20-min equilibration with 0.145 M NaCl-0.05 M Tris-HCl buffer at pH 7.6 containing 0.05% vol/vol Nonidet P-40 (Particle Data Laboratories, Elmhurst, IL), and 5% vol/vol normal goat serum. This medium was used for all subsequent washings and for diluting all antibodies and sera used in the staining procedure. The strips were next incubated for 1 h with $BRIC_{37}$, HNK_1, one of the control mabs, serum from patient M diluted 1:200, or one of the control sera diluted 1:200. Subsequently the strips were washed three times for 15 min. This was followed by a 1-h incubation with peroxidase-labeled rabbit antibodies against rat immunoglobulins, mouse immunoglobulins, or human IgM (Dako Corp., Santa Barbara, CA). Again the strips were washed three times. Location of peroxidase activity was revealed by placing the strips in 0.145 M NaCl-0.05 M-Tris-HCl buffer at pH 7.6 containing 0.01% vol/vol H_2O_2 and 0.5% wt/vol diaminobenzidine tetrahydrochloride.

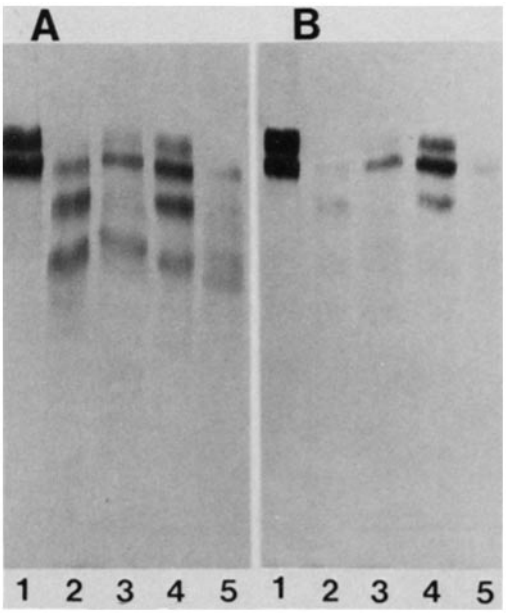

FIGURE 3 Peptide mapping of the proteins (MAG and dMAG) in adult human white matter that react with $BRIC_{37}$ and IgM from the serum of patient M. *A* and *B* are nitrocellulose sheets that contain electrophoresed proteins from (*1*) undigested white matter; (*2*) trypsin digested WM; (*3*) protease 18 digested WM; (*4*) chymotrypsin digested white matter; and (*5*) ficin digested white matter. *A* was stained with $BRIC_{37}$ and *B* was stained with IgM from the serum of patient M.

Immunohistochemical Staining with $BRIC_{37}$ and IgM from the Serum of Patient M: 6-μm sections of formalin-fixed paraffin embedded blocks from the same central nervous system material used for the immunoblots were stained with $BRIC_{37}$ using both the peroxidase antiperoxidase method of Sternberger (24) and a biotin avidin peroxidase technique (25). Adjacent sections were stained with patient M's serum using a biotin avidin peroxidase technique. Rat peroxidase antiperoxidase complex was obtained from Sternberger-Meyer Immunocytochemicals (Jarettesville, MD) and reagents for the biotin avidin peroxidase procedure, from Vector Laboratories, Inc. (Burlingame, CA).

RESULTS

The results of immunohistochemical staining obtained with $BRIC_{37}$ are identical to the results obtained through the use of serum from patient M and correspond to the previously described distribution of MAG (4). On immunoblots $BRIC_{37}$, IgM from patient M (Fig. 1), and HNK_1 (data not shown) bind to isolated human MAG and on immunoblots from homogenates of adult human spinal cord they bind exclusively to MAG and dMAG.

Immunoblots

When the three anti-MAG antibodies are applied to human CNS tissue at various stages of development, a surprising variety of proteins are found to react with the antibodies. On blots of the frontal lobe homogenate from a 31-wk-old fetus, the antibodies bind to a group of at least two very high molecular weight proteins, the smaller one with molecular weight of ~250,000 (Fig. 4*a*). In the 37-wk-old frontal lobe, in addition to the higher molecular weight proteins recognized in the 31-wk-old, two new major proteins appear, one with a molecular weight of ~200,000 and the other ~130,000. By 7 mo postnatally the 200- and the 130-kd proteins have become more prominent. In the adult frontal lobe the antibodies recognize only 100- (MAG) and 90-kd (dMAG) proteins.

In the homogenate of cerebellum from the 31-wk-old fetus, the antibodies elicit a banding pattern quite similar to the one in frontal lobe of the same age (Fig. 4*b*), but the shift towards the low molecular weight occurs at an earlier age in cerebellum than in frontal lobe.

In the spinal cord homogenates at 31 wk, in addition to the very high molecular weight proteins that were the only "MAG-related" proteins detected in the frontal lobe at 31 wk (Fig. 4*c*), the 100- and 90-kd proteins as well as the intermediate 200-kd protein have appeared. At 7 mo postnatally, the group of heaviest bands has disappeared from the spinal cord (Fig. 4*c*). In the adult spinal cord the antibodies bind to the same two bands as they do in the adult frontal lobe (100- and 90-kd) which co-migrate with isolated human MAG and dMAG (4*c*).

Examination of the results of staining the three CNS regions from the 37-wk-old fetus demonstrates that the shift towards the lower molecular weights occurs first in the areas that are myelinated earliest; it is seen first in the spinal cord, then in the cerebellum, and finally in the frontal lobe (Fig. 4*d*).

On the immunoblots there are some differences in the relative intensities of the bands elicited by $BRIC_{37}$ as opposed to the IgM from the serum of patient M. This is especially evident in Fig. 4*d*. Antibodies from the serum of patient M detect the heaviest MAG-related protein more readily than does $BRIC_{37}$ while $BRIC_{37}$ detects the 200-kd antigen more readily than do the antibodies from patient M. This variation probably reflects that the two antibodies differ in their epitope

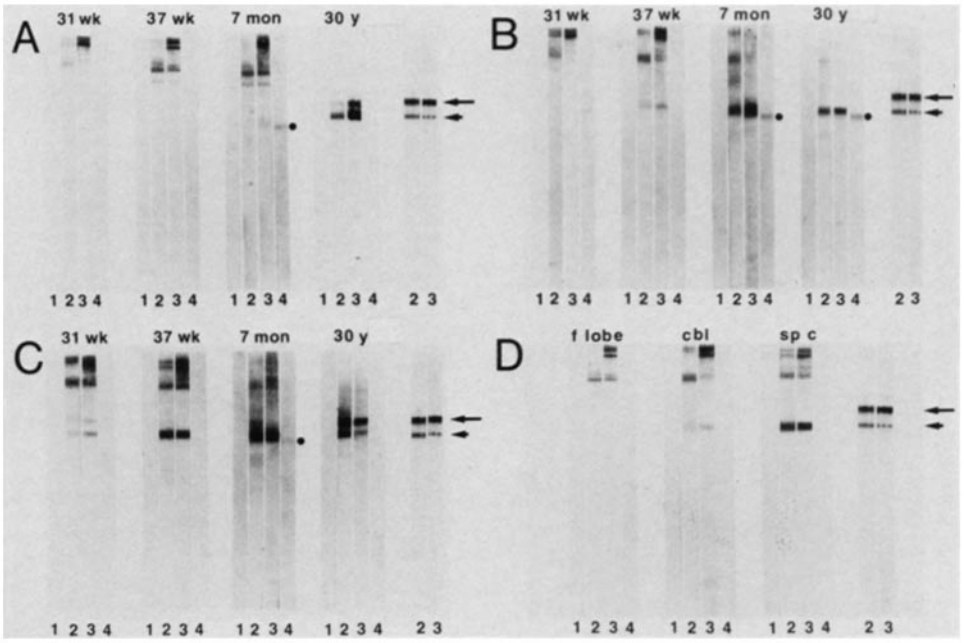

FIGURE 4 Binding of $BRIC_{37}$ and IgM from the serum of patient M to high molecular weight components of developing CNS tissue. Homogenates of frontal lobe (*A*), cerebellum (*B*), and spinal cord (C) were prepared from humans aged 31-wk gestation, 37-wk gestation, 7 mo postnatally and 30 y of age. The samples were run on SDS polyacrylamide gels and transferred to nitrocellulose. In *D*, samples of each of the three CNS regions from the 37-wk fetus are compared. The strips were stained using (*1*) control rat mab; (*2*) $BRIC_{37}$; (*3*) serum from patient M; and (*4*) control human serum followed by peroxidase-conjugated anti-rat immunoglobulin (*1* and *2*) or anti-human IgM (*3* and *4*). Western blots of isolated MAG are included at the right end of each panel to show the mobility of isolated adult MAG (long arrow) and dMAG (short arrows). In each tissue (*A*–C) there is a decrease with age in the apparent molecular weight of proteins recognized by the anti-MAG antibodies. In the 37-wk fetus (*D*) this decrease is most pronounced in the spinal cord and least in the frontal lobe which is in keeping with the stage of myelination in these areas. In *D* it can be seen that the IgM from patient M is more sensitive than $BRIC_{37}$ in detecting the >250 kd MAG-related proteins while the rat antibody is more sensitive for the 200-kd protein. The heavy chain of IgM migrates on these gels just ahead of dMAG. Endogenous IgM was detected in some of the tissue blots treated with peroxidase-conjugated anti-human IgM and is indicated with a dot. It is also noteworthy that the ratio of MAG to dMAG varies from one sample to another. In most of the fetal tissues dMAG predominates.

specificity. HNK_1 binds to the same proteins in the fetal material as do $BRIC_{37}$ and IgM from the serum of patient M. The relative intensity of the bands stained by HNK_1, is in between the patterns of $BRIC_{37}$ and IgM from the serum of patient M (data not shown). Fig. 5 shows Coomassie Blue-stained SDS polyacrylamide gels of the tissue samples used on the immunoblots.

Immunohistochemistry

Both $BRIC_{37}$ and IgM from patient M's serum stain only the periaxonal portion of myelin sheaths in adult CNS but just prior to and during myelination they also stain oligodendrocytic cytoplasm. This immunohistochemical pattern is the same as that obtained by use of polyclonal antiserum against MAG (4). However, thus far we have not been able to correlate the immunohistochemical staining of myelin sheaths versus oligodendrocytic cytoplasm with the molecular weight patterns on the immunoblots.

DISCUSSION

We have demonstrated that anti-MAG antibodies react with proteins in homogenates of CNS tissue from young human fetuses that have molecular weights much larger than the MAG found in adult myelin. During myelination the high molecular weight proteins disappear and lower molecular weight proteins reactive with the anti-MAG antibodies appear. When myelination is completed, the antibodies bind only to proteins with molecular weights identical to MAG and dMAG (Fig. 4). We have also shown that this change from high to low molecular weights occurs first in the spinal cord where myelination proceeds faster than in cerebellum and cerebrum and latest in the cerebrum where myelination is completed after the other two regions (26) (Fig. 4). We have therefore shown both by looking at the CNS from individuals at various stages of development and by looking at three regions from the CNS of each of these individuals that the shift from high to low molecular weights amongst proteins reactive with anti-MAG antibodies appears to follow myelination.

It is possible that MAG and the high molecular weight proteins reactive with the anti-MAG antibodies are unrelated except for that they react with the antibodies used in this study. However this has to be considered improbable since $BRIC_{37}$ and the mabs from patient M's serum react with different epitopes on MAG and the high molecular weight proteins and MAG are developmentally reciprocal (MAG appears when the high molecular weight proteins disappear). HNK_1 may react with a third determinant since neither $BRIC_{37}$ nor the patient's IgM react with human natural killer cells but HNK_1 does. The failure to stain natural killer cells with the IgM paraprotein and $BRIC_{37}$ may however be due to low avidity of these antibodies rather than absence of the complementary antigenic determinants from the lymphocytes and therefore does not prove that HNK_1 reacts with an epitope different from $BRIC_{37}$ or the IgM paraprotein.

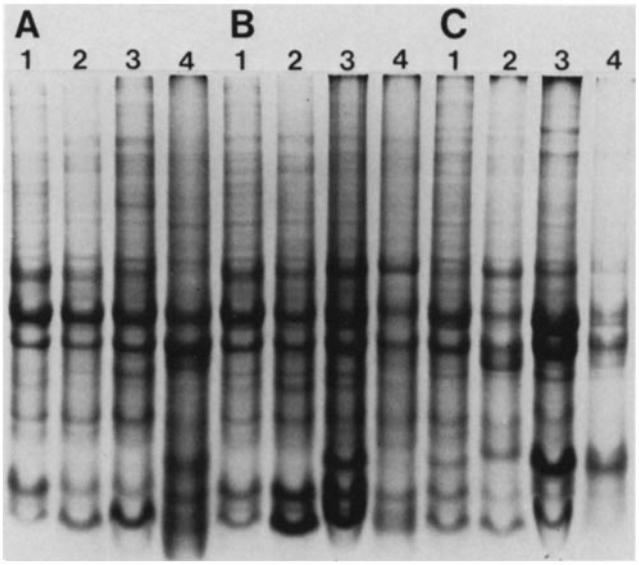

FIGURE 5 Coomassie Blue-stained SDS polyacrylamide gels of the tissue homogenates used in Fig. 4 show the total protein composition of the samples. Frontal lobe (*A*); cerebellum (*B*); and spinal cord (C): (*1*) 31-wk gestation; (*2*) 37-wk gestation; (*3*) 7-mo postnatally; and (*4*) 30 y of age.

This developmental change in molecular weights of "MAG-related proteins" is reminiscent of developmental changes that have been reported to occur in the molecular weights of myelin basic protein (MBP) or MBP-related proteins in mouse brain (27, 28). On immunoblots of homogenates of cerebrum from newborn mice, antibodies specific for MBP react only with a protein with a molecular weight of ~34,000, but as myelination proceeds, the reactivity becomes gradually confined to the four main peptides of the MBP family (21.5, 18.5, 17, and 14.0 kd). It has also been shown that the difference between molecular weights of proteins reactive with anti-MBP antibodies at various stages of development are probably not due to post-translational events (29).

Currently we have no way of predicting whether the difference between MAG and the high molecular weight proteins reactive with anti-MAG antibodies will be explained by different genes, age-dependent differences in gene expression, difference in translatability of mRNA, post-translational modification, or some other mechanism. Irrespective of how the differences between the "mature MAG" and the high molecular weight proteins reactive with the anti-MAG antibodies will be explained, the transient existence of these high molecular weight proteins during development may provide a way to monitor some of the events on the basis of which myelination and remyelination occur.

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