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The M_2 autoantigen of central nervous system myelin, a glycoprotein present in oligodendrocyte membrane

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SUMMARY

Autoantibodies with in-vitro demyelinating capacity induced in Hartley and strain 13 guinea pigs with homologous central nervous system (CNS) tissue were used to characterize the target autoantigen M_2. Using the Dot Immunobinding technique, M_2 was found to be a component of CNS myelin different from basic protein (BP) and from cerebroside. The expression of M_2 on oligodendrocytes, cells known to produce CNS myelin, also confirmed that M_2 was a component of CNS myelin. Furthermore, the autoradiography of immunoprecipitates formed with radiolabelled guinea pig myelin and analysed in sodium dodecyl sulphate gels showed that M_2 was specific to CNS myelin and absent in peripheral nervous system (PNS) myelin. On electrophoresis M_2 appeared as two CNS myelin protein bands at the 27 and 54 KD molecular weight levels, distinct from the major protein bands of proteolipid and BP. M_2 bands were of glycoprotein nature, as was demonstrated by affinity chromatography of CNS myelin on wheat germ agglutinin (WGA)-Sepharose. A monoclonal antibody induced by BP-free CNS glycoproteins recognized the same bands as anti-M_2 serum in guinea pig CNS myelin. This would imply that both M_2 bands share common determinants. M_2 bands similar to the above in guinea pig were also shown in rat, rabbit and bovine CNS myelin with guinea pig antibodies. The same type of anti-M_2 antibodies were induced in rabbit immunized with homologous CNS tissue. Although only a minor component of myelin, M_2 is strongly immunogenic compared to BP. M_2 antigen could thus be the target of chronic demyelinating processes such as experimental allergic encephalomyelitis.

Keywords Experimental allergic encephalomyelitis oligodendrocytes myelin basic protein M 2

INTRODUCTION

The antigen responsible for the induction of in-vivo acute experimental allergic encephalomyelitis (EAE), an autoimmune disease, has been shown to be the basic protein (BP) of central nervous system (CNS) myelin. In-vitro demyelinating activity has also been detected in sera from animals with whole CNS tissue-induced EAE (Seil *et al.*, 1968). In the guinea pig, this activity is due to autoantibodies to an autoantigen of myelin which is neither the BP nor a galactocerebroside, and which has been designated M_2 (Lebar *et al.*, 1976; Lebar & Lees, 1985). Moreover, the chronic form of EAE induced by whole CNS tissue or myelin in young guinea pigs with widespread in-vivo CNS

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demyelination, cannot be induced by BP alone. Treatments which eliminated the M_2 antigen activity suppressed or lowered the chronic EAE-inducing potency of CNS tissue and myelin (Lebar & Vincent, 1981a). Therefore, M_2 could play an essential role in guinea pig chronic EAE induction. It would thus seem important to identify this component. In the present work, we show that M_2 is one or more glycoprotein(s) of CNS myelin present on the oligodendrocyte membrane.

MATERIALS AND METHODS

Antigen

CNS myelin was prepared from guinea pig, rat and rabbit brains and from bovine spinal cord as previously described (Reiss, Lees & Sapirstein, 1981); it was stored at −80°C. Guinea pig PNS myelin was prepared essentially as described (Agrawal, Schmidt & Agrawal, 1983): sciatic nerves homogenized in a loosely fitting glass Potter with 0·29 M sucrose released in a fine suspension decanted from the denuded axons. This suspension (referred to as crude peripheral nervous system (PNS) myelin) was the starting material for myelin purification. The purified myelin (referred to as PNS myelin) was stored frozen at −80°C. Myelin concentration was expressed as protein content (Lees & Paxman, 1972).

Myelin basic protein (BP) was prepared from guinea pig brains as described (Lebar, Vincent & Fischer-Le Boubennec, 1979). It was stored freeze-dried and quantified on the basis of its dry weight.

Proteolipid from bovine spinal cord was a generous gift from Dr C. Nicot. Its concentration was expressed as protein content calculated from optical density measurements at 280 nm, assuming an extinction coefficient ($E^{1\%}_{cm}$) of 13·5 for proteolipid protein (Nicot *et al.*, 1973).

Bovine galactocerebroside and bovine gammaglobulin (BGG) were products from Sigma Chemical Corporation. They were quantified on the basis of their dry weight.

Immune sera

Induced in Hartley guinea pigs. Antiserum to M_2 was prepared by immunization of Hartley and strain 13 guinea pigs with homologous or isologous CNS tissues and complete Freund's adjuvant (CFA) as described (Lebar & Lees, 1985). Bleedings were tested by Dot immunobinding assay (see below) with BP and myelin as antigens and were pooled; there is a weak anti-BP activity in these sera and to ensure its complete removal the pooled antiserum was incubated with BP-Sepharose 4 B prepared from cyanogen bromide-activated Sepharose (Pharmacia).

Antisera to guinea pig PNS myelin were induced by intradermal injection: (a) in the nuchal area with an emulsion containing 3 mg of lyophilized crude PNS myelin and 0·6 mg of *Mycobacteria tuberculosis*. Six days later, 6 mg of crude PNS myelin in incomplete Freund's adjuvant (IFA) was administered in hind foot pads; (b) at multiple sites with an emulsion containing 0·6 mg (protein weight) of PNS myelin and 1 mg of *Mycobacterium tuberculosis*.

Antiserum to guinea pig BP was a pool of individual sera obtained as described (Lebar *et al.*, 1976). Antiserum to bovine gamma globulin (BGG) was a pool of sera from guinea pigs immunized in the hind foot pads with 20 μg BGG in complete Freund's adjuvant (CFA) followed 2 weeks later by a booster dose of 100 μg in IFA.

Induced in rabbits. Immune sera were prepared from two New Zealand white rabbits immunized by intradermal injection with homologous brain tissue in CFA. Animals were injected at multiple sites on the back with an emulsion containing 200 mg (wet weight) of brain homogenate and 4 mg of *Mycobacterium tuberculosis*. Ten days later 200 mg brain homogenate in IFA was given in the hind foot pads and the nuchal area. One control rabbit was injected with an emulsion of CFA alone.

8-18C5, a monoclonal antibody, was generously provided by Dr C. Linington (Linington, Webb & Woodhams, 1984). Immunoaffinity purified rabbit antibodies to galactocerebroside were kindly donated by Dr B. Zalc. Rabbit antiserum to glial fibrillary acidic protein was a generous gift from Dr C. Jaque.

Antibody detection and measurement

Galactocerebroside, BP, proteolipid and CNS myelin were the antigens dotted on nitrocellulose strips to test antibodies in immune sera, using a previously described dot immunoblotting assay (Lebar & Lees, 1985). Cerebroside was spotted as a soluble mixture in ethanol of galactocerebroside, phosphatidyl choline and cholesterol at a weight ratio of 1 : 1 : 18. BP was spotted as a solution in 10 mM Tris HCl buffered saline pH 7·4 (TBS); proteolipid solution in 2·5% sodium dodecyl sulphate (SDS) was diluted in water; myelin was suspended in TBS. Negative controls of specificity of antigen-antibody reaction were guinea pig antiserum to BGG or rabbit antiserum to CFA.

Demyelinating activity test

Rat embryo spinal cord taken on the 15th gestational day was cultivated in Leighton tubes as previously described (Hauw *et al.*, 1972; Hauw *et al.*, 1974). Briefly, two explants of hemisectioned spinal cord were layered on a collagen-coated coverslip in a Leighton tube. Serum-free medium (0·25 ml per tube) (J. M. Boutry *et al.*, unpublished) was the nutrient medium; it was changed twice a week. At 25 days *in vitro* the well-myelinated cultures were selected and used for demyelination experiments as described (Lebar *et al.*, 1976).

Antigen characterization: immunoprecipitation and autoradiography

Preparation of radiolabelled antigen. Purified myelin, 250 μg to 1 mg (protein weight), was suspended in 0·5 to 1 ml TBS in a test tube (12 × 70 mm) previously coated with 50 μg glycoluril (iodogen: Pierce Chemical Company). Two hundred and fifty to 500 μCi Na^{125} I were added. The mixture was allowed to stand 20 min at room temperature with occasional stirring. The radiolabelled myelin suspension was then washed twice with 20 volumes cold TBS containing 0·25 M KI plus 0·1% (w/v) BSA. The pellet of myelin was solubilized at 4°C in the dissolving buffer: TBS containing 1% (w/v) NP 40, 0·1% (w/v) BSA and protease inhibitors (2 to 5 mM EDTA, 2 mM phenyl methyl sulphone fluoride, 20 mM iodoacetamide and 10 iu/ml aprotinin), and centrifuged at 100,000 *g* for 20 min. The supernatant, counted for radioactivity (10 to 70×10^6 ct/min of trichloroacetic acid-TCA-precipitable activity per mg of myelin), was the antigen used for immunoprecipitation.

Preparation of antibody. In order to link IgG to protein A Sepharose immune sera were suspended in Protein A Sepharose Cl 4B (Pharmacia) gel (1 ml immune serum per ml of gel) at 4°C for 30 min. After centrifugation the pellet was washed twice with 10 mM Tris HCl buffer pH 7·4 containing 1% NP 40 and 0·5 M NaCl.

Immunoprecipitation. The washed pellet of IgG-Protein A Sepharose was suspended in the desired amount of radiolabelled solubilized myelin (usually 10 μl of IgG-Protein A-Sepharose for 10^6 to 10^7 ct/min of TCA-precipitable radioactivity), made to 0·5 M in Na Cl and allowed to stand 2 h at 4°C with gentle stirring in order to permit complex formation. Immune complexes linked to Protein A-Sepharose were washed with the following solutions: (a) TBS containing 1% nonidet P 40 (NP 40) and 0·5 M NaCl; (b) TBS containing 1% NP 40 and 0·1% SDS; (c) 10 mM Tris HCl buffer containing 0·1% NP 40. They were then eluted and dissociated in boiling SDS sample buffer for electrophoresis: 0·0625 M Tris HCl buffer pH 6·8 containing 2% (w/v) SDS, 10% (v/v) glycerol, and in some experiments 0·1 M dithiothreitol.

Electrophoretic analysis. Eluted complexes were run in SDS polyacrylamide (gradipore 10 to 22% Acrylamide) slab gels 1 or 1·5 mm thick according to Laemmli (1970). Gels were then stained with 0·04% (w/v) Coomassie Blue G-250 in 3·5% (w/v) perchloric acid as described (Reisner, Nemes & Bucholtz, 1975), destained in 41% methanol 7% acetic acid, and dried at 80°C under vacuum in a BioRad dryer. Autoradiography of dried gels was performed at −80°C with Kodak-Omat H-X ray film and Dupont intensifying screens (Quanta III). Molecular weights were calculated according to Poduslo & Rodbard (1980) by reference to the mobilities of low molecular weight standard proteins (Sigma or Pharmacia kits). Other standards were used including guinea pig CNS myelin, guinea pig BP, bovine proteolipid.

Affinity chromatography on WGA-Sepharose

In order to isolate glycoproteins of myelin, affinity chromatography was carried out on WGA-

Sepharose 6 MB (Pharmacia) with radiolabelled CNS myelin. Elution of the material absorbed on the ligand was achieved by incubation with TBS containing 1% (w/v) NP 40 and 2·5% (w/v) N-acetyl glucosamine overnight at 4°C. Both materials unabsorbed on and eluted from WGA-Sepharose were separately immunoprecipitated, run in gel and autoradiographed.

Indirect immunofluorescence studies on glial cell cultures

Oligodendrocytes were isolated from adult rat brains, using a Percoll density gradient, as described by Lisak *et al.* (1981). They were cultivated in MEM. Live cells were incubated at room temperature: 1, with first antiserum for 25 min; 2, with the second fluorescein or rhodamine-conjugated anti-immunoglobulin reagent for 25 min. They were then fixed in methanol at −20°C for 10 min, mounted and observed with a fluorescence microscope.

RESULTS

Characterization of M_2 antiserum

Specificity of antiserum to M_2 tested by immunodot

When tested in a dot immunobinding assay, guinea pig anti-M_2 serum was devoid of anti-cerebroside, anti-proteolipid (a very weak, questionable, trace of reactivity to proteolipid was observed, as has already been shown (Lebar & Lees, 1985)) and anti-BP activity; (cerebroside, proteolipid and BP being major components of CNS myelin). On the other hand, anti-M_2 serum reacted with guinea pig CNS myelin, even at a high dilution of serum. Guinea pig antiserum to BGG was a negative control (Fig. 1).

Demyelinating capacity of M_2 antiserum

M_2 antiserum used in the present study was tested on long-term cultures of rat spinal cord explants.

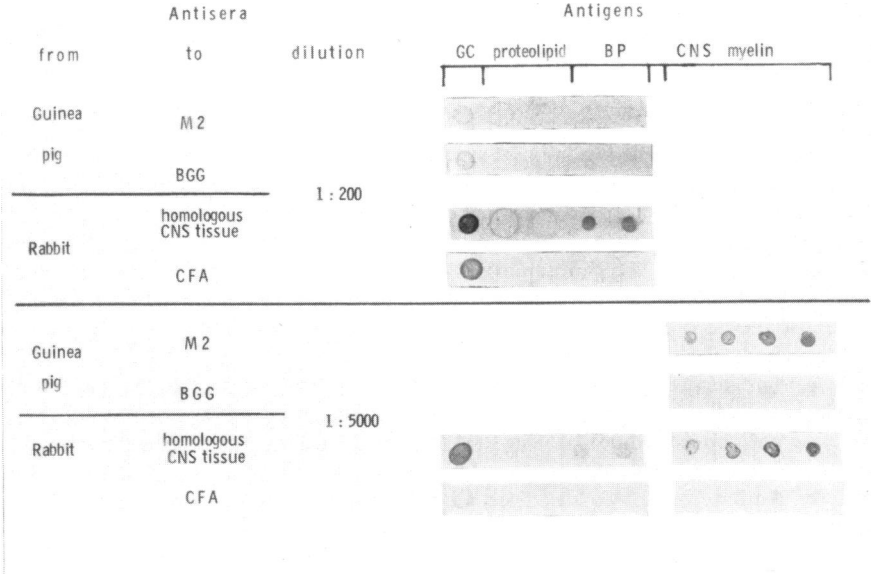

Fig. 1. Reactions of guinea pig and rabbit immune sera against myelin by the dot immunobinding assay. Bovine galactocerebroside 1 μg was applied to the nitrocellulose strips. Other antigens were spotted, from left to right, as 3-fold increasing amounts, beginning at 0·1 μg for bovine proteolipid and guinea pig BP, 0·08 μg for guinea pig CNS myelin. Non-specific reactions were not seen with soluble antigens at the tested concentrations except for a slight background with cerebroside. With insoluble myelin, the background was a little greater.

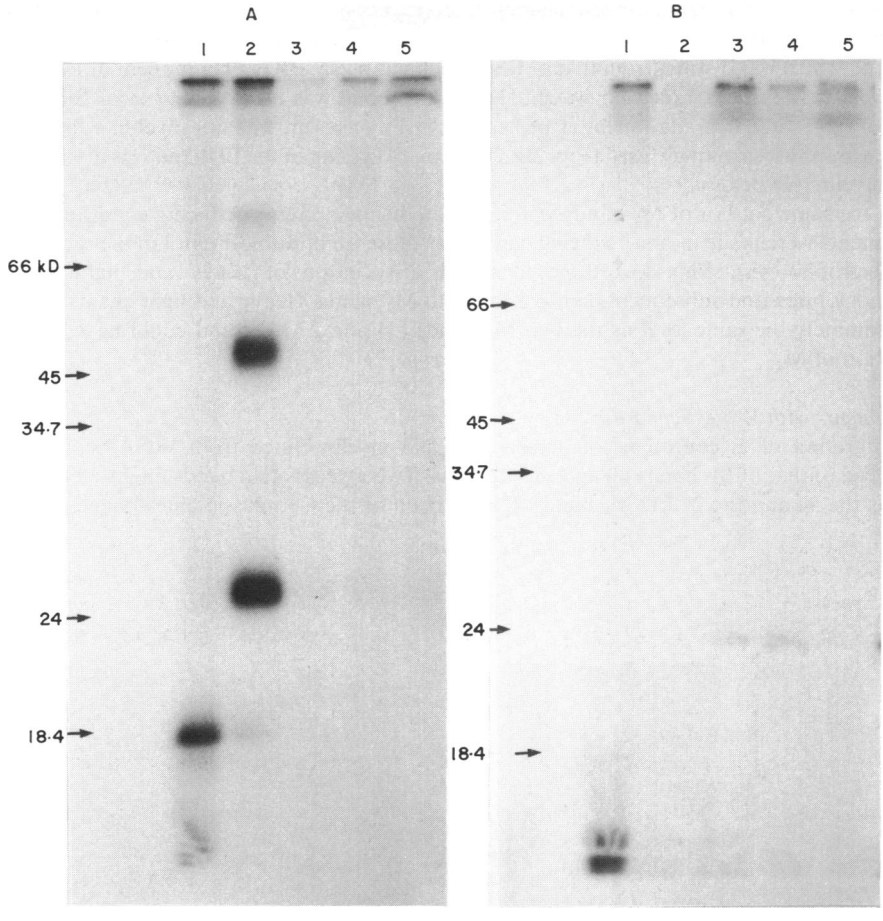

Figure 2. Autoradiographs of radiolabelled guinea pig myelin immunoprecipitated with guinea pig sera and run in SDS acrylamide gel.
(A) Autoradiography of CNS myelin antigens (B) Autoradiography of PNS myelin antigens. Lane 1, immunoprecipitation with BP antiserum; Lane 2, immunoprecipitation with M_2 antiserum; Lane 3, immunoprecipitation with individual antiserum to PNS myelin; Lane 4, immunoprecipitation with individual antiserum to crude PNS myelin; Lane 5, immunoprecipitation with BGG antiserum. Arrows indicate the level of molecular weight markers.

Demyelination occurred in the presence of complement. BGG antiserum had no effect (data not shown).

Characterization of M_2 antigen

CNS and PNS myelin: electrophoretic migration of antigens
Immunoprecipitation of radiolabelled guinea pig myelin with Hartley guinea pig immune sera was followed by autoradiographic analyses of the electrophoretically migrated antigens.

CNS myelin-associated antigens. M_2 appeared as two bands in CNS myelin migrating to the 54 kD ($54{\cdot}18 \pm 4{\cdot}53$) and 27 kD ($27{\cdot}06 \pm 1{\cdot}89$) levels, clearly distinct from the 18·5 kD BP band (Fig. 2A). The same pattern was seen in reducing and non-reducing conditions, and with M_2 antiserum from strain 13 guinea pigs immunized with isologous CNS tissue. There was no reaction of CNS myelin with anti-PNS myelin and control anti-BGG sera (Fig. 2A).

PNS myelin-associated antigens. There was no reaction of PNS myelin with M_2 and control BGG antisera. Antisera against PNS myelin precipitated one band. BP antiserum also precipitated one band which was distinct from that of PNS myelin antisera: BP is a component of PNS myelin. However, the apparent molecular weight level of this band was lower than that of BP. This was probably due to a degradation by a protease, possibly present in PNS myelin, which resisted protease inhibitors routinely used (Fig. 2B). The same lowering of the BP band was often seen with CNS myelin (see below).

As the same pattern of M_2 bands was seen in reducing and non-reducing conditions, further experiments were made in non-reducing conditions (absence of dithiothreitol in the sample buffer for electrophoresis). This was done to avoid dissociation of heavy and light chains of immunoglobulin and subsequent comigration with M_2 bands. Heavy and light chains migrate to approximately the same level as the two M_2 bands (54 and 27 kD), and could have altered the migration of M_2.

Experiments with WGA-Sepharose

The migration of glycoproteins of guinea pig CNS myelin eluted from WGA-Sepharose was compared to that of M_2 bands on autoradiographs. Two glycoprotein bands migrated to the same level as the 54 and the 27 kD M_2 bands. Comparison of the Coomassie blue-stained gel with its

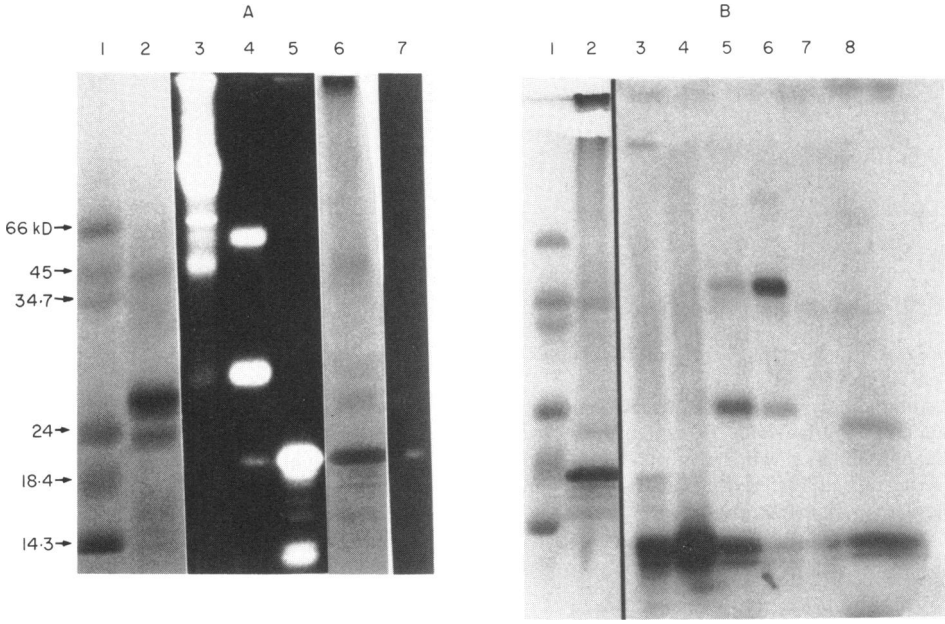

Fig. 3. Comparison of M_2 bands with other CNS myelin protein bands; identification as glycoprotein bands. Stained gels have been lined up with their autoradiographs. (A) Lanes 1, 2 and 6: gel stained with Coomassie blue. These components were run in reducing conditions. 1, molecular weight markers; 2, bovine proteolipid, 50 μg; 6, CNS myelin, 75 μg. Lanes 3, 4, 5 and 7: autoradiographs of radiolabelled CNS myelin components, run in non reducing conditions; 3, eluted from WGA-Sepharose; 4, immunoprecipitated with M_2 antiserum; 5, immunoprecipitated with BP antiserum; 7, immunoprecipitated with control BGG antiserum. (B) Lanes 1 and 2: stained gel. 1, molecular weight markers (the same as in Fig. 3 (A); 2, guinea pig CNS myelin, 160 μg. Lanes 3 to 7: autoradiograph of radiolabelled guinea pig CNS myelin components. 35×10^6 ct/min of TCA precipitable activity of CNS myelin were incubated with 300 μl of WGA-Sepharose for 3 h at 4°C. Immunoprecipitation of both unabsorbed and absorbed fractions were then carried out. 3 to 5: immunoprecipitation of 6×10^6 ct/min unabsorbed fraction with: 3, control BGG antiserum; 4, BP antiserum; 5, M_2 antiserum; 6 and 7 immunoprecipitation of $1{\cdot}5 \times 10^5$ ct/min of absorbed fraction with 6, M_2 antiserum; 7, BP antiserum. Lane 8: Whole radiolabelled CNS myelin control.

autoradiograph showed the stained proteolipid protein band to be clearly distinct from the autoradiographed 27 kD M_2 band (Fig. 3A).

To prove the glycoprotein nature of M_2 bands, immunoprecipitation of both CNS myelin fractions (unabsorbed on and eluted from WGA-Sepharose) was done. It revealed M_2 bands in each fraction. M_2 was partially retained on WGA-Sepharose; the relative intensity of the two M_2 bands was reversed in the eluted fraction compared to the unabsorbed one, which can be interpreted as a higher affinity of the 54 than the 27 kD M_2 band for WGA. The protein control of non-absorption on WGA was BP, which is not a glycoprotein. BP was immunoprecipitated by BP antiserum in the unabsorbed fraction while it was not in the eluted fraction. Actually, there was a proteolysis of BP, which led to two major effects. (1) A peptide fragment from BP was immunoprecipitated instead of whole BP: the band revealed by anti-BP serum with the myelin fraction unretained on WGA-Sepharose, as well as the BP band of whole radiolabelled CNS myelin both had faster migration than the Coomassie blue stained BP band of unlabelled control myelin. (2) Apparently, there was a non specific precipitation of peptides from BP with M_2 and BGG antiserum in the unabsorbed fraction, which was however less pronounced than the specific one with BP antiserum. In the fraction retained on WGA-Separose, the same non specific binding was definitely much weaker

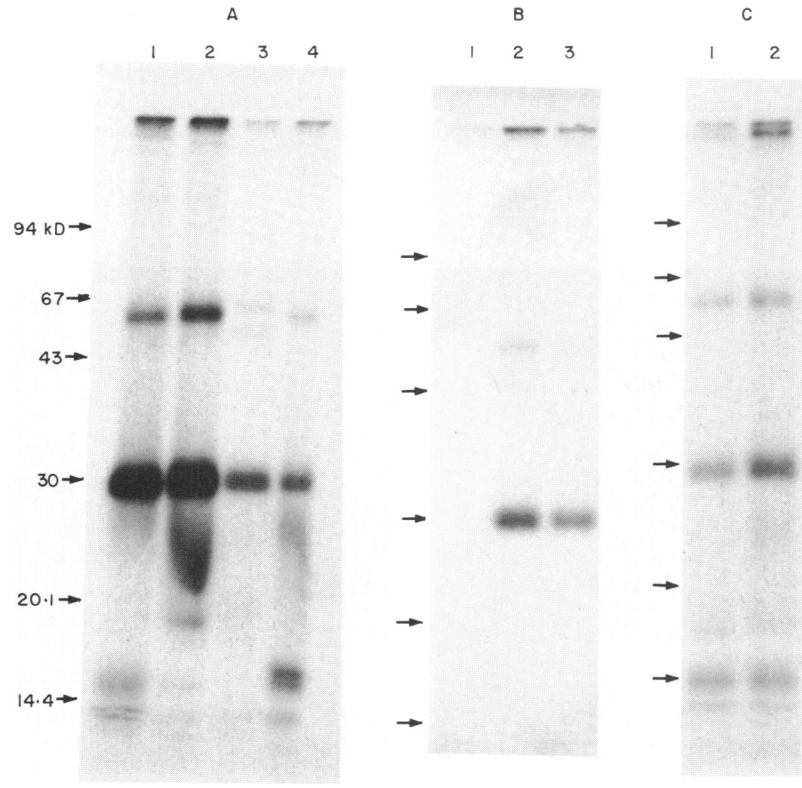

Fig. 4. Autoradiography of immunoprecipitation with radiolabelled CNS myelin. (A) Guinea pig, rat, rabbit and bovine CNS myelin immunoprecipitated with guinea pig M_2 antiserum. Lanes 1 to 4: Immunoprecipitation with M_2 antiserum and radiolabelled CNS myelin from: 1, guinea pig; 2, rat; 3, rabbit; 4, bovine. (B) Guinea pig CNS myelin immunoprecipitated with rabbit and guinea pig antisera. Lane 1, immunoprecipitation with a control individual serum from a rabbit immunized with CFA only, taken 32 days after immunization; Lane 2, immunoprecipitation with an individual rabbit antiserum to homologous CNS tissue, 32 days after immunization; Lane 3, immunoprecipitation with guinea pig M_2 antiserum. (C) Guinea pig CNS myelin immunoprecipitated with 8-18C5 and guinea pig M_2 antibodies. Lane 1, immunoprecipitation with 8-18C5; Lane 2, immunoprecipitation with M_2 antiserum; The same molecular weight markers were used in A, B and C

(Fig. 3B). The suspected proteolysis of myelin BP, accompanied by non specific precipitation of peptides from BP, was a technical difficulty in all our experiments, but was usually resolved by the rapidity of the experiment. However the experiment including affinity chromatography on WGA-Sepharose was relatively long and thus favoured proteolysis, and shifting of BP bands. Solubilization of myelin probably enhanced proteolysis, perhaps due to the neutral protease present in isolated myelin (Sato, Quarles & Brady, 1982), which resisted protease inhibitors.

M_2 antigen in myelin from different mammals

Radiolabelled guinea pig, rat, rabbit and bovine myelin were immunoprecipitated with M_2 antiserum from guinea pig. The same M_2 appeared on the same migration levels in electrophoresis, except for the rabbit upper M_2 band which seemed to be composed of two close bands situated around the level of the 54 kD M_2 guinea pig band (Fig. 4A).

Antiserum to homologous CNS tissue in rabbit

In the dot assay, rabbit antiserum to homologous CNS tissue at a low dilution (1:200) had a trace reaction with proteolipid and a clearly positive reaction with cerebroside, BP and CNS myelin. At high dilution of the antiserum (1:5000), there was no longer any reaction with proteolipid and the reaction with BP became very weak but strong positive reactions with cerebroside and CNS myelin remained. Control rabbit antiserum to CFA reacted with cerebroside at 1:200 dilution and showed no reaction at 1:5000 dilution (Fig. 1). On immunoprecipitation with radiolabelled guinea pig CNS myelin, rabbit antiserum to homologous CNS tissue behaved like guinea pig M_2 antiserum: the same bands appeared with both antisera. Reactivity of rabbit serum to proteolipid and BP was probably too weak to produce any proteolipid protein and BP bands on autoradiograph. The

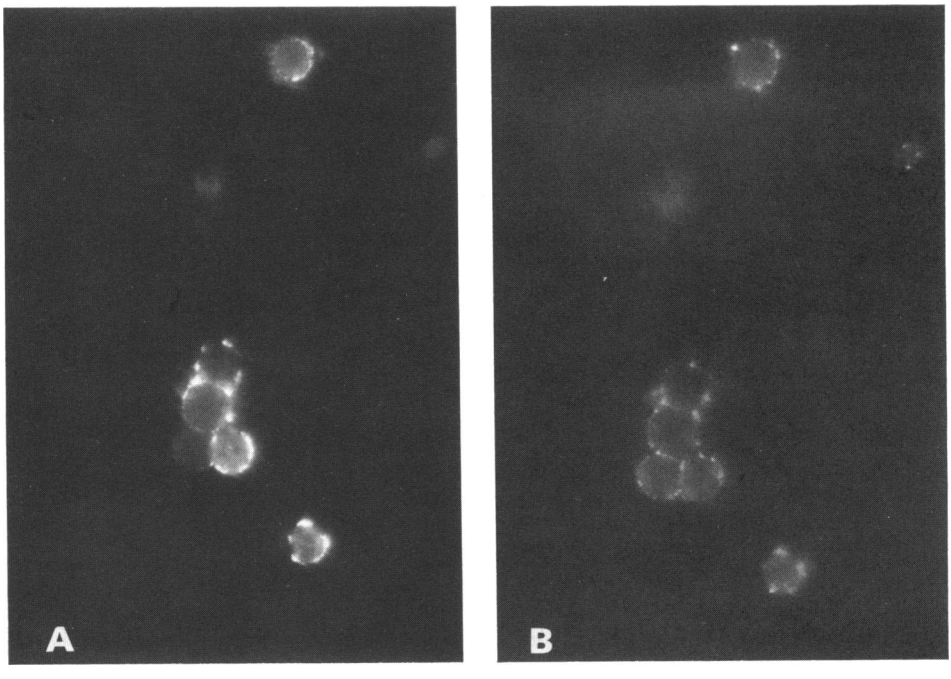

Fig. 5. Immunofluorescence staining of adult rat oligodendrocytes 24 h after isolation. Double labelling with: (A) Guinea pig antiserum to M_2 diluted 1:100 and fluorescein-conjugated sheep antiserum to guinea pig immunoglobulin diluted 1:100. (B) Rabbit antibodies to galactocerebroside at 4–8 μg/ml concentration and rhodamine-conjugated sheep antiserum to rabbit immunoglobulin diluted 1:10.

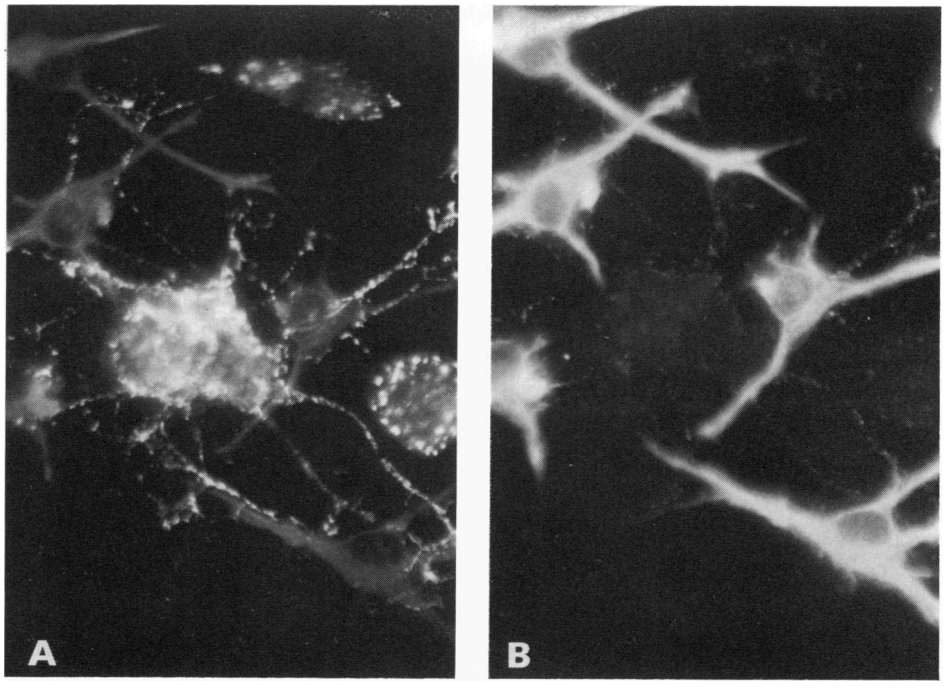

Fig. 6. Immunofluorescence staining of rat glial cells after 8 days in culture. Double labelling with: (A) Guinea pig antiserum to M_2 diluted 1:100 and fluorescein-conjugated sheep antiserum to guinea pig immunoglobulins diluted 1:100. (B) Rabbit antiserum to glial fibrillary acidic protein diluted 1:100 and rhodamin-conjugated sheep antiserum to rabbit immunoglobulin diluted 1:10.

cerebroside, not being macromolecular, could not be expected to form a band. On the other hand, a strong reactivity to M_2, which was not expected (the positive reaction to CNS myelin in the immunodot assay could have been attributed to a reactivity solely against cerebroside), was revealed on autoradiograph. There was no reaction of control rabbit antiserum to CFA (Fig. 4B).

Immunoprecipitation with a monoclonal antibody

8-18C5 is a monoclonal antibody induced in mice immunized with rat CNS glycoproteins (Linington, Webb & Woods, 1984). On immunoprecipitation with radiolabelled guinea pig CNS myelin, the same bands appeared with 8-18C5 and with M_2 antiserum as antibodies (Fig. 4C).

Cellular localization of M_2 antigen

Isolated rat oligodendrocytes were cultivated in MEM. At 24 h of culture 85 to 90% of the cells expressed galactocerebroside and were thus identified as oligodendrocytes (Raff *et al.*, 1978). Double immunofluorescence labelling with M_2 antiserum (fluorescein-labelling) and rabbit antibodies to cerebroside (rhodamine-labelling) showed that the same cells were stained with both reagents (Fig. 5). There was no staining with control BGG antiserum. After 8 days, the proportion of oligodendrocytes in culture fell to 60–70% of total cells. They were no longer round-shaped and showed extended processes. Contaminating cells were mainly astrocytes, identified by the specific expression of glial fibrillary acidic protein. At that time double labelling with M_2 antiserum and rabbit antiserum to glial fibrillary acidic protein displayed a green staining of oligodendrocytes and a red staining of astrocytes (Fig. 6). M_2, present on oligodendrocytes, was absent on astrocytes.

DISCUSSION

M_2 appeared as a pair of protein bands migrating to the 54 kD and 27 kD levels in SDS gel. The protein nature of M_2 has already been suggested by its trypsin susceptibility in CNS myelin, which is enhanced by pretreatment with phospholipase A or C (Lebar & Vincent, 1981b). However, the high content of lipids (about 70%) in myelin makes it probable that some lipids were labelled with Na ^{125}I by glycoluril (the reagent routinely used in this work), although the majority of the labelling was on exposed tyrosine in proteins. On the other hand, the sharpness of the M_2 bands ruled out the existence of glycolipid micelles which would have formed diffuse bands. Moreover, when labelling was done using iodine monochloride, which minimizes incorporation to lipid (Shepherd, Bedford & Morgan, 1976), or was done by the Bolton and Hunter reagent which only binds to primary amino groups of proteins, the migration pattern of M_2 was the same (R. Lebar, unpublished).

Poduslo, Harman & McFarlin (1980) demonstrated the existence of CNS myelin-associated glycoprotein bands using electrophoretic migration of CNS myelin in SDS gradipore polyacrylamide followed by application of radiolabelled wheatgerm agglutinin (WGA) lectin. Two major bands were at the 26·1 and 48·8 kD levels, reminiscent of the M_2 band levels. In experiments reported here, M_2 bands appeared after immunoprecipitation with the CNS myelin material specifically eluted from WGA-Sepharose. M_2 proteins were concentrated in this eluted fraction, whose M_2 bands were visible using 40 times less material (in terms of TCA precipitable radioactivity) than in the unabsorbed fraction. A specific association of a glycolipid with the protein can be excluded, from the observation (Poduslo *et al*, 1980) that WGA-binding proteins situated at the same apparent molecular weight levels as M_2, were insoluble in chloroform: methanol.

Coincident with a proteolysis of BP, a non specific precipitation of peptide fragments from BP was present in this experiment. These peptides probably bind non-specifically to IgG-Protein A Sepharose much more intensely than does non-proteolysed BP. M_2 banding pattern did not seem to be affected by that proteolysis: in some experiments the immunoprecipitated BP band was situated lower than the BP band of control myelin standard; M_2 bands remained at the same 54 and 27 kD levels as when the BP band was in its normal position (see Fig. 3).

The same M_2 banding pattern was seen using rat, rabbit and bovine, instead of guinea pig CNS myelin, although in rabbit myelin a doublet of bands seemed to replace the unique 54 kD in the same position. The cross reactivity between these species was an expected result (Lebar *et al.*, 1979).

M_2 antigen has not been found in non-nervous tissue such as liver or kidney. It was identified as a component of CNS myelin since in-vitro demyelinating guinea pig antibodies were induced, detected and absorbed by CNS myelin (Lebar *et al.*, 1976). In the present work, the presence of M_2 on rat oligodendrocyte membrane was a confirmation that M_2 is a component of CNS myelin, as CNS myelin is known to be a membrane specialization of oligodendrocyte. Furthermore, M_2 bands were shown to be specific to CNS myelin: M_2 antiserum did not reveal any band with PNS myelin. Conversely, antisera to homologous PNS myelin did not form any band with CNS myelin, while they did form one band with PNS myelin, whose significance has not yet been established.

M_2 protein bands were distinct from the BP band, as expected. The lower M_2 band was situated above the proteolipid protein band, a result which again lessened the possibility of a relationship between M_2 and proteolipid (Lebar & Lees, 1985). The same banding pattern of M_2 in reducing and non-reducing conditions also ruled out association of M_2 with CNS myelin cyclic nuclteotide phosphodiesterase or Wolfgram proteins. The latter have apparent molecular weights close to that of M_2 upper band only in reducing conditions (Lees & Sapirstein, 1983). M_2 seemed to constitute only a minor proportion of CNS myelin protein since proteolipid protein, BP and Wolfgram proteins together make up at least 80% of CNS myelin proteins.

Immunoprecipitation of guinea pig CNS myelin with the monoclonal antibody 8-18C5 produced exactly the same binding pattern as immunoprecipitation with M_2 antiserum. Both M_2 bands were revealed with 8-18C5, which would signify that both upper and lower M_2 bands share common determinants. The lower band could be a degradation product of the upper one (Linington *et al.*, 1984). Alternatively, it could be another glycoprotein of CNS myelin with some or all sequences shared by that of the upper hand. Basic proteins (prelarge and large in the guinea pig) as well as proteolipid and DM 20, in CNS myelin, are examples of this type. In any case the M_2 lower

band probably includes fewer sugars and/or sialic acids (Hedo, 1984) than the M_2 upper band, because of its lesser affinity to WGA.

Using the imunodot technique (Lebar & Lees, 1985), we were unable to find in our M_2 antiserum pools the ELISA-detected antibodies to galactocerebroside described recently in guinea pigs immunized with homologous CNS tissue showing signs of chronic EAE (Schwerer, Lassman & Bernheimer, 1984; Tabira & Endoh, 1985). On the other hand, with the same immunodot technique cerebroside antibodies were often detected even in unimmunized rabbits (data not shown). Immunization of rabbits with CFA alone increased the reactivity versus cerebroside, which however did not reach the level obtained in rabbits immunized with CNS tissue. These anti-cerebroside antibodies are known to demyelinate CNS tissue cultures (Fry *et al.*, 1974) and cerebroside has been claimed to be the major target of demyelinating antibodies induced in rabbit with CNS tissue (Raine *et al.*, 1981). The present work in a rabbit immunized with homologous CNS tissue confirmed the presence of these antibodies along with antibodies to other myelin components. In particular, with radiolabelled guinea pig CNS myelin as antigen, this rabbit serum formed two bands, exactly on the same level as the M_2 bands formed with the M_2 antiserum. This result extends the autoantigenic character of M_2 to other species, and suggests that M_2 could be a general target of in-vitro demyelinating antibodies.

Although M_2 is only a minor quantitative component of myelin, using CNS tissue as immunogen, M_2 is a potent inducer of antibodies compared to BP. Cellular immunity to M_2 could not be tested since M_2 has not yet been isolated. This strong immunogenicity of M_2, its probable situation on the myelin surface (Lebar *et al.*, 1979), the demyelinating effect of M_2 antibodies, all suggest a role of M_2 in chronic EAE induction. In the guinea pig, chronic EAE is not induced by BP alone but can be induced with CNS tissue and myelin and treatments which suppress M_2 antigen ativity in CNS tissue or myelin reduce or abolish their capacity to induce EAE (Lebar & Vincent, 1981a). By contrast, in the mouse, BP alone is able to induce chronic EAE (Fritz, Chou & McFarlin, 1983), but it is possible that invasion of CNS tissue by mononuclear cells following BP sensitization could lead to a secondary autosensitization to M_2 and to the subsequent development of chronic EAE.

Although survival of oligodendrocytes has been shown in demyelinating plaques of chronic EAE (Wayne *et al.*, 1984), these cells might also be a target for demyelinating antibodies, as we have also found M_2 antigens on oligodendrocyte membranes.

In human multiple sclerosis, the in-vitro demyelinating effect of patient sera is much weaker than the demyelinating effect of experimental EAE sera and could not even be attributed to antibodies (Grundke-Iqbal & Bornstein, 1980). M_2 antibodies might be present in the target tissue but undetectable in serum, as the triggering mechanism of multiple sclerosis is far from being understood as well as that of chronic EAE.

In conclusion, to demonstrate the role of M_2, particularly in chronic EAE, the next necessary step is that of isolation of the antigen. Further work on this point is clearly needed.

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