A specific immunological probe for the major myelin proteolipid

Confirmation of a deletion in DM-20

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Major myelin proteolipid (MMPL, also called PLP) and DM-20 are the two major intrinsic membrane proteins of CNS myelin. A specific immunological probe was obtained for MMPL by raising antibodies against the synthetic tridecapeptide 117–129 of MMPL. Antibodies against this peptide reacted with the MMPL but dit not cross react with DM-20, while both proteolipids had been shown previously to be recognized by antibodies directed against the C-terminal hexapeptide of MMPL. This is in accordance with previous findings showing that DM-20 differs only from MMPL by a deletion of residues 100–140 (±few units). Furthermore, this site-specific immunological probe also recognizes MMPL in its native form in oligodendrocytes in primary glial cell cultures.

Site specificity *Synthetic peptide* *Myelin* *Proteolipid* *Oligodendrocyte* *Membrane protein*

1. INTRODUCTION

Central nervous system myelin contains several intrinsic membrane proteins. The two major ones are known to be the major myelin proteolipid (MMPL) and DM-20. The primary structure of bovine brain MMPL has been established by Lees' and Stoffel's groups [1,2]. Recently a cDNA clone coding for rat brain MMPL was isolated and comparison of the encoded sequence with bovine MMPL showed a complete homology except for 4 amino acid residues [3]; on the other hand total sequence homology was described for the human MMPL [4].

The amino acid sequence of DM-20 has not been completely elucidated, but it has been shown to have a high degree of structural similarity with the MMPL [5–7] and recently Trifilieff et al. [8] reported that bovine DM-20 only differs from bovine MMPL by a deletion of residues 100–140 (± few units).

According to the primary sequence of the MMPL, two models of its integration in the myelin membrane have been proposed but with a significant difference: in Lees' model [9], the main hydrophilic loop (residues 90–151) is oriented towards the cytoplasmic space, while in Stoffel's model [10] it is oriented towards the extracytosolic side. Immunocytochemical investigations with specific antibodies directed against this hydrophilic loop could be of help for defining the orientation of the MMPL in the myelin. Furthermore a specific immunological probe for the MMPL that does not cross react with DM-20 can be obtained if antibodies are directed against a polypeptide sequence included in the deletion.

As it is known that antibodies against a peptide can react with the corresponding amino acid se-

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quence in the intact protein [11], we synthesized a peptide which comprises residues 117–129 of the MMPL. Antibodies directed against this peptide indeed reacted with the MMPL after electroimmunoblotting but not at all with the DM-20, while both proteolipids were recognized by antibodies directed against the C-terminal hexapeptide of the MMPL [7]. We also showed that this specific serum labels the MMPL in its native form in oligodendrocytes in primary glial cell cultures.

2. MATERIALS AND METHODS

2.1. *Materials*

Keyhole limpet hemocyanin (KLH) was obtained from Calbiochem Behring Corp.; sheep (anti-rabbit IgG) antibodies conjugated to horseradish peroxidase and FITC-labeled sheep (anti-rabbit IgG) antibodies were from Biosys (Compiègne, France); nitrocellulose paper (0.45 μm pore size) was from Millipore. All other reagents were obtained from the usual commercial sources at the highest available degree of purity.

Total bovine brain proteolipids and pure bovine MMPL were obtained as described in [12]. Pure bovine DM-20 was purified as described in [12] or [13]. Myelin and total rat brain proteolipids were obtained from adult Wistar rats as reported [14, 15].

2.2. *Synthesis of the tridecapeptide 117–129 of MMPL and coupling to KLH*

Synthesis of the tridecapeptide H-Thr-Gly-Gly-Gln-Lys-Gly-Arg-Gly-Ser-Arg-Gly-Gln-His-OH was performed by the solid-phase method of Merrifield [16] on aminobenzyl resin [17,18] with a substitution of 1 mmol NH_2/g. The Boc-amino acids were coupled with dicyclohexylcarbodiimide (DCCD) in methylene chloride in 2.5 excess over the free amines, except glutamine which was coupled by DCCD and 1-hydrozybenzotriazole in dimethylformamide [19]. The side chain protections were benzyl ether for threonine and serine, benzyloxycarbonyl for lysine, tosyl for arginine and histidine. The lysine and histidine derivatives were purchased as their dicyclohexylamine salts and neutralized just before the coupling step. After total assembly of the peptide on the resin, cleavage and simultaneous deprotection of the side chains were achieved by anhydrous hydrogen fluoride (1 h, 0°C). The crude peptide was purified by gel filtration on Biogel P-2 in 0.1 M acetic acid followed by ion-exchange chromatography on CM-52 (Whatman) with a conductivity gradient in ammonium acetate buffer. The peptide eluted at 18 mS at room temperature. Its homogeneity was checked by HPLC and amino acid analysis after hydrolysis in 6 N HCl at 110°C for 24 h (uncorrected values: Thr 0.8, Ser 0.7, Glu 1.8, Gly 5.0, His 1.1, Lys 1.0, Arg 2.1).

This peptide (10 mg) was coupled to KLH (60 mg) in the presence of glutaraldehyde for 90 min as described in [20].

2.3. *Immunization*

Two rabbits were injected subcutaneously on days 0, 20 and 48 with 300 μl of a solution containing 1 mg of synthetic peptide 117–129 coupled to KLH and 300 μl of either Freund's complete adjuvant (day 0) or incomplete adjuvant (days 20 and 48). The rabbits were bled on day 64 and the serum was kept frozen at −20°C. The antisera were used without removal of KLH carrier antibodies.

2.4. *Polyacrylamide gel electrophoresis*

Electrophoresis was performed according to the method of Laemmli [21] consisting of a linear gradient of acrylamide (10–20%) containing 0.1% SDS. The stacking gel was made up of 4% acrylamide. A constant voltage of 110 V was applied for 7 h.

2.5. *Immunoblotting*

Proteins were electrophoretically transferred from slab gel to nitrocellulose sheets according to the method of Towbin et al. [22]. Nitrocellulose sheets containing sample proteins were incubated overnight in PBS containing 3% (w/v) BSA and 2.5% (v/v) normal sheep serum (buffer A). Rabbit sera were added at a concentration of 1/2000 and incubation was conducted for 2 h at room temperature. The nitrocellulose sheets were rinsed several times with PBS and afterwards incubated in buffer A containing sheep (anti-rabbit IgG) antibodies conjugated to horseradish peroxidase (1/1000 diluted). After an additional washing step, the antibody binding was detected with 4-chloro-1-naphthol and hydrogen peroxide [23].

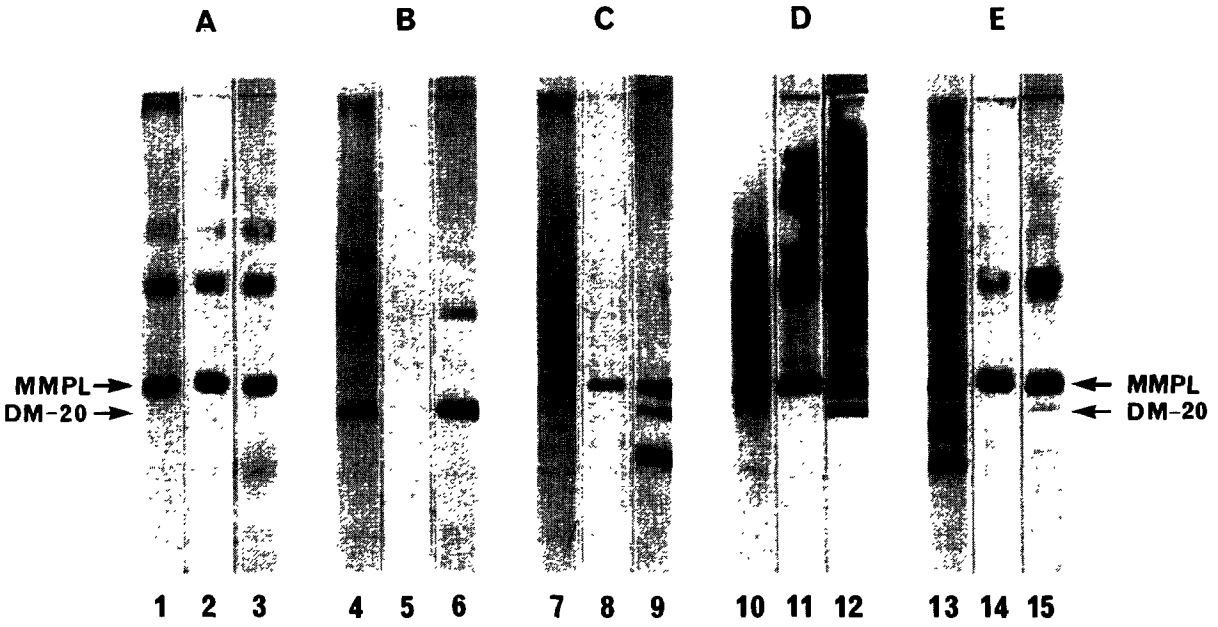

Fig.1. Electroimmunoblot analysis of pure MMPL (A), pure DM-20 (B), total bovine brain proteolipids (C), total rat brain proteolipids (D) and rat brain myelin proteins (E) with the anti-tridecapeptide 117–129 and the anti-C-terminal hexapeptide antisera. Approximative protein amounts (estimated by the Lowry method [27]) applied onto the gel: (A) 20, (B) 20, (C) 20, (D) 95, (E) 40 μg. Lanes: 1, 4, 7, 10 and 13, gel strips stained after electrophoretic transfer; 2, 5, 8, 11 and 14, nitrocellulose strips after visualization of the peroxidase activity following incubation with the anti-tridecapeptide antiserum; 3, 6, 9, 12 and 15, nitrocellulose strips after visualization of the peroxidase activity following incubation with the anti-C-terminal hexapeptide antiserum.

2.6. *Cell culture and immunofluorescence procedures*

Primary glial cell cultures enriched in oligodendrocytes were prepared and maintained as reported earlier [24]. Immunofluorescence experiments were performed either on fixed or live cell cultures following respectively methods A and B described in [25] using appropriate dilutions of the anti-tridecapeptide antiserum and FITC labeled sheep (anti-rabbit IgG) antibodies.

3. RESULTS AND DISCUSSION

The tridecapeptide 117–129 of the MMPL, H-Thr-Gly-Gly-Gln-Lys-Gly-Arg-Gly-Ser-Arg-Gly-Gln-His-OH was synthesized, coupled to KLH and used to immunize rabbits. 48 days after the first inoculation, anti-tridecapeptide antibodies were detected by the dot-immunobinding assay [23].

The specificity of the anti-tridecapeptide antibodies was checked by electroimmunoblotting experiments. Pure MMPL (fig.1A) and pure DM-20 (fig.1B) were examined first; after immunoreaction, only pure MMPL could be detected (fig.1A, lane 2); no antibody binding occurred with pure DM-20 (fig.1B, lane 5).

The specificity of this antiserum for MMPL was further confirmed by electroimmunoblotting of total bovine brain proteolipids, total rat brain proteolipids and rat brain myelin proteins (fig.1C–E). In the 3 samples, only one strongly stained band was detected at the level of the MMPL band. Confirmation of this specificity was also given by comparison of these results with electroimmunoblots of the same samples obtained with the anti C-terminal hexapeptide of the MMPL antiserum prepared by Nussbaum et al. [7]: pure MMPL (fig.1A, lane 3) and pure DM-20 (fig.1B, lane 6) were positive after immunoreaction as well as in the different samples of total bovine brain proteolipids (fig.1C, lane 9), total rat brain proteolipids (fig.1D, lane 12) and rat brain myelin proteins (fig.1E, lane 15). Additional immunoreactive bands are also visible on the different immuno-

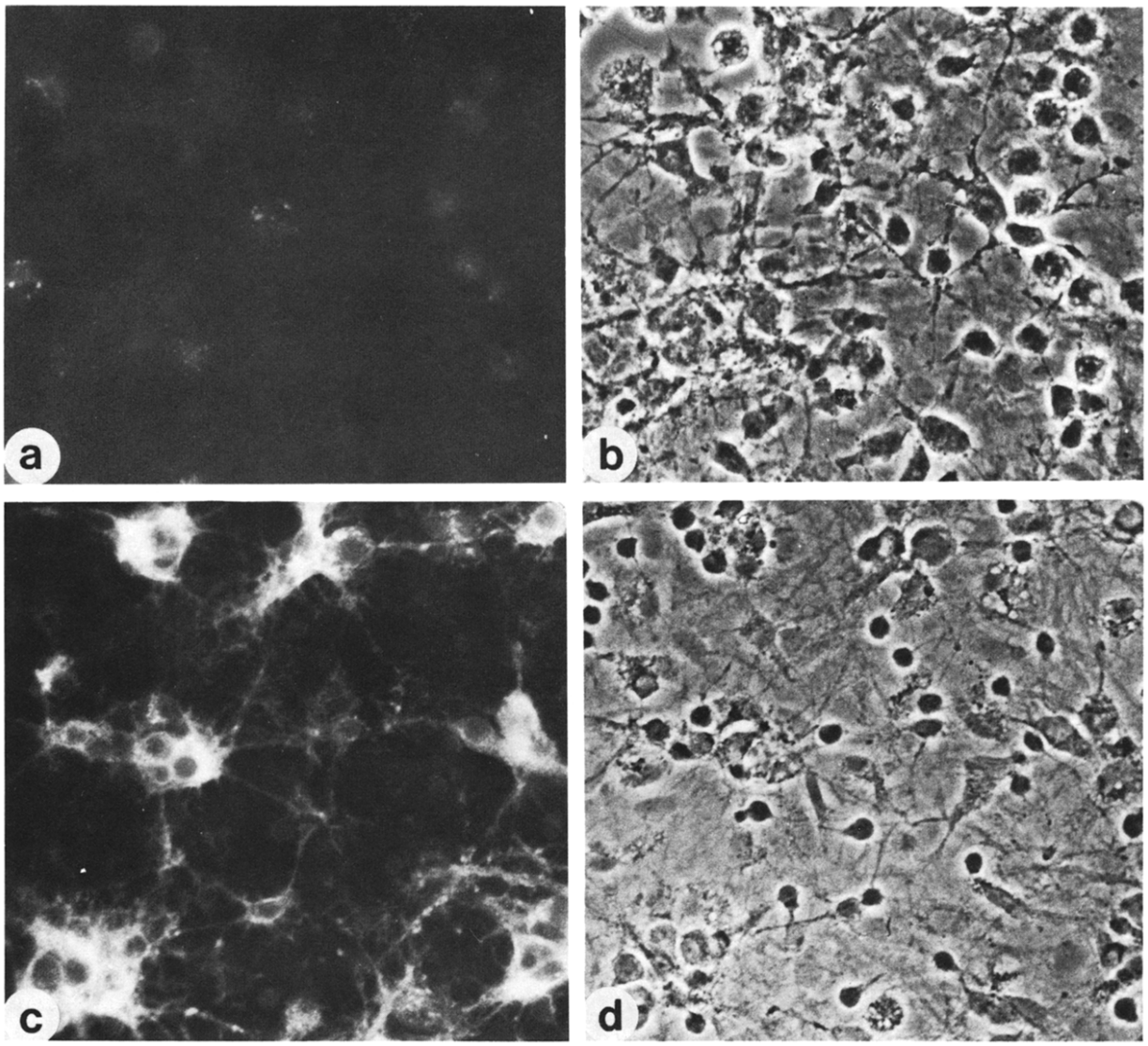

Fig.2. Immunofluorescence (a,c) and respective phase-contrast (b,d) micrographs of live (a,b) and fixed (c,d) primary glial cell cultures from newborn rat brain hemispheres (17 days after plating) incubated with the anti-tridecapeptide antiserum. No immunolabeling was observed on live cells; cells identified as oligodendrocytes, lying on the top of a continuous layer of astrocytes, are positively stained only after preliminary permeabilization. × 280.

blots; high molecular mass bands are due to MMPL and DM-20 aggregates; the presence of a stained band of ~16 kDa (fig.1C, lane 9) is in accordance with the existence in bovine brain of small minor proteolipids [12] structurally related to the MMPL (to be published).

These experiments show unambiguously that the anti-tridecapeptide antiserum reacts with the MMPL but does not cross react with DM-20; this antiserum therefore constitutes a specific immunological probe for the MMPL. These results are in good agreement with our previous work on pure DM-20 and confirm that DM-20 differs from the MMPL by a single deletion of residues 100–140 (± few units) [8].

This specific immunological probe directed against residues 117–129 of the main hydrophilic loop of the MMPL might be very useful in studies

concerning the synthesis, transport and incorporation of this proteolipid in the myelin membrane. We therefore started immunofluorescence studies on primary glial cell cultures enriched in oligodendrocytes, cells which are responsible for the synthesis of CNS myelin [26]. Since no immunolabeling was observed on live cells (fig.2a,b), we concluded that the hydrophilic loop of the MMPL is not accessible, under our experimental conditions, at the surface of the oligodendrocytes. Labeling was then performed on identical cell cultures after preliminary permeabilization of the cells with ethanol/PBS (95/5, v/v) and acetone at −70°C [25]; fig.2c,d shows that the oligodendrocytes lying on the top of the astrocyte layer are highly labeled, particularly at their periphery; staining is also observed along their processes. This immunofluorescence pattern is similar to that obtained with the anti C-terminal hexapeptide antiserum [24], and therefore confirms that the anti-tridecapeptide antiserum recognizes the MMPL in its native form.

Immunocytochemical studies on myelin membrane with the present specific immunological probe will enable a greater refinement of MMPL localization in myelin. In addition, it is now possible, using double immunolabeling experiments, to study comparatively the synthesis, transport and incorporation of both MMPL and DM-20 in the myelin membrane.

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