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# Human monoclonal IgM with autoantibody activity against two gangliosides (GM1 and GD1b) in a patient with motor neuron syndrome

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## SUMMARY

Small amounts of oligoclonal immunoglobulins were detected by Western blotting in the serum from a patient with motor neuron syndrome. The prominent one, a monoclonal IgMλ, reacted strongly with the gangliosides GM1 and GD1b and more weakly with asialo GM1, as shown by immunoenzymatic staining of thin-layer chromatograms of gangliosides, ELISA on purified glycolipid coats and immunoadsorption with purified GM1. Affinity-chromatography with purified GM1 resulted in the purification of monoclonal IgMλ. This purified IgM and its Fab fragments showed the same pattern of reactivity with gangliosides as that observed with whole serum. Such monoclonal IgM could be responsible for motor neuron diseases in some patients with overt or barely detectable monoclonal gammopathies.

**Keywords** motor neuron syndrome monoclonal IgM gangliosides autoantibodies

## INTRODUCTION

In certain patients affected with predominantly sensory peripheral neuropathy and IgM monoclonal gammopathy, reactivity of serum monoclonal IgM with myelin components was reported, thus suggesting an immunological mechanism for peripheral nerve demyelination. Among target antigens of these monoclonal IgM, the most common ones are myelin-associated glycoprotein (Braun, Frail & Latov, 1982; Ilyas *et al.*, 1984), which is a predominant component of central nervous system (Quarles, 1984), and sulphated glucuronyl glycolipids (Chou *et al.*, 1985, 1986; Ilyas *et al.*, 1985a; Jauberteau *et al.*, 1988). The latter are known to be present only in peripheral nerve in adults. IgM seems to bind carbohydrate epitopes that are shared by these two antigens. In some cases, IgM also reacts with a glycolipidic antigen present in human white matter (Jauberteau *et al.*, 1989). Reactivity against intermediate filaments has been described by Dellagi *et al.* (1982). The identification of these monoclonal IgM is of clinical significance since the neurological disease can be dramatically improved by plasma exchanges (Lassoued *et al.*, 1985; Haas & Tatum, 1988). Gangliosidic antigens have been reported less frequently in predominantly sensory demyelinating neuropathies. IgM reactivity was directed against gangliosides containing a disialosyl group (Ilyas *et al.*, 1985b), and against sialosyllactosaminyl paragloboside (Miyatani *et al.*, 1987). So far, reactivity with the two gangliosides GM1 and GD1b has been reported in only a few cases of IgM gammopathy associated with motor-dominant neuropathy (Kusunoki *et al.*, 1989) or motor neuron disease (Freddo *et al.*, 1986; Ito & Latov 1988; Nardelli *et al.*, 1988).

We report on a case of motor neuron syndrome associated with several monoclonal immunoglobulins (Ig), as detected by a Western blotting method. Only one of them, a monoclonal IgMλ, had a detectable antibody activity against a determinant of GM1 and GD1b.

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## MATERIALS AND METHODS

*Patient*

A 77-year-old man progressively developed an asymmetric upper motor weakness over a 6-year period. Tendon reflexes were abolished. No sensory disturbances or bulbar signs were observed. Electrophysiological studies showed severe muscle denervation. Motor and sensory nerve conduction velocities were initially normal. After 6 years, a moderate reduction of right cubital nerve motor conduction velocity was detected. Radial cutaneous nerve and superficial peroneal muscle biopsies showed a severe denervation atrophy. Except for the presence of serum monoclonal Ig, the usual laboratory investigations were normal without evidence of underlying diseases.

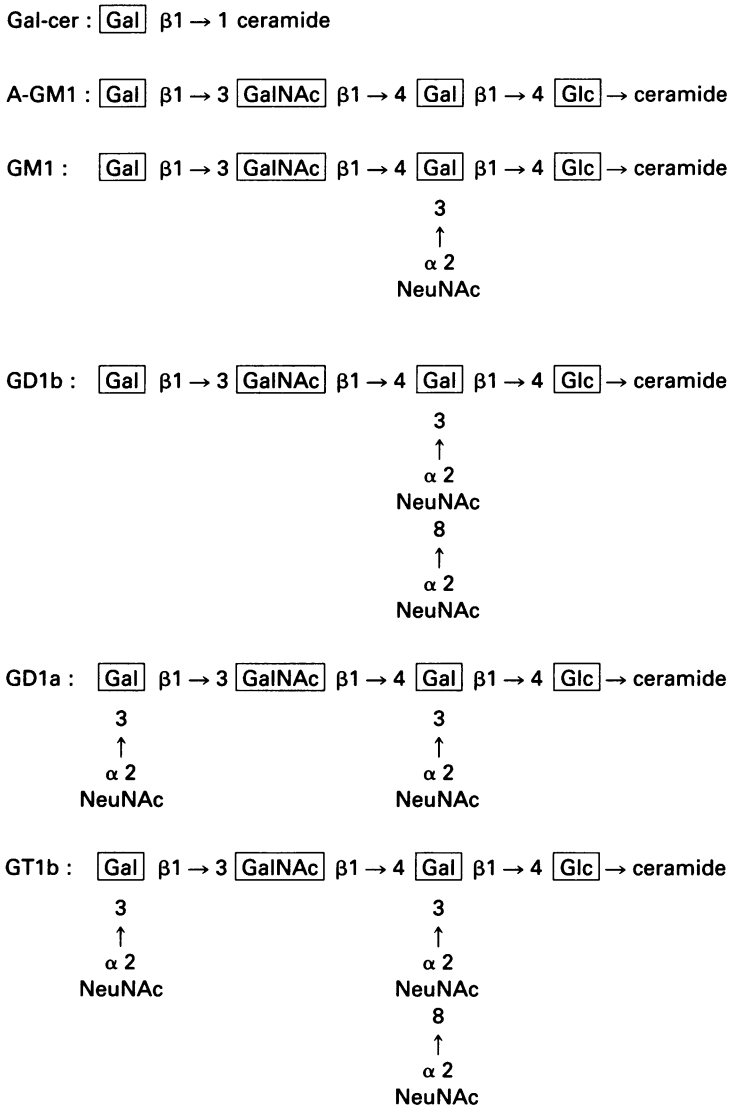


**Fig. 1.** Structure of glycosphingolipids tested by ELISA. Gal-cer, galactocerebroside; Gal, galactose; A-GM1, asialo GM1, GalNac, N-Acetyl-galactosamine; Glc, glucose; NeuNAc, N-acetylneuraminic acid.

*Immunochemical methods*

The patient's serum samples, collected 6 years after the beginning of the symptoms, were studied using the usual immunochemical techniques (electrophoresis, immunoelectrophoretic analysis with polyvalent and monospecific anti-Ig sera, isolation of IgM by gel filtration and measurement of Ig levels by laser nephelemetry). In addition, monoclonal Ig in small amounts were searched for using a sensitive Western blot technique (Aucouturier *et al.*, 1987; Aucouturier & Preud'homme, 1987; Briault *et al.*, 1988). Briefly, serum diluted 1/50 to 1/200 was separated by thin-layer agarose electrophoresis (Paragon, Beckman, Gagny, France) and transferred to nitrocellulose sheets by pressure blotting. After saturation for 1 h with 5% powdered skimmed milk, the blots were revealed with alkaline phosphatase-coupled antibodies specific for human $\gamma$, $\alpha$, $\mu$, $\chi$, and $\lambda$ chains at the appropriate dilutions in 0·01 M phosphate, 0·15 M NaCl, pH 7·4, buffer containing 2% bovine serum albumin (BSA). The specificity of these reagents was controlled by the same method using known myeloma and macroglobulinemia Ig.

*Purification of gangliosides*

Gangliosides were purified from normal human brain, spinal cord and peripheral nerve obtained at autopsy. After lipid extraction in chloroform-methanol (2:1, v/v) and partition performed according to Folch, Lees & Sloane-Stanley (1957), the gangliosidic fraction was prepared using a reversed-phase C18–silicic column (prepared and kindly donated by J. Portoukalian, INSERM U218, Lyon, France) as described by Williams & McCluer (1980). Neuraminic acid (NeuNAc) content was determined according to Svennerholm (1957). Purified standard gangliosides from bovine brain were supplied by Sigma (St Louis, MO) for gangliosides GM1, GD1a, GT1b, galactocerebrosides and asialo GM1. Bovine brain ganglioside mixture and standard GD1b were a gift from Fidia (Abano Terme, Italy). These standard glycolipids purified from bovine brain are known to share carbohydrate determinants similar to human glycolipids.

Thin-layer chromatography (TLC) of gangliosides was performed on aluminum-backed TLC plates (silica gel 60;

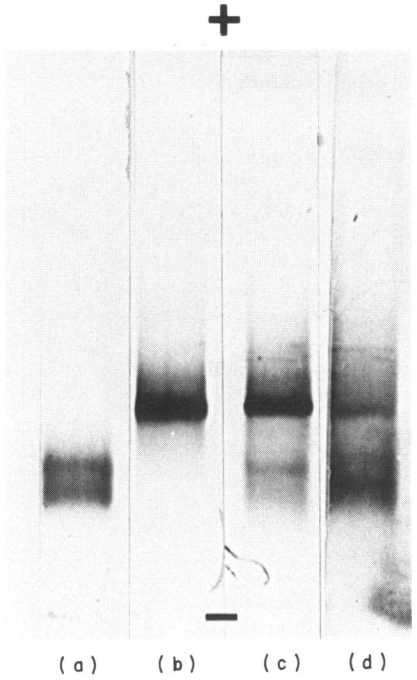


**Fig. 2.** Western blotting of thin-layer agarose electrophoresis of the patient's serum revealed by anti-$\gamma$ (lane a), anti-$\mu$ (b), anti-$\lambda$ (c) and anti-$\chi$ (d) conjugated sera. A diffuse pattern was observed after revelation with anti-$\alpha$ antibodies (not shown).

Merck, Darmstadt, FRG) in a solvent system made up of chloroform-methanol-0·25% $CaCl_2$ in water (50:40:10, v/v/v). Glycolipids were detected by colorimetry using alpha-naphtol reagent (Esselman, Laine & Sweeley, 1972).

*Detection of antibodies to glycolipids on TLC plates*

This technique was developed on the basis of the procedures of Magnani *et al.* (1982) and Harpin *et al.* (1985). Briefly, after chromatography, the dried plates were dipped in a solution of 0·05% polyisobutyl methacrylate (Aldrich, Milwaukee, WI) in n-hexane and then saturated with a solution of a 0·01 M phosphate, 0·15 M NaCl, pH 7·4, buffer (PBS), containing 1% gelatin (Merck) and 10% inactivated horse serum (GIBCO, Paisley, UK), for 30 min at room temperature with continuous low speed agitation. The plates were overlayed with the patient's serum diluted 1:100 in the same buffer and incubated at 37°C for 2 h. After washing with PBS, the plates were further incubated for 1 h at room temperature with peroxidase-conjugated goat antibodies monospecific for $\gamma$, $\mu$, $\alpha$, $\chi$ or $\lambda$ chains (Cappel, West Chester, PA) diluted 1:1000 for the anti-heavy chain and 1:500 for the anti-light chain sera. After washing, the reaction was revealed with 4-chloro-l-naphtol. Control experiments with normal sera show no detectable reactivity.

*ELISA*

Binding of serum IgM to the bovine standard glycolipids (Fig. 1) was measured by ELISA according to Freddo *et al.* (1986). Microtitration plates (Immunoplate I; Nunc, Roskilde, Denmark) were coated with 1 $\mu$g/well of each glycolipid in 20 $\mu$l of methanol and the methanol was removed by evaporation. The wells were saturated with 200 $\mu$l of 1% BSA in PBS, during 2 h at room temperature, and washed with 0·05% Tween 20 in PBS. One-hundred microlitres of serial dilution of patient's or control sera in 1% BSA, 0·05% Tween 20 in PBS were added to each well and incubated overnight at 4°C. After washing, 100 $\mu$l of peroxidase-conjugated goat anti-$\mu$ antibodies, diluted 1:1000, were added for 1 h at 37°C. The reaction was developed with O-phenylenediamine. Optical densities (OD) were read at 492 nm in an ELISA reader (Titertek Multiskan; EFLAB, Helsinki, Finland). Control sera were obtained from 18 patients (mean age 73 years) with monoclonal IgM (12 $\chi$ and 6 $\lambda$), without neurological disease, and 17 patients (mean age, 63 years) with a motor neuron disease (amyotrophic lateral sclerosis), without detectable serum monoclonal Ig.

*Immunoadsorption*

Further study of gangliosidic reactivity was performed by immunoadsorption on bovine standard GM1 according to Coulon-Morelec (1972). This GM1 immunoadsorbent consisted of GM1, lecithin and cholesterol in a ratio of 1:2:10 by weight. Briefly, 14 mg of cholesterol and 2·8 mg of lecithin in ethanol were dispersed in 2·2 ml of water, then 1·4 mg of GM1 in 1% ethanol were added. After stirring, 0·15 M NaCl was added up to a final volume of 9 ml. After centrifugation at 6000 ***g*** for 30 min, the pellet was resuspended in 1 ml of decomplemented patient's serum diluted 1:20 in 0·15 M NaCl and incubated overnight at 4°C with gentle stirring. After centrifugation, the supernatant was used in immunodetection assays on TLC and ELISA. It was also studied by Western blotting as above. An immunoadsorbent prepared with an irrelevant glycolipid (galactocerebroside and cholesterol in a ratio of 1:70 by weight) was used as a negative control.

*Antibody purification and Fab reactivity*

The patient's monoclonal IgM$\lambda$ was purified by affinity chromatography according to Hirabayashi *et al.* (1983). Briefly, octyl–Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was washed with a methanol-water (1:1, v/v) solution containing 0·1 M KCl, and the packed gel (1·5 ml) was coated with 1 mg of GM1 dissolved in the same solution. The patient's serum was applied to this column and incubated for 16 h at 4°C. The antibody adsorbed to the beads was eluted with 3·0 M KSCN in PBS. The eluate contained only IgM $\lambda$ by 7·5% SDS–PAGE according to Laemmli (1970) and Western blotting (Towbin, Staehelin & Gordon, 1979). After dialysis against PBS, the purified antibody was subjected to enzymatic cleavage by trypsin to obtained Fab fragments (Plaut & Tomasi, 1970). Porcine trypsin (bicrystallized, type III; Sigma) dissolved in 0·1 M Tris-HCl buffer, pH 7·8, containing 0·02 M $CaCl_2$, was added to the antibody at a ratio of 200 (protein/enzyme) and incubated for 18 h at 37°C. The enzymatic digestion was stopped by the addition of soybean trypsin inhibitor (Sigma). The digestion products were controlled as above and only Fab fragments were detectable. The anti-ganglioside reactivity of purified antibody and Fab fragments was tested by ELISA as described above.

## RESULTS

Serum Ig levels were normal (IgM: 2·2 mg/ml). Agarose electrophoresis showed very small amounts of a monoclonal

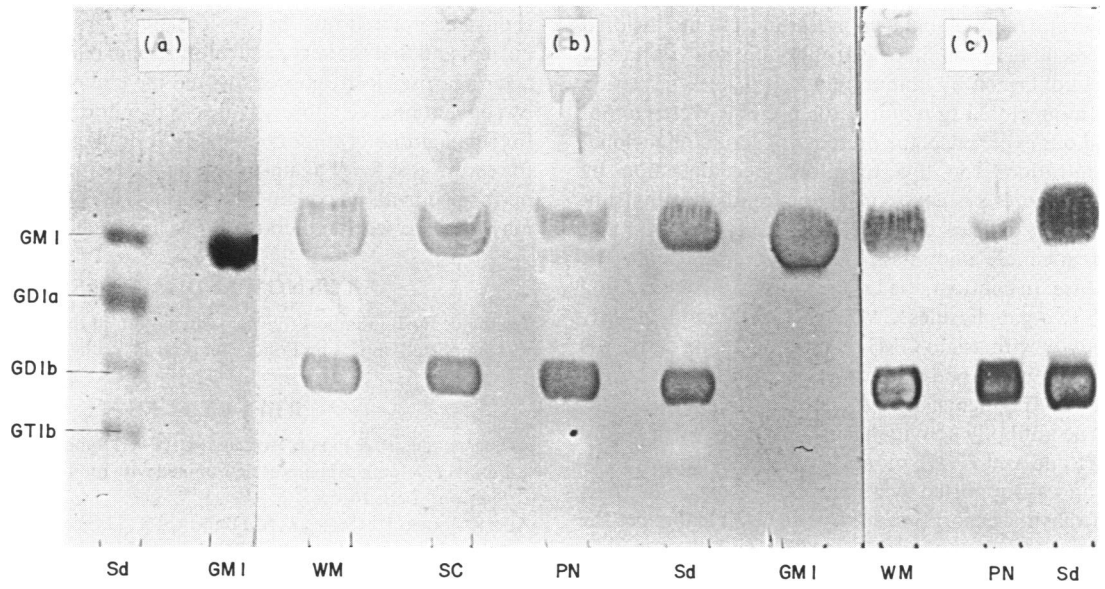


**Fig. 3.** Thin-layer chromatograms of a standard preparation (Sd) containing a mixture of purified gangliosides from bovine brain (10 $\mu$g), standard bovine brain GM1 (5 $\mu$g) and human ganglioside fractions (10 $\mu$g NeuNAC) from human white matter (WM), spinal cord (SC) and peripheral nerve (PN). (a) Alpha-naphtol staining, (b) and (c) indirect immunoenzymatic staining by the patient's serum diluted 1/100 and conjugated anti-$\mu$ (b) and anti-$\lambda$ (c) antibodies.

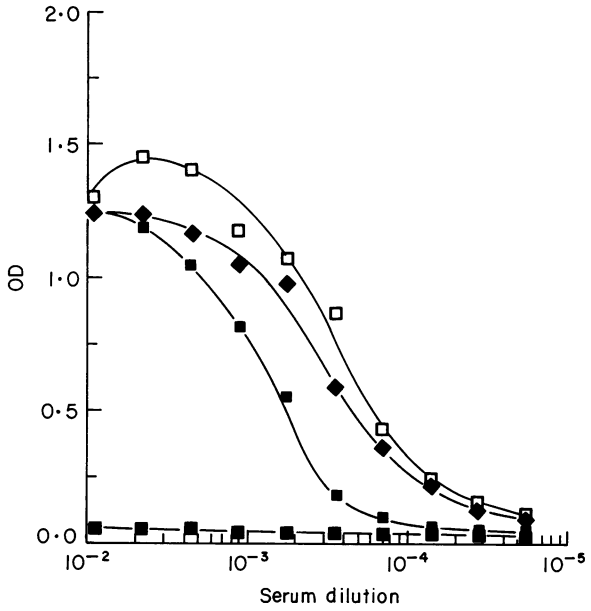


**Fig. 4.** ELISA performed with various dilutions of the patient's serum and peroxidase-coupled anti-$\mu$ antibodies, using various bovine glycolipid coats: GM1 (□), GD1b (◆), asialo GM1 (■), GD1a and GT1b (■).

band of $\beta$2 mobility. IgM was not increased by immunoelectrophoresis but its precipitation line was curved. Immunoelectrophoretic study of IgM isolated by gel filtration showed a predominant IgM $\lambda$. Molecules bearing $\chi$ light chain, which were present in much smaller amounts, also appeared to be homogeneous. Western blot analysis confirmed that the prominent abnormality was an IgM$\lambda$. It was associated with even smaller amounts of IgM$\chi$, IgG$\chi$ and IgG$\lambda$ (Fig. 2).

TLC of gangliosidic fractions from various parts of the human nervous system or of bovine standard ganglioside preparations were incubated with the diluted patient's serum, then with peroxidase-tagged anti-$\mu$ antibodies (Fig. 3b). Two bands were consistently stained. They could be identified as GM1 and GD1b from their migration by comparison with that of a mixture of known gangliosides (Fig. 3a) and using purified gangliosides. The same gangliosides were stained when the patient's serum was revealed by anti-$\lambda$ antibodies (Fig. 3c). In contrast, there was no staining by antibodies to the other Ig isotypes. These data were confirmed by ELISA: the patient's serum reacted with GM1 and GD1b up to dilutions as high as 1:12·800 (Fig. 4). A slightly weaker reactivity was observed with asialo GM1 but none with any of the other gangliosides tested. The various control sera fail to show any reactivity detectable by ELISA with the panel of gangliosides (not shown).

The binding activity of the serum to GM1 and GD1b on TLC plates and by ELISA was completely abolished after immunoadsorption with GM1. Immunoblotting of the adsorbed serum showed the complete and selective removal of the monoclonal IgM$\lambda$, the other serum fractions, including the other oligoclonal Ig, being unaffected. The control experiments performed with the galactocerebroside adsorbent showed no change of the reactivity with GM1 and GD1b in the immunoenzymatic studies and no modification of the Western blot pattern of the adsorbed serum. The monoclonal IgM$\lambda$ was recovered in pure form by affinity-chromatography with insolubilized GM1. This purified IgM and its Fab fragments displayed the same reactivity against GM1, GD1b and asialo GM1 as the patient's whole serum.

## DISCUSSION

The present report deals with a case of a progressive motor neuron syndrome involving the arms, associated with small

amounts of several monoclonal Ig in the serum. The monoclonal IgM$\lambda$ was detectable by immunoelectrophoretic analysis and its presence was confirmed by immunoblotting. Characterization of the other monoclonal Ig required the use of a Western blot technique. In our experience, the presence of serum oligoclonal Ig in concentrations too low to allow their detection by immunoelectrophoresis is common in apparently healthy subjects of more than 75 years of age. Hence, the IgM$\chi$, IgG$\chi$ and IgG$\lambda$ present in minute amounts are likely to be irrelevant to the patient's disease. In contrast, the IgM$\lambda$ showed a strong binding activity with two gangliosides GM1 and GD1b and a slightly weaker reactivity with asialo GM1. That the binding activity is actually due to an antibody activity is shown by the strong reactivity of Fab fragments. To the best of our knowledge, this proof of a true antibody activity has not been provided before for anti-GM1 and anti-GD1b monoclonal IgM. The specificity of this binding was supported by various control experiments. It was confirmed by the adsorption study with GM1 leading to the purification of the monoclonal IgM$\lambda$ together with the complete disappearance of the reactivity with GM1 in the adsorbed serum. Immunoadsorption on GM1 also removed the reactivity with GD1b and the affinity-chromatography purified IgM and Fab fragments reacted with GM1 and GD1b. Thus, the monoclonal IgM binds an epitope shared by the two gangliosides. Comparing the structures of several glycolipids (Fig. 1) and their pattern of reactivity with the patient's monoclonal IgM suggests that the reactive epitope could be the carbohydrate sequence including the disaccharide Gal ($\beta$1$\rightarrow$3) GalNAc which is found in GM1, GD1b and asialo GM1. Two similar cases of motor neuron disease with a serum monoclonal IgM reacting with the same determinant were described by Ito & Latov (1988). This epitope could be localized in the human gray matter, in the anterior horn of the spinal cord and in the neuromuscular junction (Latov *et al.*, 1988). Antibody binding to motor neurons was also described in experimental models of motor neuron disease in guinea-pigs immunized with isolated spinal motor neurons (Engelhardt & Joo, 1986). Antibodies to these components could act directly at their site of attachment or become internalized by axonal uptake (Ritchie *et al.*, 1986). It has not yet been demonstrated whether the anti-ganglioside GM1 and GD1b antibodies are implicated in the motor neuron disease. Low titre serum antibodies to GM1 have rarely been reported in apparently neurologically healthy individuals and some higher titres have also occurred in multiple sclerosis, lupus erythematosus and after central nervous damage (Endo *et al.*, 1984; Latov *et al.*, 1988; Pestronk *et al.*, 1988a, b). Overall, there is a strong correlation between the selective motor nerve involvements accompanying monoclonal IgM gammopathies and the occurrence of high titre anti-GM1 and anti-GD1b antibodies (Freddo *et al.*, 1986; Latov *et al.*, 1988; Kusunoki *et al.*, 1989). The improvement of neurological symptoms in a patient following therapeutic reduction of the serum antibody concentration (Latov *et al.*, 1988) may support the role of monoclonal IgM in this neurological disease. The mechanism of the impairment of the motor neurons in these processes remains to be determined. Its elucidation could perhaps also provide some insight into a better understanding of the pathogenesis of other motor neuron diseases such as amyotrophic lateral sclerosis, in which an immunological participation has been suggested by the finding of low titres of anti-ganglioside antibodies (Rowland, 1987; Pestronk *et al.*, 1988a, 1989). It should be pointed out that the patient's serum IgM level was normal and that the very small monoclonal band visible on thin-layer agarose electrophoresis would have remained undetected by conventional cellulose acetate electrophoresis. Hence, search for small amounts of monoclonal IgM by Western blotting and for serum reactivity against glycolipid determinants should be performed in all unexplained motor neuron syndromes, since it may indicate specific therapy by plasma exchanges.

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