Serum IgG antibodies to P0 dimer and 35 kDa P0 related protein in neuropathy associated with monoclonal gammopathy

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Background: Peripheral neuropathies (PN) associated with monoclonal gammopathy (MG) are widely considered as autoimmune disorders, but the putative role of incriminated antigens is still not understood. **Objective:** Fifty five patients with PN associated with MG were studied to investigate whether new antigens could be found, and to evaluate their relation to clinical manifestations.

Methods: An immunological study was conducted on patient sera to identify autoreactivities against nerve proteins by western blotting. Antigen proteins were purified and analysed by proteomic tools. Correlation with ultrastructural and clinical features was then studied.

Results: Of the 55 patients suffering from PN associated with MG, 17 exhibited IgG autoantibodies directed against peripheral nerve proteins of 35, 58, and 60 kDa. N-terminal microsequencing and mass spectrometry analyses of the 35 kDa protein revealed perfect peptidic matching with 47% of the amino acid sequence of P0, whereas the 58 and 60 kDa proteins were identified as the reduced and non-reduced forms of a P0 dimer. Deglycosylation did not affect IgG binding to the 35 kDa P0 related protein, suggesting a peptidic epitope. In contrast, deglycosylation abolished IgG recognition of the P0 dimer protein, so that a carbohydrate moiety may be implicated in the epitope formation. This confirmed the existence of two different types of IgG, one recognising the 58 and 60 kDa proteins and one directed against the 35 kDa protein.

Conclusions: This is the first report of antibody activity directed against the dimeric association of P0. Although P0 oligomerisation and adhesion properties play a crucial part in the myelin sheath compaction, the pathogenic significance of these autoantibodies needs further investigations to be elucidated.

Peripheral neuropathies (PN) are commonly associated with monoclonal gammopathy (MG) or paraproteinaemia, and the clinical spectrum ranges from acute to chronic disorders.^{1–3} Monoclonal proteins are six to ten times more frequent in patients with idiopathic peripheral neuropathies than in the general population⁴ suggesting a pathogenic role for these antibodies. More than 50% of patients with peripheral neuropathy and IgM MG have monoclonal IgM antibodies that bind to myelin associated glycoprotein (MAG).⁵ The recognised epitope is the carbohydrate HNK-1/L2, which is expressed by MAG but also by other myelin components.^{6–8} Such epitopes are rather common, so that MAG is not the only antigen molecule recognised by these IgM autoantibodies.^{9–10} Gangliosides¹¹ and other neural cell adhesion molecules such as L1, N-CAM, and J1 contain an identical or closely related epitope.^{12–14}

Autoantibodies have been identified against a few other antigens of the peripheral nervous system (PNS) such as peripheral myelin protein 22,¹⁵ myelin basic protein, protein 2^{9–10} or protein 0 (P0).^{16–17} However, the aetiology of many neuropathies associated with MG remains undetermined.

We immunologically studied 55 patients suffering from PN associated with MG to investigate whether new antigens could be found. Seventeen cases exhibited IgG autoantibodies against PNS proteins of 35, 58, and 60 kDa. We performed antigen identification by proteomic methods, and we looked for correlations with clinical and morphological data.

METHODS

Patients

Sera from 55 patients with PN associated with MG were collected from 1989, and stored at –20°C until screening for

antibodies directed against peripheral myelin proteins. There were 44 IgM MG, 9 IgG MG, and 2 biconal IgG and IgM gammopathies. To assess antibody specificity, we tested sera from patients with IgM MG without neuropathy (12 cases with non-nephrotic proteinuria) and from patients with neurological disorders without MG (diabetic neuropathy in three, Guillain-Barré syndrome (GBS) in three, neuropathy of unknown aetiology in 10). Control sera consisted of 20 healthy subjects.

Morphological studies

Most patients with PN and MG underwent a superficial peroneal nerve biopsy on the antero-external surface of the leg. One fragment was immediately fixed by immersion in 2.5% buffered glutaraldehyde and postfixed in osmium tetroxide. Epon embedded ultrathin sections were prepared for electron microscope examination. A second peripheral nerve specimen was frozen, and direct immunofluorescence was performed on cryostat sections using anti-IgA, anti-IgG, and anti-IgM sera, as well as anti-kappa and anti-lambda light chain sera (Dako, Trappes, France).

Enzyme linked immunosorbent assay

Anti-MAG IgM antibody kit (Bühlmann Laboratories, Allschwil, Switzerland), was used for detection of IgM anti-MAG in the patient sera. In brief, standard sera and patient sera, diluted

Abbreviations: PN, peripheral neuropathies; MG, monoclonal gammopathy; MAG, myelin associated glycoprotein; PNS, peripheral nervous system

1/1000, were added in duplicate to the pre-coated plate and incubated for two hours at 4°C. Enzyme labelled secondary antibody solution was added and incubated for another two hours. Tetramethylbenzidine substrate solution was pipetted to each well, and incubated in dark for 30 minutes before the plate was read at absorbance 450 nm in a microplate reader. Duplicate average absorbance values were plotted against the Bülhman titres units of the standards using line/log graph paper.

Immunoblot studies

Immunoblot studies were first carried out on human PNS extracts, and then because of the large amounts of protein needed for further characterisation; extensive immunoblot studies, protein isolation and characterisation were performed on rat PNS extracts. Human sciatic nerves were obtained at necropsy of a non-neurological patient. Rat PNS was obtained from adult Sprague-Dawley rats bred in our animal house. Sciatic nerves were ground up in TRIS buffered saline containing anti-proteases (1 mM PMSF, 1 µg/µl aprotinin, 1 µg/µl leupeptin). Protein extracts (480 µg) were loaded in a 6 cm wide one well comb and separated by SDS-PAGE in 10% polyacrylamide gels, then transferred electrophoretically onto Immobilon P nylon membranes (Millipore, St Quentin, France) for immunoblot analysis. After saturation for one hour in TRIS buffered saline containing 3% milk, blots were cut into 0.5 cm wide stripes, so that 40 µg of PNS proteins were separated per lane. Blots were incubated overnight with the different patient sera diluted 1:100. After washing, immunolabelling was visualised with peroxidase conjugate goat antihuman IgG and/or IgM diluted 1:1000 (Bio-Rad, Marnes-la-coquette, France). MAG immunolabelling was performed with anti-MAG goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA), secondary antibodies were rabbit anti-goat IgG peroxidase-conjugate (Sigma, St Quentin, France). Negative control consisted in omitting the patient serum.

Protein isolation

To highly enrich in fractions the proteins recognised by the autoantibodies, we realised protein electro-elution as previously described.¹⁸ Briefly, we separated PNS protein extracts by SDS-PAGE on 12% acrylamide large size gel to emphasise protein separation in the molecular weight range of 20 to 80 kDa. After migration, gels were stained with Coomassie blue (R250 brilliant blue, Sigma, St Quentin, France) for two hours, excessive dye was washed out with a solution of water/methanol/acetic acid (13/6/1, v/v/v) until enough contrast was reached. Then, antigen protein bands were cut off and sliced in 2 mm³ cubes to enable maximum soaking buffer (0.4 M NH₄HCO₃, 0.05% SDS) absorption. After 12 hours of immersion, gel fragments were placed in the electro-elution unit for five to eight hours to reach complete elution of proteins. Proteins were reduced by 2% beta-mercapto-ethanol addition and heating at 100°C for five minutes. Deglycosylation was performed by protein incubation at 37°C for 18 hours with 50 mM/µg peptide-N-glycosidase F (PNGase F, Roche Diagnostics, Meylan, France).

N-terminal microsequencing

N-terminal amino acid sequence analyses were done on an automatic sequencer (Applied Biosystems 491 Procise) and phenylthiohydantoin-amino acids were identified with an online HPLC system.

Sequence cycles were run according to the standard protocol provided by the manufacturer.

Mass spectrometry analysis

Protein digestion

The electroeluted samples were digested by trypsin in digestion buffer TRIS-HCl 0.2 M pH 9.0. The digestion was carried out for four hours at 28°C and stopped by freezing the samples.

Sample preparation

The proteolytic digests were desalted and concentrated by a micro-chromatography over C18 ZipTips (Millipore, St Quentin, France) for desalting and concentrating. The peptides were eluted with 2 µl of solvent 70% acetonitrile/0.1% Tri-fluoro-acetic acid (TFA) in water. α -cyano-4-hydroxycinnamic acid (Sigma, St Quentin, France) was used as a matrix, prepared as a saturated solution in 50% acetonitrile/0.1% TFA in water. Peptide mixtures were mixed 1:1 with the matrix solution. Samples were prepared with the dried drop-let method on a stainless steel target with 26 spots.

MALDI mass spectrometry

Mass spectrometry analyses were performed on a Bruker REFLEX III MALDI-TOF in the reflectron mode with a 20 kV acceleration voltage and a 23 kV reflector voltage. External mass calibration was achieved with a mixture of eight peptides having masses ranging from 961 Da (fragment 4–10 of adrenocorticotrophic hormone) to 3495 Da (β chain of oxidised bovine insulin). For PSD (post source decay) experiments, the reflector voltage was stepped down in 10 to 12 steps, starting from 30 kV, in order to collect fragment ions from the precursor to immonium ions.

Database searching for rat proteins

Monoisotopic mass values were used to search the SwissProt database with a 0.2 Da tolerance by means of the MSFit search engine (<http://prospector.ucsf.edu>) while restricting the species to *Rattus Norvegicus*. Confirmation of the P0 myelin protein identification was obtained by means of the MASTag search engine from the same web site, using the average mass values of fragment ions obtained from PSD spectra.

RESULTS

Immunological and morphological findings are summarised in table 1 with clinical, haematological, and electrophysiological data.

Immunological findings

Immunoblot studies evidenced serum autoantibodies that reacted with PNS myelin proteins of different molecular weights. Of the 55 patients suffering from PN associated with MG, 17 (31%) exhibited IgG autoantibodies directed against peripheral nerve proteins of 35, 58, and 60 kDa on both human and rat PNS extracts (fig 1 lane b and fig 2 lanes a, b). These 17 patients were selected for further immunological investigations. IgG antibodies directed against the protein doublet of 58 and 60 kDa were found in all the 17 cases (fig 1 lane b and fig 2 lane c). Determination of a titre for this 58 and 60 kDa antibody activity was performed by serial patient serum dilutions; titres' ranges from 1:400 to 1:1600. In contrast, IgG reactivity against the 35 kDa protein (fig 1 lane b and fig 2 lane c) was found in 10 of 55 (18%) patients, and in 4 of the 17 selected cases (numbers 5, 7, 9, 17). No IgM antibodies directed to these antigens were detected in any patient serum.

IgG directed against the protein doublet of 58 and 60 kDa were not found in any of the 10 patients with MG and no PN; they were present in 1 of 13 (8%) patients with PN and no MG (PN of unknown aetiology). IgG directed against the 35 kDa protein were found in 2 of 10 (20%) patients with MG and no PN and in 3 of 13 (23%) patients with PN and no MG (one GBS, two of unknown aetiology). No IgG directed against the 35 kDa protein or the protein doublet of 58 and 60 kDa were found in any of the 20 healthy subjects.

By χ^2 analysis, the frequency of IgG directed against the 35 kDa protein was significantly higher in patients with PN associated with MG than in healthy subjects ($p < 0.05$), but not than in patients with PN and no MG or those with MG and no PN. The frequency of IgG directed against the protein doublet of 58 and 60 kDa was significantly higher in patients with PN

Table 1 Clinical, immunological, and morphological data

Case	Sex	Age (y)	PN clinic	Electro	Haematol	LC	MAG /titre (BTU)	Other nerve proteins (kDa)	USE		IF
									WML	E deposits	
1	M	63	SM	DM	MGUS	κ	Yes / 124544	58 and 60	++	–	M-IgM
2	M	80	SM	AX	MGUS	κ	Yes / 23003	58 and 60	++	–	M-IgM
3	M	50	SM	AX/DM	MGUS	κ	Yes / 237925	58 and 60	+	–	M-IgM
4	M	86	SM	AX	MGUS	κ	Yes / 131639	58 and 60	++	–	M-IgM
5	F	94	SM	AX/DM	MGUS	κ	Yes / 129146	35, 58, 60	+	–	M-IgM
6	F	60	SM	AX/DM	WM	κ	Yes / 150093	58 and 60	+	–	Negative
7	F	58	SM	DM	MGUS	κ	Yes / 1363	35, 58, 60	ND	ND	ND
8	F	53	SM	AX/DM	WM	κ	No	58 and 60	–	–	Negative
9	M	47	SM	AX/DM	MGUS	κ	Yes / 125798	35, 58, 60	+	–	Negative
10	M	69	SM	AX	WM	κ	No	58 and 60	–	Granular	E-IgM
11	M	82	SM	AX	WM	κ + cryo	No	58 and 60	–	Tubular	E-IgM
12	M	64	SM	AX/DM	MGUS	κ	Yes / 90316	58 and 60	+	–	M-IgM
13	F	62	SM	AX/DM	MGUS	κ	Yes / 184962	58 and 60	+	–	M-IgM
14	F	63	PRN	AX/DM	BGUS	κ + λ	Yes / 80700	58 and 60	+	–	Negative
15	F	76	SM	AX/DM	MGUS	λ	Yes / 135406	58 and 60	+++	–	M-IgM
16	F	81	SM	DM	MGUS	λ	Yes / 86867	58 and 60	+	–	Negative
17	F	64	SM	AX/DM	WM	κ	Yes / 133533	35, 58, 60	++	–	M-IgM

AX, axonal; BGUS, biconal gammopathy of undetermined significance; BTU, Bühlmann titre unit; cryo, cryoglobulin; DM, demyelinating; E deposits, endoneurial deposits; E-IgM, endoneurial IgM deposits; electro, electrophysiological study; haematol, haematological status; IF, immunofluorescence study; LC, light chain type; MAG, antibodies directed against myelin associated glycoprotein; MGUS, monoclonal gammopathy of undetermined significance; M-IgM, IgM binding on myelin sheaths; ND, not done; other nerve proteins, antibodies directed against other peripheral nerve proteins; PN clinic, clinical type of peripheral neuropathy; PRN, polyradiculoneuritis; SM, sensitive-motor; USE, ultrastructural examination; WM, Waldenström macroglobulinaemia; WML, widened myelin lamellae, lesions are graded from +++ (great number of typical figures) to – (absence of typical figures).

Figure 1 Immunoblot studies on human PNS extract. SDS-PAGE and western blot of human peripheral nerve protein extract. Patient no 5 serum IgG or IgM binding were revealed on lanes b and c respectively. Anti-MAG immunolabelling was performed on lane d (arrow head). Incubation with secondary antihuman IgG and IgM antibodies alone, revealed high molecular weight proteins corresponding to immunoglobulins contained in the human PNS extract (lane a).

associated with MG than in healthy subjects ($p<0.005$) or patients with PN and no MG ($p<0.05$) or those with MG and no PN ($p<0.05$).

In 14 of these 17 selected cases, an intense IgM autoreactivity was observed on human PNS extract with a protein band migrating approximately at 90 kDa (fig 1 lane c), and comigrating with MAG (fig 1 lane d). These results were identical on rat PNS extracts (fig 2 lanes a, b) including comigration with MAG (fig 2 lane e). Autoantibodies directed against this protein were of the IgM isotype (fig 2 lane d). This result was confirmed by the anti-MAG ELISA assay.

Figure 2 Immunoblot studies on rat PNS extract. SDS-PAGE and western blot of rat peripheral nerve protein extract. Lanes a and b, patient no 1 and no 5 serum IgG + IgM binding; patient no 5 serum IgG or IgM binding were revealed on lanes c and d respectively. Anti-MAG immunolabelling was performed on lane e. No binding was observed after incubation with control serum (lane f) or secondary antibodies alone (lane g).

Protein characterisation studies
Protein doublet of 58 and 60 kDa

In absence of reducing agent, after SDS-PAGE from PNS protein extract, we detected a protein doublet of 58 and 60 kDa on Coomassie blue staining. N-terminal microsequencing of the 58 kDa showed that the sequence of the first five amino acids was IVVYT. When run against the protein database, this sequence revealed 100% identity to rat P0. Mass spectrometry analysis of this protein after trypsin digesting gave a P0 signature. The P0 protein has the capacity to oligomerise and form homodimers, trimers, and tetramers, so that the 58 kDa protein would be the dimer form of P0.

So as to confirm this result, we purified the monomeric form of P0 and run it on SDS-PAGE in both native and reducing conditions, and we visualised P0 oligomer products by silver nitrate staining. In native conditions, we observed a protein band of 30 kDa and a protein doublet of 58 and 60 kDa (fig 3 lane a). In reducing conditions, the 30 kDa protein shifted to 32 kDa and the doublet band became a single band

Figure 3 Antigen proteins isolation. Silver nitrate staining after SDS-PAGE. Monomeric P0 protein was purified by electroelution and oligomer products were separated in native conditions (lane a), after reduction by beta-mercapto-ethanol (lane b), or after both deglycosylation by PNGase F and reduction (lane c).

migrating at 58 kDa (fig 3 lane b). The mass spectrometry analysis of these proteins gave a classic P0 signature. This experiment favours the fact that the 58 kDa protein is a dimer of P0 and, that the 60 kDa protein is the non-reduced form of that P0 dimer. So as to finally confirm the immunoreactivity of the reduced and non-reduced forms of P0 dimer, we blotted on the same membrane the P0 oligomerisation products, in native and reducing conditions, as well as a PNS protein extract. After incubation with a patient serum, we observed an immunoreactivity with a doublet band migrating at 58/60 kDa in both the PNS protein extract and the P0 oligomerisation products formed under non-reducing conditions (fig 4 lanes d and a respectively). In the oligomerisation products formed in reducing conditions, only the 58 kDa band was revealed by patient autoantibodies (fig 4 lane c). We demonstrated here the presence of a specific epitope on the P0 dimer that is absent on the other oligomeric forms of P0. To elucidate the structural origin of that epitope, we analysed the effect of deglycosylation on the P0 dimer immunoreactivity. After deglycosylation and reduction, the P0 oligomer products were separated on SDS-PAGE and silver nitrate staining revealed two protein bands at 30 and 52 kDa, corresponding to the monomeric and dimeric forms of P0 after a shift of respectively 2 and 6 kDa (fig 3 lane c). Immunoblot analysis of these two protein bands with patient serum revealed no immunoreactivity (fig 4 lane b), so that deglycosylation, without destroying the P0 dimer, abolished the epitope recognition by autoantibodies of patient serum.

Protein of 35 kDa

N-terminal amino acid analysis of the 35 kDa protein showed that the sequence was IVVYTD in the first six amino acids, producing a 100% identity with rat P0. With mass spectrometry analysis, we managed to demonstrate perfect peptidic matching with 47% of the amino acid sequence of P0. To clarify the relation between these two proteins, we analysed the changes in their apparent molecular weights after reduction and deglycosylation. As previously described^{19,20} deglycosylation produced an approximately 4 kDa decrease on both P0 and the P0-like protein, whereas reduction caused a same 2 kDa shift in migration of both proteins, still without change in reactivity with the patient serum.

Clinical, electrophysiological, and morphological findings

The 17 patients (31% of 55) presenting autoreactivities against the P0 dimer doublet of 58 and 60 kDa had either an IgM

Figure 4 Antigenicity of P0 oligomer products. SDS-PAGE and western blot of P0 oligomer products in native conditions (lane a), after reduction by beta-mercapto-ethanol (lane b), or after both deglycosylation by PNGase F and reduction (lane c), peripheral nerve protein extract in native conditions (lane d). Membrane was incubated with patient no 3 serum and IgG binding was revealed by peroxidase conjugate antibodies.

monoclonal gammopathy of undetermined significance (IgM-MGUS) (11 cases), Waldenström macroglobulinaemia (WM) (five cases) or IgM biclonal gammopathy (one case) as detected by immunofixation electrophoresis. There were eight men and nine women, and their age ranged from 47 to 94 years. At electrophysiological examination, 16 patients presented a sensori-motor peripheral neuropathy that was purely axonal in four cases, prominently demyelinating in three, and myelino-axonal in nine. One patient suffered from a myelino-axonal polyradiculoneuritis. Of these 17 patients, the four cases presenting autoreactivities against the 35 kDa P0 related protein corresponded to three MGUS (cases no 5, 7, 9) and one WM (case no 17).

In 16 of 17 patients, nerve biopsy specimens were available. Electron microscopic examination revealed demyelination with a widening of myelin lamellae in 13 cases (no 1 to 6, 9, 12 to 17) corresponding to those with serum anti-MAG activity. This pattern was mild in eight cases (no 3, 5, 6, 9, 12–14, 16), marked in four (no 1, 2, 4, 17), and prominent in one (no 15). Among these cases, nine (no 1 to 5, 12, 13, 15, 17) presented a binding of IgM on myelin sheaths at immunofluorescence study. In the three other patients (cases no 8, 10, 11), without serum anti-MAG activity, electron microscopic examination showed axonal lesions. At immunofluorescence study, cases no 10 and 11 presented IgM endoneurial deposits. These endoneurial deposits proved to be tubular at electron microscopic examination in the patient with WM and cryoglobulin (no 11).

DISCUSSION

The 35 kDa protein is a P0-like protein sharing a large homology in amino acid sequence with P0 but the immunogenic epitope is still undetermined.^{19,20,22,23} The 35 kDa protein was very immunoreactive on western blot although Coomassie's blue staining of SDS-PAGE from PNS extracts revealed a minor quantity of that protein compared with the P0 protein.

The 58 kDa protein gives a perfect P0 signature when analysed by both N-terminal sequencing and mass spectrometry analysis after protease digesting. The myelin protein P0 is a transmembrane protein with an extracellular domain, 124 amino acids long, and with characteristic features of an immunoglobulin variable domain.^{24,25} The capacity of P0 monomers to oligomerise has well been described^{26,27} and its tetrameric association is probably the functional form of the protein in the myelin sheath.²⁷ Thus, the P0 tetramer from one

membrane surface of the spiral wraps of the myelin sheath is linked to another P0 tetramer on the opposite membrane surface. The linkage of these tetramers together should be attributable to both homophilic protein-protein contact and direct membrane intercalation of a tryptophan side chain, permitting a perfect intermembrane spacing.²⁷ Therefore, P0 is the major molecule involved in myelin sheath compaction. Autoreactivity against the P0 monomer was previously described,^{16, 17} and the authors assumed that it was attributable to the presence of the HNK-1/L2 carbohydrate epitope on P0. Thus, the IgM anti-MAG antibodies directed against this very same epitope would also recognise the protein P0. Bollensen *et al* and Yan *et al*, described autoreactivity against the P0 monomer but no reactivity was found against the P0 dimer or tetramer. In our study, we only observed IgG reactivity against the P0 dimer but not against either the monomer or the tetrameric form of P0. To our knowledge, this is the first time that immune reactivity against the P0 dimer is demonstrated. This observation may indicate the existence of a particular epitope on the P0 dimer structure. Thermodynamic analysis conducted by Shapiro *et al* on the P0 oligomerisation revealed temperature non-dependent dimerisation of P0 whereas its tetramerisation is temperature dependent, suggesting that the two associations may have very different structural origins. This difference may explain the existence of a conformational epitope on the P0 dimer that does not exist on the other forms of P0. We showed that in native PNS extracts, both the reduced and non-reduced forms of the P0 dimer are immunoreactive, indicating that the conformational modification induced by reduction is not sufficient to avoid epitope recognition. In contrast, reduction followed by deglycosylation results in the disappearance of immunogenicity of the P0 dimer, causing a shift of 6 kDa without destroying dimer formation. This latter result may indicate either that the epitope is totally or partially on the carbohydrate moiety, or that the structural changes resulting from the deglycosylation alter this specific epitope.

Serum anti-MAG autoreactivity was found in 14 of the 17 selected patients and was associated with a widening of myelin lamellae in patients who underwent a peripheral nerve biopsy. Conversely, three patients had neither anti-MAG antibodies nor widened myelin lamellae, as usually observed.²¹ In contrast with serum anti-MAG autoantibodies that are always associated with widened myelin lamellae at ultrastructural level, autoantibodies directed against the 35 kDa P0 related protein or against the P0 dimer doublet of 58 and 60 kDa are not yet correlated with any clinical or morphological features.

We found a significantly higher frequency of IgG to the 35 kDa P0 related protein in patients with PN associated with MG than in healthy subjects, but not than in patients with PN and no MG or those with MG and no PN. Thus, the pathological significance of IgG to the 35 kDa P0-like protein in PN associated with MG has still to be determined.

We found a significantly higher frequency of anti-P0 dimer IgG in patients with PN associated with MG than in healthy subjects or patients with PN and no MG or those with MG and no PN. Although a previous study¹⁷ demonstrated the demyelinating effects of anti-P0 IgG antibody injections in rat sciatic nerves, the pathogenic significance of the anti-P0 dimer autoantibodies needs further investigations to be elucidated.

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