Peripheral neuropathy associated with monoclonal IgM anti-Pr₂ cold agglutinins

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Abstract

A patient with a chronic, large fibre sensory neuropathy had an immunoglobulin M λ monoclonal paraprotein reactive at titres in excess of 1/10⁵ with NeuNAc(a2-8)NeuNAc(a2-3)Gal configured disialosyl groups present on the gangliosides GD1b, GT1b, GQ1b, and GD3. The paraprotein showed weaker reactivity with GD1a, GM3, and LM1 but no reactivity with GM2, GM1, or asialo-GM1. In addition, the paraprotein had cold agglutinating activity with anti-Pr₂ specificity, Pr₂ being an antigenic determinant on membrane glycoproteins or glycolipids in erythrocytes or both. A large fibre sensory neuropathy with monoclonal anti-disialosyl antibodies is an increasingly recognised form of paraproteinaemic neuropathy.

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Many recent studies have focused on peripheral neuropathy syndromes associated with immunoglobulin (IgM) paraproteinaemia.¹ The specificity of the paraproteins is almost invariably directed to carbohydrate determinants present on different glycoproteins and glycolipids distributed in neural tissue.² Recognised clinical and serological associations include (a) a chronic demyelinating sensorimotor neuropathy associated with the paraprotein directed against a sulphated glucuronic acid epitope shared by sulphated glucuronyl paragloboside, myelin-associated glycoprotein and the myelin P0 protein,¹ and (b) a multifocal motor neuropathy with the paraprotein directed against GM1 ganglioside and related glycoconjugates containing a terminal Gal(β 1-3)GalNAc configuration.³ Although well described, these associations are not absolute or exclusive in that substantial overlap exists between the clinical and serological features of these patients.⁴

Among patients with paraproteinaemic neuropathy in general, there may yet be other clinical and serological associations that remain to be identified. In this report we describe a patient with a large fibre neuropathy with prominent sensory symptoms and ataxia, and clinically absent motor involvement in whom an IgM paraprotein is present that reacts predominantly with disialosyl groups present on gangliosides found in neural tissue. In addition, the paraprotein reacts with an antigenic determinant termed

Pr₂, which is present on glycoproteins and glycolipids in red blood cell membranes.⁵ The specificity of this paraprotein is similar to that of the mouse monoclonal A2B5 which is widely used as a marker for differentiating populations of neuronal and glial cells.^{6,7} The clinical and serological features of this case are remarkably similar to four other cases that have been recently reported.⁸⁻¹¹ Taken together, these reports suggest that this association may represent another phenotype within the spectrum of paraproteinaemic neuropathy.

Materials and methods

CASE REPORT

This 61-year-old lady was first noted by her local blood transfusion laboratory to have cold agglutinins when examined in 1980, aged 49; these are still present. She presented in 1986, aged 55 with paraesthesiae and numbness affecting initially her feet and later her hands. She also had difficulty with fine finger movements and unsteadiness while walking, particularly in the dark. She was only able to walk with the aid of a walking frame. She does not have symptoms or signs of cold agglutinin disease.

On examination she had an area of perioral sensory loss to pinprick but otherwise normal cranial nerves. In the limbs, there was no wasting or weakness. She was areflexic. Glove and stocking sensory loss to light touch and pinprick was present with an additional area of sensory loss over the mid-abdominal region and sternum. Joint position sense was markedly impaired in the digits, and vibration sense absent to the elbows and ribs. Deep pain and thermal sensation was normal. She had prominent pseudoathetosis of the outstretched hands and gait ataxia with a positive Romberg test.

Electrophysiological examination (kindly performed by Dr G Jamal) of motor nerves revealed prolonged distal motor latencies and F wave latencies (right common peroneal latency: 6.9 ms, F wave: 76.4 ms; right tibial latency: 5.8 ms, F wave: 83.9 ms; right median latency: 5.2 ms, F wave: 43.2 ms). Motor conduction velocities in the leg and arm were reduced (right common peroneal: 34 m/s; right tibial: 30.5 m/s; right median: 37.5 m/s). All sensory nerve action potentials examined (right sural, median, ulnar, and radial) were absent. Needle electromyography revealed borderline neurogenic changes. Visual evoked potentials were normal. CSF

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examination was normal with a protein of 0.36 g/l.

Sural nerve biopsy (kindly performed by Dr D Doyle) demonstrated a severe reduction in large myelinated fibres with preservation of the unmyelinated small fibre population. No active fibre degeneration or demyelination was observed although there was a degree of axonal regeneration with some isolated fibres surrounded by thin myelin sheaths suggestive of remyelination. There were no hypertrophic changes and no inflammatory cells or immunoglobulin deposits were noted.

Laboratory tests revealed a normal full blood count, an elevated erythrocyte sedimentation rate at 96 mm/h, elevated serum IgM at 6.4 g/l with an IgM λ paraprotein and normal serum IgG and IgA. Bone marrow examination, urine analysis for Bence Jones proteinuria and skeletal survey were normal.

Studies on the serological reactions of her cold agglutinins demonstrated that they: (a) strongly agglutinated all 'common phenotype' red blood cells including cord cells; (b) did not agglutinate neuraminidase-treated human or canine red blood cells; (c) very weakly agglutinated papain-modified red blood cells; (d) very weakly agglutinated M^aM^b red blood cells; (e) strongly agglutinated 'common phenotype' red blood cells modified by mild treatment with sodium metaperiodate and (f) strongly agglutinated canine red blood cells both before and after modification with papain. These characteristics are typical of anti-Pr₂ specificity.⁵

She was treated with a single course of plasma exchange (total exchange 15 litres) followed by low dose oral cyclophosphamide (150 mg per week in three divided doses). Although she reports an improvement in her sensory symptoms, hand function, and mobility after eight months of treatment, there is no quantifiable evidence of this and she remains significantly disabled.

ANTI-GANGLIOSIDE ANTIBODY ASSAYS

Polystyrene multiwell plates (Immulon 2, Dynatech Laboratories) were coated with the purified gangliosides or glycolipids GM1, GM2, GM3, GD1a, GD1b, GT1b, GD3, asialo-GM1 (Sigma Chemical Co., Poole, United Kingdom), and GQ1b (Calbiochem UK, Nottingham) by the evaporation at room temperature of 200 ng glycolipid in 100 μ l of methanol per well. GT1b (purchased from Sigma) was noted to contain trace amounts of GQ1b. Wells were filled with 200 μ l PBS-BSA (pH 7.4, 3% bovine serum albumin) and left for two hours at 4°C, emptied, and 100 μ l of serially diluted test serum in PBS-0.1% BSA was added in duplicate to glycolipid coated and to blank wells which had been soaked in methanol and blocked in the same way. The plate was left to incubate with serum overnight at 4°C, washed 10 times in PBS, and 100 μ l of a 1/3000 dilution of peroxidase-conjugated rabbit anti-human IgM or IgG (Dako) or a 1/2000 dilution of anti-human κ or λ light chain antibody (Sigma)

was added. The plate was incubated for a further four hours at 4°C, washed 10 times in PBS and the plate developed by adding substrate solution consisting of one 20 mg o-phenylenediamine tablet (Sigma) in 60 ml 0.1M citrate buffer pH5.5 with 20 μ l of 30% hydrogen peroxide. The reaction was stopped with 20 μ l of 4M sulphuric acid after 20 minutes and the optical densities read in an MR5000 plate-reader (Dynatech). Antibody titres were calculated by subtracting the optical densities of the blank well from those of the ganglioside-coated well and plotting the resultant optical density versus the dilution on semi-log paper. The dilution at which the baseline optical density (defined as three standard deviations above that obtained with second antibody alone) was crossed was assigned as the antibody titre.

THIN LAYER CHROMATOGRAPHY (TLC) OVERLAY

Gangliosides were separated on aluminium backed Kieselgel 60 WF₂₅₄ S HPTLC plates (Merck) in the solvent system chloroform:methanol:0.25% potassium chloride in a volume ratio of 50:40:10. An aliquot of a standard ganglioside mixture containing LM1 was kindly provided by Dr D Marcus, Baylor College, Houston, Texas, United States. The chromatograms were air-dried, dipped in 0.1% polyisobutylmethacrylate beads for 20 s at 40°C, air dried, blocked with 1% BSA in PBS pH 7.5 for one hour, incubated with the test serum in appropriate dilutions in blocking buffer for four hours at 4°C, washed five times in blocking buffer, incubated for 2–3 hours at 4°C with peroxidase-conjugated rabbit anti-human IgM diluted 1/3000 (Dako) and washed five times in blocking buffer. Plates were developed autoradiographically for 5–30 s using an enhanced chemiluminescence procedure according to manufacturer's instructions (Amersham, United Kingdom).

IMMUNOABSORPTION AND PURIFICATION STUDIES

Cold agglutinating antibodies were purified from normal human red blood cells as follows: group O red blood cells were washed four times in PBS (0.01M, pH 7.4). One volume of packed cells was then incubated with one volume of plasma at 4°C with occasional mixing for one hour. This produced an aggregated pellet which was centrifuged at 4°C (five minutes, 2500 rpm) and washed six times in ice cold PBS with mechanical deagglutination at each wash. Following the last centrifugation, the wash supernatant was removed and replaced by one volume of PBS and the cells were gently mixed at 40°C for five minutes. This mixture was centrifuged immediately (in buckets containing water at 40°C) and the supernatant, which contained a fine milky precipitate was transferred to a clean tube. This material is referred to as RBC-IgM. Immunoabsorption studies were also carried out by pre-incubation of appropriate dilutions of RBC-IgM with enzyme-linked immunosorbent assay (ELISA) plates

Table 1 Ganglioside structures

Compound	Carbohydrate sequence
GD1b	Gal(β 1-3) GalNAc(β 1-4) Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc(α 2-8) NeuNAc
GT1b	Gal(β 1-3) GalNAc(β 1-4) Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc(α 2-8) NeuNAc
GQ1b	Gal(β 1-3) GalNAc(β 1-4) Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc(α 2-8) NeuNAc
GD3	Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc(α 2-8) NeuNAc
GD1a	Gal(β 1-3) GalNAc(β 1-4) Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc
GM3	Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc
LM1 (= SPG)	Gal(β 1-4) GlcNAc(β 1-4) Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc
GM1	Gal(β 1-3) GalNAc(β 1-4) Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc
GM2	GalNAc(β 1-4) Gal(β 1-4) Glc(β 1-1) Ceramide NeuNAc(α 2-3) NeuNAc
Asialo-GM1	Gal(β 1-3) GalNAc(β 1-4) Gal(β 1-4) Glc(β 1-1) Ceramide

coated with GD3 and using the harvested supernatant for TLC overlay studies. Immunoglobulin isoelectric focusing and immunoblotting was based on standard techniques.¹²

Results

Table 1 shows the structures of the gangliosides discussed below for reference. Examination of the patient's serum by ELISA

Figure 1 Enzyme linked immunosorbent assay (ELISA) demonstrating binding of red blood cell purified immunoglobulin M (RBC-IgM) to a panel of gangliosides.

demonstrated IgM antibodies reactive with gangliosides containing NeuNAc(α 2-8) NeuNAc(α 2-3) configured disialosyl groups at titres in excess of 1/10⁵. The highest titres were thus obtained with GD1b (1/850,000), GQ1b (1/450,000), GT1b (1/300,000), and GD3 (1/260,000). Weak reaction was observed with GD1a (1/570) and GM3 (1/660) which contain a terminal NeuNAc(α 2-3)Gal(β 1-) structure. The monosialo series gangliosides GM1 and GM2 gave no reaction; neither did asialo-GM1. Although the range of gangliosides available for screening was not extensive, these results indicate that the NeuNAc(α 2-8)NeuNAc(α 2-3) moiety is the most dominant part of the antigenic determinant and that weaker reactivity occurs with the terminal NeuNAc(α 2-3)Gal(β 1-) determinant.

ELISA assays using κ and λ light chain specific antibodies showed the reactivity to these gangliosides to be in the λ light chain fraction, consistent with the typing of the patient's serum paraprotein as IgM λ . There were no IgG anti-ganglioside antibodies.

The red blood cell purified IgM fraction (RBC-IgM) focused in IEF gels as a single paraprotein at the same isoelectric point as the paraprotein present in the serum and also exhibited the same heavy and light chain type (IgM λ) as the serum paraprotein (data not shown). In ELISA (fig 1) and TLC (fig 2) studies, the RBC-IgM reacted with GD1b,

Figure 2 Thin layer chromatogram (TLC) of mixed gangliosides immunostained with the patient's red blood cell purified immunoglobulin M (RBC IgM). Panel A is an orcinol stained TLC of a known mixture of 7 µg of each ganglioside and 10 µg of asialo-GM1 (GA1), loaded into 2 lanes to aid identification. Lanes 1 and 3 contain GM3, GM2, GM1, GD1a, GD1b, and GT1b (also containing trace amounts of GQ1b); lanes 2 and 4 contain GA1 and GD3. Panel B is the autoradiogram of the immunostained TLC, identical to panel A except that 1 µg of each ganglioside was loaded. RBC-IgM was used at a concentration of 100 ng/ml. GD3, GD1b, GT1b, and GQ1b are strongly stained; GM3 and GD1a are weakly stained; and there is no staining of GM1, GM2, or GA1.

Table 2 Clinical and serological features of reported cases of sensory neuropathy with anti-disialosyl antibodies.

	Current case	Ilyas et al	Duane et al	Arai et al	Obi et al
Reference number	-	8	9	10	11
Age (years)	62	61	67	53	77
Sex	F	M	F	M	M
Duration of symptoms (years)	7	25	1	0.5	1
Paraprotein isotype	IgM λ	IgM κ	IgM λ	IgM κ	IgM κ
Serum IgM (g/l)	8.1	↑	Normal	6.4	1.35
Cold agglutinins	anti-Pr ₂	NC	NC	anti-Pr ₂	NC
<i>Paraprotein specificity</i>					
GD1b	++	++	++	++	++
GD3	++	++	+	+	++
GT1b	++	++	-	++	++
GQ1b	++	ND	+	ND	++
GD2	ND	++	++	ND	ND
GD1a	+	-	-	++	++
GM3	+	-	-	+	+
LM1 (SPG)	+	-	-	+	+
GM2	-	ND	-	-	-
GM1	-	-	-	-	-
<i>Clinical features</i>					
Paraesthesiae	+	+	+	+	+
Gait ataxia	+	+	-	+	+
Rombergism	+	+	+	+	-
Proprioceptive loss	+	+	+	+	+
Vibration loss	+	+	+	+	+
Areflexia	+	+	+	+	+
Motor weakness	-	-*	-	++	++
Tremor	-	-	-	+	-
<i>Electrophysiology</i>					
SNAPs	Absent	Absent	Absent	Absent	Absent
Motor NCVs	Reduced	Reduced	Normal	Reduced	Normal
DMLs	Prolonged	ND	Prolonged	ND	ND
F wave latencies	Prolonged	ND	Normal	ND	ND
EMG changes	Mild	Present	Absent	ND	Mild

*Peculiar fluctuating ptosis of right eye and upward gaze palsy.

†Mild upper limb weakness.

NC = not commented on and thus possibly not specifically evaluated; ND = not determined; SNAPs = sensory nerve action potentials; NCV = nerve conduction velocity; DML = distal motor latency.

GT1b, GQ1b, GD3, GD1a, and GM3 gangliosides in a similar pattern to that observed with whole serum. In addition, TLC studies demonstrated that the RBC-IgM reacted with purified LM1 with a similar signal intensity to GD1a and GM3. As the LM1 that we had available was pre-mixed with other gangliosides for use as a 'standard', we have been unable to determine the LM1 titre by ELISA. Absorption of the RBC-IgM fraction by pre-incubation with GD3 ganglioside followed by TLC overlay with the supernatant demonstrated complete removal of identifiable binding to GD3, GD1b, GT1b, GQ1b, GD1a, and GM3. These results indicate that (a) cold agglutination of red cells with the patient's serum yielded a single species of monoclonal antibody (RBC-IgM) that corresponded to the IgM λ paraprotein identifiable in whole serum and (b) this RBC-IgM antibody accounted for the anti-ganglioside antibody activity as described within the patient's serum.

As the serum paraprotein was known to possess strong cold agglutinating activity, we wished to establish the binding properties to gangliosides (the potential nerve target antigens *in vivo*) over a range of temperatures including body temperature. Using GD1b as antigen, the binding observed at 4°C was 100-fold greater than that at 37°C and 10-fold greater than at 22°C for the serum and the RBC-IgM. For example, at 37°C, the serum titred out at 1/10³, compared with 1/10⁵ at 4°C. Thus, although the antibody titre drops considerably when assayed at body temperature, substantial antibody binding is still retained.

Discussion

The clinical features of this case are very similar to four others that have been previously reported with this serological pattern of reactivity (table 2): all patients have symptoms comprising paraesthesiae and numbness with prominent ataxia and areflexia, suggestive of a large fibre sensory neuropathy. Sensory nerve action potentials have been uniformly absent in all cases. Although motor involvement has been sparse clinically, electrophysiological tests in our case and in others have demonstrated clear evidence of slowed motor conduction velocities with reduced amplitude, dispersed muscle action potentials, and prolonged distal motor latencies with little or no EMG evidence of denervation. These findings indicate a primary demyelinating process in motor nerves. In sensory nerves, sural nerve biopsies from other cases have demonstrated loss of large myelinated fibres, occasional axons denuded of myelin, thinly myelinated fibres suggestive of remyelination,⁸⁻¹¹ and segmental demyelination on teased fibre analysis.^{8,10} From the clinical and electrophysiological features, it is not possible to exclude the possibility that in sensory nerves there may be primary pathology of dorsal root ganglion neurons in addition to a distal axonopathy and segmental demyelination.

It would thus appear that a large fibre predominantly sensory neuropathy with anti-disialosyl antibodies may represent an homogeneous entity within the spectrum of paraproteinaemic neuropathy. This clinical pattern is not entirely unique to this serological specificity as there are clearly overlaps

with other disease patterns. For example, sensory features usually predominate in the sensorimotor neuropathy associated with anti-myelin associated glycoprotein antibodies or anti-sulfatide antibodies.¹⁴

IgG antibodies to GQ1b have recently been observed in the acute phase sera of patients with a variant of Guillain-Barré syndrome termed Miller-Fisher syndrome.^{15,16} In this disease, sensory ataxia and areflexia are prominent features and motor signs, except for ophthalmoplegia, are absent. GQ1b has two pairs of NeuNAc(a2-8)NeuNAc(a2-3) configured on terminal and internal galactoses and anti-GQ1b antibodies thus clearly have the potential to crossreact with other polysialylated gangliosides, as is the case with our paraprotein. Of the six Miller-Fisher serum samples positive for GQ1b antibodies reported by Chiba *et al*,¹⁵ only one cross-reacted with GD1b. Of the four cases reported by our group,¹⁶ three crossreacted to varying degrees with GT1b, GD1b, GD3, and GD1a, whereas one was entirely specific for GQ1b. The fine specificities of Miller-Fisher serum samples thus vary but have some similarities to the paraproteins described here. Interestingly, the case reported by Ilyas *et al*⁸ had a fluctuating ptosis and upward gaze palsy affecting the right eye which could represent an element of Miller-Fisher syndrome. Anti-GD1b IgG antibodies identified in an earlier study of Guillain-Barré serum samples were not associated with purely sensory syndromes, although their fine specificity was again slightly different from the current cases.¹⁷ A patient with acute relapsing sensory neuropathy, anti-disialosyl antibodies and an IgM κ paraprotein has also been described recently,¹⁸ and shares many of the features of our patient and the other four cases discussed here.

The acute (including Guillain-Barré syndrome) and chronic neuropathies associated with anti-GM1 antibodies are generally motor, although sensory features may occur. Although the motor neuropathy associated with anti-GM1 antibodies may react exclusively with GM1, they may also cross-react with GD1b by virtue of their common terminal Gal(β 1-3)GalNAc configuration. It is thus paradoxical that reactivity with GD1b may be associated on the one hand with motor neuropathy via the Gal(β 1-3)GalNAc moiety and on the other hand with sensory neuropathy via the NeuNAc(a2-8)NeuNAc(a2-3) moiety. Accordingly, it is equally important to think of anti-carbohydrate antibodies in terms of their reaction with the simplest structural denominator in addition to the parent molecule bearing that determinant.

The reactivity of anti-Pr₂ monoclonal IgM antibodies with gangliosides has been recognised for many years, and in human erythrocytes the Pr₂ determinants are carried by sialylated glycoproteins.^{19,20} Although clearly similar, many of these anti-Pr₂ antibodies differ very slightly in fine specificity from each other. For example, the anti-Pr₂ antibody

described by Uemura *et al*²¹ had a higher affinity for the monosialosyl structure NeuNAc(a2-3)Gal(β 1-4)Glc- or NeuNAc(a2-3)-Gal(β 1-4)GlcNAc- (present, for example, on GM3, GD1a, and LM1) compared with the disialosyl structure NeuNAc(a2-8)NeuNAc(a2-3)Gal- (present, for example on GD1b, GT1b and GD3). In contrast, the anti-Pr₂ antibodies described by us, Arai *et al*⁹ and Obi *et al*¹⁰ react more strongly with the disialosyl structure NeuNAc(a2-8)NeuNAc(a2-3)Gal- than the monosialosyl structure NeuNAc(a2-3)-Gal(β 1-4)Glc-. Thus the paraproteins among the neuropathy patients tend to have preference for the disialosyl structure as the dominant reactive determinant (see table 2). Whether these preferences can be related to antibody binding to putative target antigens *in vivo* is unknown.

The composition of gangliosides in central and peripheral nervous tissue varies greatly between species. Whole human peripheral nerve and peripheral nerve myelin contain GD1b and GT1b,²² which react with our paraprotein and the monoclonal antibody A2B5.⁶ In addition, LM1 is a major peripheral nerve ganglioside²³ which also reacts with our paraprotein, albeit less strongly than with the disialosyl gangliosides. Human nerve thus has target antigens for anti-disialosyl antibodies. It is therefore surprising that no immunoglobulin deposits were noted in the sural nerve biopsy of our case or the other four similar cases that have been described, despite the fact that extensive pathology was evident in the biopsies. Human and rodent cell culture studies have demonstrated that dorsal root ganglion neurons are A2B5 positive, as are many CNS glial and neuronal cells, whereas Schwann cells are negative.^{6,24} Using our patient's RBC-IgM in parallel with A2B5 and a panel of neuronal and glial specific markers, we performed immunofluorescence studies on sections of human and rat peripheral nerve and a wide range of cultured rat neuronal and glial cells and observed a very similar pattern of paraprotein binding to that seen with A2B5 (data not shown). In particular, no staining was seen in peripheral nerve sections. It is therefore possible that the relevant determinant(s) on gangliosides are shielded from antibody binding *in situ* in sural nerve but are accessible on other structures such as dorsal root ganglion neurons or sensory structures in the skin.

It is clearly important to consider the possibilities regarding pathogenesis in this and other related cases. It may be that this clinical and serological combination is purely coincidental and that no such relationship would be shown to exist if good case control studies could be performed. Alternatively, we can assume that such a relationship does exist and offer the following possible explanations: (a) the clinical pattern dictates the antibody specificity; (b) the antibody specificity dictates the clinical pattern; and (c) the clinical pattern and the antibody specificity are dictated by a higher unifying principle or

mechanism. Which of these will turn out to be the case awaits further investigation.

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