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H. 知的財産権の出願・登録状況(予定を含む)

なし

厚生労働科学研究費補助金(こころの健康科学研究事業) 平成16年度分担研究報告書

分担研究者 結城伸泰 獨協医科大学内科学(神経)助教授

感覚性運動失調型ニューロパチーにおけるガングリオシドの機能的役割

研究要旨

感覚性運動失調型ニューロパチーでIgG抗GD1b抗体やb系列ガングリオシド(GD3, GD2, GD1b, GT1b, GQ1b)に対するIgM抗体が、小脳性運動失調型ニューロパチーでIgG抗GQ1b抗体が検出される。本症では自己抗体の標的となるガングリオシド、特にb系列ガングリオシドの関与により、運動失調をきたすと考えられるが、未だ明らかにされていない。本研究では、ラットにおける固有感覚を司る後根神経節(DRG)大型ニューロンに発現するb系列ガングリオシドに注目し、マウスIgG抗GD1bモノクローナル抗体を作製し、神経細胞に発現しているガングリオシドの局在を免疫組織化学より解明した。その結果、患者血中に存在する抗GD1b抗体はDRGにおける大型ニューロンのGD1bと結合することがわかった。つまり感覚性運動失調型ニューロパチーにおける抗b系列ガングリオシド抗体はDRGの大型ニューロンに発現しているガングリオシドに結合し、大型ニューロンが細胞死を惹き起こし、神経障害を起こすことが示唆された。

A. 研究目的

感覚性運動失調型ニューロパチーでは筋力低下や表在感覚障害は目立たず、位置覚が著しく障害される。このニューロパチーによる運動失調は、感覚性と小脳性とに分けられ、感覚性運動失調型ニューロパチー患者のb系列ガングリオシドに対するIgM抗体が、ラットDRGニューロンに結合し、細胞死を惹き起こし(Ohsawa *et al.*, ***Neurosci Lett*** 1993)また、小脳性運動失調を呈し、IgG抗GQ1b抗体が上昇するフィッシャー症候群患者血清でも、細胞死が惹き起こされるということが報告されている(Yuki *et al.*, ***Muscle Nerve*** 1993)。しかしながら、この細胞死はアポトーシスが強く示唆されるが、確認されていない。また、b系列ガングリオシドGD1bをウサギに感作して感覚性運動失調型ニューロパチーのモデルを作成し(Kusunoki *et al.*, ***Ann Neurol*** 1996)、DRGにおけるTrk CのmRNA発現が低下していることを示した報告(Hitoshi *et al.*, ***Neurosci Lett*** 1999)もあり、抗ガングリオシド抗体が抗原となるガングリオシドの局在に対応した特異的な分布を示すものである。

抗ガングリオシド抗体がラットのDRGニューロンに結合し、細胞死を惹き起こすことが確認され、形態学的にアポトーシスであることが予想される。ラットのDRGニューロンの初代培養を用いて、患者血清による細胞死を確認する。また、これらの抗体が正常に発現しているガングリオシドに結合することにより神経の機能を障害しているものと推定されるため、ラットのDRGとモノクローナル抗体を用いて免疫組織化学を行い、神経細胞におけるガングリオシドの局在を明らかにする。

B. 研究方法

(1) 成熟Wistarラット脊髄DRGニューロンをラミニン、ポリL-リジンコートしたプレートに撒いて分散培養し、最終濃度1%になるよう患者血清を添加し、2日後生細胞を数えて生存率を求めた。

(2) ラットDRGニューロン細胞を用いて、細胞膜破壊で生じるホスファチジルセリンの原形質膜の内側から外側への転座に関係するアネキシンVの親和性とDNA染色剤であるヨードプロピディウムを染色し、アポトーシス細胞とネクローシス細胞の判別を蛍光顕微鏡で観察した。

(3) 1%パラホルムアルデヒドで灌流固定したラットDRGを用いて免疫組織化学を行った。抗体はGD3合成酵素ノックアウトマウスにガングリオシドを感作して得た抗GD1bモノクローナル抗体と固有感覚を司るニューロンのマーカーであるパルブアルブミンの免疫組織化学染色を行った。また小型ニューロンに特異的に反応する植物由来イソレクチンB4(IB4)を用いて、ニューロンの染め分けを行った。

C. 研究結果

(1) DRGニューロンの初代培養

ラットDRGニューロン細胞に至適条件下で、正常対照(n=7)、運動ニューロンが選択的に変性する筋萎縮性側索硬化症(ALS)(n=5)、抗アセチルコリン受容体抗体により生ずる神経筋接合部の病である重症筋無力症(MG)(n=5)の血清を添加した際、ALSでは91%、MGでは96%程度生存していた。これに対してb系列ガングリオシドに対するIgM抗体陽性感覚性運動失調型ニューロパチー患者(n=7)血清では21%、フィッシャー症候群(n=10)では生存率は31%であった。b系列ガングリオシドに対するIgM患者血清では、大型ニューロンに特異的に、また、高率に細胞死が惹起されることを確認した。

(2)アポトーシスの判別

ラットDRGニューロン細胞にIgM抗体陽性感覚性運動失調型ニューロパチー患者血清を添加した細胞の方がわずかにアポトーシス細胞が多く観察された。しかしながら、定量的検出はできなかった。

(3)免疫組織化学

DRG の大型ニューロンに、作製した抗 GD1b モノクローナル抗体を用いて GD1b と固有感覚を司り、大型ニューロンのマーカーであるパルブアルブミンとが共存することを示した。また、小型ニューロンに特異的な IB4 との染め分けも確認した。

D. 考察

感覚性運動失調型ニューロパチーにおいて b 系列ガングリオシド(GD1b)に対する IgM 抗体が DRG の大型ニューロンのガングリオシドに結合し、神経の機能を障害していると示唆された。しかしながら、この細胞死がアポトーシスであるか定量的には検出できなかった。1 次感覚ニューロンの細胞死の機序を究明できれば特異性の高い新しい治療薬の開発の糸口が見つかり、平衡感覚を司る 1 次感覚ニューロンを機能的に細分類する分子マーカーを神経解剖学の分野へ提供できる。

E. 結論

感覚性運動失調型ニューロパチーにおける b 系列ガングリオシド(GD1b)に対する IgM 抗体が DRG の大型ニューロンのガングリオシドに結合し、神経の機能を障害していると示唆された。

F. 健康危険情報

なし

G. 研究発表

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なし

厚生労働科学研究費補助金（こころの健康科学研究事業）
分担研究報告書

ヒト外眼筋支配脳神経における GQ1b 糖鎖抗原の subcellular localization の検討

分担研究者　千葉厚郎　杏林大学医学部　第一内科・助教授

研究要旨：通常の免疫組織染色による観察において外眼筋を支配する脳神経の傍絞輪部付近への特異的集積が示唆されている GQ1b 糖鎖抗原について、さらにその集積部位における局在を subcellular レベルで検討した。ヒトの動眼神経または外転神経の凍結切片を、Fisher 症候群患者血清血清抗 GQ1b 抗体と同じ微細反応性を有する抗 GQ1b モノクローナル抗体 7F5 で免疫染色し、共焦点レーザー顕微鏡・電子顕微鏡により観察した。共焦点レーザー顕微鏡の連続切片画像の３次元再構成では染色性は中央でくびれた中空の円筒状の分布を示した。ミエリンと反応する抗 CD57 (HNK-1) 抗体との多重染色による検討では、シリンダー状に染め出されるミエリンの端をキャップするようなミエリンと co-localization しない分布を示した。免疫電顕による検討では、ミエリン二重層が消失し泡沫状の膜構造物が集簇している部位において、その膜構造物に最も強い染色を認めた。またミエリン二重層の最外部を取り囲む膜状の構造物にも弱い染色を認めた。共焦点顕微鏡像と合わせて考えると、GQ1b 糖鎖抗原はヒトの外眼筋を支配する脳神経の Schwann 細胞の細胞膜に局在し、特にミエリン二重層構造が消失し Schwann 細胞の細胞膜が絨毛状に重なり合っている傍絞輪部の細胞膜上に高度に集積していると考えられた。

A．研究目的

我々はこれまでにガングリオシド GQ1b を認識する抗体と Guillain-Barré 症候群や Fisher 症候群などで見られる、感染後の急性外眼筋麻痺との臨床な強い相関を示すと共に、免疫組織化学的に GQ1b 糖鎖抗原がヒトの外眼筋を支配する脳神経の傍絞輪部と思われる部位に特異的に高度に集積していること、また生化学的にそれらの脳神経においてはガングリオシド GQ1b の含有量が他の末梢神経に比して有意に高いことを示してきた。また昨年度の研究においては抗 GQ1b 抗体が神経症状出現前に既に上昇していることを示し、本抗体が組織障害の二次的結果として上昇する自己抗体ではないことを明らかにした。*Campylobacter jejuni* 感染後の Fisher 症候群においてはその先行感染病原体に患者抗 GQ1b 抗体が交叉反応を示す GT1a 糖鎖構造の存在が示されている。以上の知見を総合的に考えると、本抗体が先行感染病原体に対する免疫反応により上昇し、それが GQ1b 糖鎖抗原が集積している外眼筋支配神経の傍絞輪部に何らかの作用を及ぼすことにより外眼筋麻痺が発症するという病態機序が強く示唆されるが、しかし抗体の作用機所の詳細は明らかにはなっていない。

自己免疫性末梢神経障害における抗糖脂質抗体の病因的意義についても、それを支持する知見が集積されつつあるが、その作用部位と作用機序の詳細についてはまだ解明されていない点が多い。末梢神経障害発症における抗糖脂質抗体の作用機序解明のための一つのステップとして、我々がこれまで研究を進めてきた GQ1b を一つのモデルとして、ヒトの外眼筋支配脳神経における GQ1b 糖鎖抗原の局在を subcellular レベルで明らかにすることを目的として、今回以下の検討を行った。

B．研究方法

剖検にて得られたヒトの動眼神経または外転神経の凍結切片を、冷アセトンあるいは冷アセトン-4%パラフォルムアルデヒド等で固定し、Fisher 症候群患者血清中の抗 GQ1b 抗体と同様に GT1a とも交叉反応する抗 GQ1b モノクローナル抗体 7F5 で免疫染色し、共焦点レーザー顕微鏡・電子顕微鏡により観察した。共焦点レーザー顕微鏡による観察においては、通常の断層像の他、スライス面に対して垂直方向の連続断層像を撮影しそれを３次元再構成して得られる画像から、抗原の立体的分布を検討した。電子顕微鏡用のサンプルはジアミノベンチジン (DAB) で発色を行った。また、ミエリンとの相互関係を検討するために抗 CD57 (HNK-1) 抗体、Ranvier 絞輪との相互関係を検討検討するためにビオチン標識コレラ毒素 B サブユニットとの二重染色による検討を行った。

C．研究結果

これまでの 7F5 による免疫染色の DAB 発色/明視野による観察では、傍絞輪部への染色性の集積が示されていたが、今回の蛍光免疫染色像の暗視野での観察において染色性は傍絞輪部に集積しているのみではなくそれに続く神経線維の外表面に沿っても低密度ながら局在していることが認められた。その染色性は均一ではなく、一部点状に傍絞輪部程で

はないが比較的強く染まる部分が散在するような不均一な分布を示していた。

共焦点レーザー顕微鏡の連続切片画像の３次元再構成画像では中央でくびれた中空の円筒状の分布を示す染色性を認めた。抗 CD57 (HNK-1) 抗体との二重染色による検討では、シリンダー状に染め出されるミエリンの端にそこをキャップするようなミエリンとはco-localizationをしない分布を認めた。コレラ毒素 B サブユニットとの二重染色では、円筒のくびれの部分に 7F5 とコレラ毒素Bサブユニットによる染色性の co-localization を認めた。

7F5 による免疫染色性を得られかつ電子顕微鏡による超微細形態の観察に耐え得るような固定条件の検討を行ったが、抗原性の検出には切片をパラホルムアルデヒドなどのアルデヒド系の固定剤での固定前に冷アセトン処理が必要であった。冷アセトン処理後に 4%パラフォルムアルデヒド処理においては抗原性の検出は可能であったが、その強さは冷アセトン処理単独のものよりも弱かったため、免疫電顕での観察には冷アセトン処理単独のものを用いた。免疫電顕による検討では、ミエリンの二重層構造が失われてその神経線維長軸方向の延長に泡沫様の膜構造物が集簇している部位において、最も強い染色を認めた。その部分においては泡沫状の膜同士が接する境界部分において最も強い染色を認めた。その他、ミエリンに密着してその周囲を取り囲む膜状の構造物にも染色を認め、その膜状構造物とミエリンとの結合が部分的に緩んでいる箇所においてはその染色性がより強くなっていたが、その染色性は全般にミエリン二重層が失われた部分での染色性よりも弱かった。また軸索とミエリンの境界部にもミエリン周囲と同程度の染色を認めた。

D．考察

今回の共焦点レーザー顕微鏡および免疫電顕での観察結果は、これまで明視野光学顕微鏡観察での形態的特徴から推定されていた、GQ1b 糖鎖抗原は傍絞輪部に高密度に集積しているという知見と合致している。

免疫電顕において最も強い染色性を認めたミエリンの層構造が失われて泡沫様の膜構造物が集簇している部位はその位置関係からは、傍絞輪部においてミエリンの二重層構造が失われてSchwann細胞の細胞膜が細絨毛状に広がっている部位ではないかと考えられる。現在傍絞輪部における GQ1b 糖鎖抗原の集積の機能的な意味は不明であるが、その部位において泡沫状の膜同士が接する境界部分により強い染色を認めた点からは、GQ1b 糖鎖抗原がその部位の膜表面に高密度に局在し、接する細胞膜間での何らかの相互作用に関与している可能性も推測される。

今回の免疫電顕像において傍絞輪部だけではなくミエリン二重層の最外部に膜状の染色が認められたが、これは部位的に考えてミエリンの最外部を覆うSchwann細胞の細胞膜と考えられる。この結果は蛍光染色像での神経線維の外表面に沿って低密度ながら染色性が認められている結果と一致している。またその表面に点状に散在する比較的強い蛍光染色部位は、免疫電顕にて認められた膜状構造物とミエリンとの結合が部分的に緩んで染色性がより強くなっている箇所に相当するのではないかと思われる。この分布の不均一性が生理的分布を反映したものであるのか、あるいはアセトン処理などによる部分的構造破壊によって抗原への抗体のアプローチがしやすくなったた結果として見かけ上の抗原性が高まった結果に過ぎないのかに関しては、今後の検討を要する。

ミエリンの最内層部と軸索の境界部の染色に関しては、その部位がミエリン側の最内層部か軸索膜側かは、今回の観察では識別できなかった。

7F5 による GQ1b 糖鎖抗原性の検出には切片のアセトン処理を要することから電顕による超微形態の評価には限界があるが、今回の検討からは GQ1b 糖鎖抗原はヒトの外眼筋を支配する脳神経のSchwann細胞の細胞膜に全般に局在していること、またそのSchwann細胞の細胞膜上の分布は均一ではなく、傍絞輪部の膜上にはより高度に集積していることが示された。

E．結論

GQ1b 糖鎖抗原は外眼筋支配脳神経の Schwann 細胞の細胞膜上に局在しており、特に傍絞輪部のSchwann細胞の細胞膜が絨毛状に広がっている部分において、膜と膜とが重なり合う部分により高度に集積している。このような分布からはGQ1bが細胞膜間の相互作用に何らかの寄与をしている可能性も推定され、また同部位への抗体の作用により膜間の相互作用が弱まり、それにより有髄神経の刺激伝導に障害をきたす可能性も考えられる。

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G．知的所有権の取得状況

該当無し

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Effects of phospholipids on antiganglioside antibody reactivity in GBS

Minako Hirakawa[a,b], Daiji Morita[a,b], Shoji Tsuji[b], Susumu Kusunoki[a,*]

[a]*Department of Neurology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka 589-8511, Japan*
[b]*Department of Neurology, Division of Neuroscience, Graduate School of Medicine, University of Tokyo, Japan*

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Abstract

Serum antibody activities to mixtures of a ganglioside and various phospholipids were compared with those to a ganglioside alone in 30 anti-GM1 IgG-positive GBS patients and 30 anti-GQ1b IgG-positive Miller Fisher syndrome (MFS) patients. Anti-GM1-positive sera had higher antibody reactivities against a mixture of GM1 and several phospholipids including PA, PI and PS, than against GM1 alone. In contrast, in case of anti-GQ1b antibody, no phospholipid provided significant enhancement. Sphingomyelin provided decrease of the activity for both anti-GM1 and anti-GQ1b IgG. The effects of phospholipids must be considered to determine the pathogenetic role of antiganglioside antibodies in GBS and MFS.

Keywords: GBS; Antiganglioside antibody; Phospholipids

1. Introduction

Antiganglioside antibodies frequently are present in patients with Guillain-Barré syndrome (GBS) and its variant, Miller Fisher syndrome (MFS). Anti-GM1 IgG antibodies are associated in particular with the pure motor variant of GBS (Visser et al., 1995), or with acute motor axonal neuropathy (Hafer-Macko et al., 1996), whereas anti-GQ1b IgG antibodies are associated with MFS (Chiba et al., 1992). Antiganglioside antibodies are useful diagnostic markers as well as possible pathogenetic factors in GBS.

Recently, we reported that IgG antibody in acute GBS sera had a higher titer against a mixture of GM1 and phosphatidic acid (PA) than against GM1 alone (Kusunoki et al., 2003). The use of a mixture antigen of GM1 and a phospholipid may provide us with an improved method of the antibody assay for the diagnosis of GBS. To investigate which phospholipid is the most effective for the enhancement of the anti-GM1 IgG activities and whether such an enhancement is generally observed in the antiganglioside antibodies in GBS or its variant, we examined IgG reactivities against GM1 or GQ1b with and without various phospholipids.

2. Materials and methods

2.1. Serum samples

Sera were obtained from 30 GBS patients who had anti-GM1 IgG antibodies and from 30 MFS patients who had anti-GQ1b IgG antibodies.

2.2. Antibody assay

Antiganglioside antibody reactivities were measured by the enzyme-linked immunosorbent assay (ELISA), as described elsewhere (Kusunoki et al., 1994). Nine phospholipids were used: phosphatidic acid (PA), phosphatidylethanolamine (PE), phospatidylinositol (PI), phosphatidylserine (PS), lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), phosphatidylcholine (PC), sphingomyelin

* Corresponding author. Tel.: +81 72 366 0221x3553; fax: +81 72 368 4846.
E-mail address: kusunoki-tky@umin.ac.jp (S. Kusunoki).

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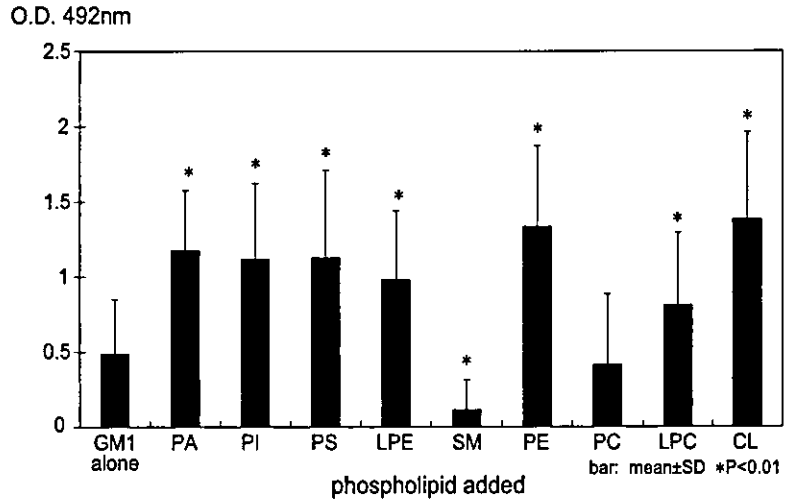

Fig. 1. Means of the antibody activities for 30 anti-GM1 IgG-positive GBS patients. Activity increased when a mixture of GM1 and PA, PI, PS, LPE, PE, LPC or CL was the antigen and decreased when a mixture of GM1 and SM was.

(SM) and cardiolipin (CL) (all purchased from DOOSAN Serdary Research Laboratories, USA).

Wells of 96-well microtiter plates were coated with 200 ng of ganglioside (GM1 or GQ1b), an antigen mixture of 100 ng each of ganglioside and phospholipid, or 200 ng of phospholipid only. An uncoated well was the control. Each OD value was corrected by subtraction of the control well OD. Serum with a corrected OD of more than 0.1 was considered positive. The antibody activity of each patient's serum was expressed as the mean of the corrected ODs of two independent assays.

2.3. Statistics

Antibody activities against a ganglioside and against a mixture of ganglioside and phospholipid were compared by the Wilcoxon signed-ranks test. Significance was considered positive if $p<0.01$.

3. Results

The binding activity of anti-GM1 IgG-positive GBS sera to a mixture of GM1 and a phospholipid was compared to that of GM1 alone. The mean antibody activity against a mixture of GM1 and phospholipid was significantly higher than against GM1 alone when the phospholipid was PA, PI, PS, LPE, PE, LPC or CL, whereas it was significantly lower when the antigen was a mixture of GM1 and SM (GM1/SM) (Fig. 1). Of the individual sera, more than 70% of the GBS patients had higher antibody activity against a mixture of GM1 and the phospholipid PA, PI, PS, LPE, PE, LPC or CL than against GM1 alone. In contrast, almost all had significantly lower antibody activity against GM1/SM than against GM1 alone. The antibody activity against the mixture of GM1 and SM remained decreased even when a mixture of 200 ng GM1 and 100 ng phospholipid per well was used instead of 100 ng each.

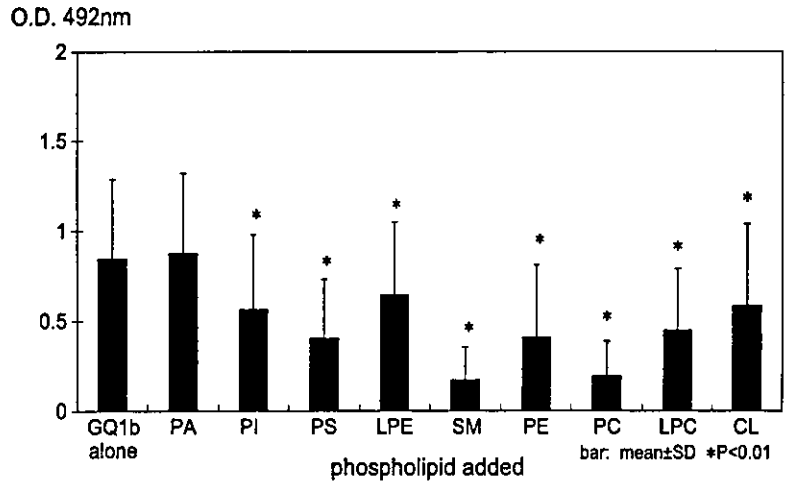

Fig. 2. Means of the antibody activities for 30 anti-GQ1b IgG-positive MFS patients. Means of the antibody activities increased only when a mixture of GQ1b and PA was the antigen. The other phospholipids produced no enhancement.

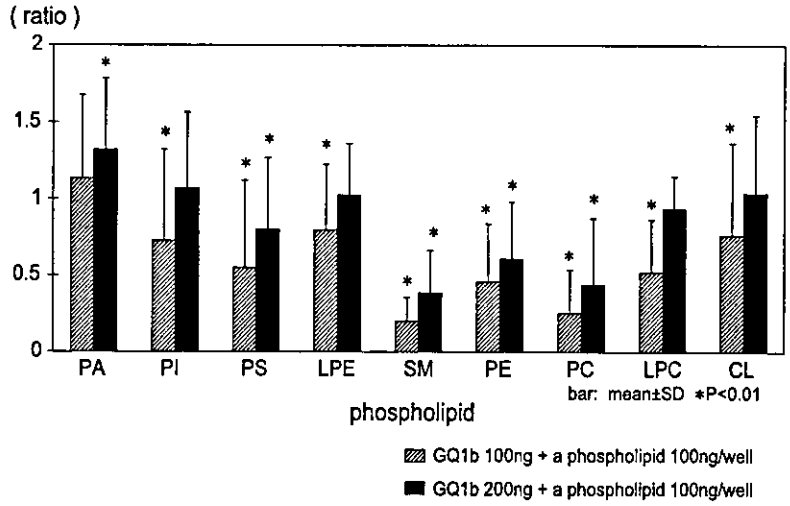

Fig. 3. Antibody reactivity against a mixture divided by that against GQ1b alone. Columns give the means of the ratios. Activities in MFS against mixtures (except GQ1b and PA) were lower than against GQ1b alone. When the mixture contained 200 ng of GQ1b and 100 ng of phospholipid per well, instead of 100 ng each, binding activity against the mixture was restored to a level similar to that against GQ1b alone (200 ng/well) for PI, LPE, LPC and CL.

Results for the anti-GQ1b IgG-positive sera clearly differed from those for anti-GM1 IgG-positive sera; only PA had a slight enhancing effect, but it did not reach statistical significance. The other phospholipids did not have an enhancing effect. Antibody activities against mixtures were lower than against GQ1b alone (Fig. 2).

When a mixture of 200 ng GQ1b and 100 ng phospholipid per well was used instead of 100 ng each, serum antibody binding activity against a mixture was restored to a level similar to that against GQ1b alone for the phospholipids PI, LPE, LPC and CL but not PS, SM, PE or PC (Fig. 3).

The control experiments showed that IgG antibodies against phospholipids were negative in most cases, indicative that the reaction of the IgG antibody with phospholipid did not account for the phospholipid enhancement of anti-GM1 IgG antibody activity described above. Some GBS patients had low antibody activity titers against LPE, PE, PC, LPC and CL. However, the antibody reactivities against mixture antigens (GM1/LPE, GM1/PE, GM1/PC, GM1/LPC and GM1/CL) were still much higher than those against GM1 alone even if ODs of phospholipid coated wells were subtracted from those of GM1/phospholipid wells.

Of the 30 patients with GBS, 4 had both anti-GM1 IgG and anti-GM1 IgM. We examined the reactivity of the IgM antibodies from those four patients with a mixture of GM1 and phospholipid. No enhancing effect like that obtained with IgG antibodies occurred with the IgM antibodies. When GM1 was mixed with SM, IgM activity decreased like that for IgG.

4. Discussion

In the previous study, maximal binding activities were observed at a GM1/PA ratio of 1:1 when varying ratios of GM1 and PA of mixture antigen were used (Kusunoki et al., 2003). In this study, we therefore investigated antibody activities against a mixture at a ganglioside/phospholipid ratio of 1:1. Anti-GM1 IgG-positive GBS sera had higher antibody activities against a mixture of GM1 and the phospholipid PI, PS, LPE, PE, LPC or CL, as well as PA, than against GM1 alone. In our preliminary study, some anti-GM1 IgG-negative patients had IgG reactivity against a mixture of GM1 and such phospholipids as PI, PS, CL as well as PA. A mixture antigen of GM1 and such a phospholipid as PA, which provided significant enhancement of the anti-GM1 IgG reactivity in GBS sera, may be more useful than GM1 alone in ELISA for the diagnostic test of GBS.

Effects of the addition of a phospholipid on the activities of anti-GQ1b IgG-positive sera differed. A phospholipid addition to GQ1b antigen did not produce a significant increase in antibody activity, and activity was decreased when a mixture of GQ1b and a phospholipid (except PA) was the antigen.

The cause of the difference in the anti-GM1 IgG and anti-GQ1b IgG reactivities remains to be determined. It is possible that the physiochemical differences of GM1 and GQ1b make the differences of the effects of phospholipids on binding activities of antibodies. However, anti-GM1 antibody activities are not necessarily enhanced by the addition of PA to the antigen, as seen in some GBS sera and in the rabbit antisera (Kusunoki et al., 2003). The most probable reason may be the difference in the preceding infection. The major phospholipid constituents in Gram-negative bacteria are PE, phosphatidylglycerol and CL. PS and PA are present as minor components (Huijbregts et al., 2000), but these bacteria have no SM (Zien et al., 2001; Carman and Henry, 1999). This indicates that anti-GM1 IgG-positive sera reactivity increased when a phospholipid present in *Campylobacter*

jejuni, a Gram-negative bacterium, was added to the antigen mixture. Respiratory rather than gastrointestinal infections, however, precede onset in most anti-GQ1b IgG-positive patients; those with Miller Fisher syndrome. The difference in the preceding infectious agent may, at least in part, explain why the reactivity of anti-GQ1b IgG-positive serum was not enhanced by the addition of those phospholipids that increased the reactivity of anti-GM1 IgG-positive serum.

Whether antibodies with high reactivity against a mixture of GM1 and phospholipid rather than against GM1 alone recognize a conformational epitope formed by GM1 and a phospholipid has yet to be clarified. The antibodies possibly have specificity for GM1 which undergoes conformational change in association with a phospholipid, as reported for β2-glycoprotein I and anticardiolipin antibodies (Matsuura et al., 1994). Phospholipids also may affect the binding force between antibodies and GM1. Because the phospholipids composition surrounding ganglioside antigen can influence antibody binding, susceptibility to injury can be contributed by membrane phospholipids content. The composition and distribution of each phospholipid at such possible target sites as the paranodal myelin, axolemma, and neuromuscular junction, needs to be clarified in future studies.

The effect of SM in decreasing the antibody activity of both anti-GM1 IgG-positive and anti-GQ1b IgG-positive sera seems to be of importance. Gangliosides are widely distributed throughout the nervous system, individual ones sometimes being dense at a particular site. For example, GQ1b is present in the spinal roots and in all the cranial nerves (Chiba et al., 1997), but is densely localized in the paranodal regions of oculomotor, trochlear and abducens nerves. Ophthalmoplegia may be due to the specific binding of anti-GQ1b antibodies to these regions (Chiba et al., 1993). The decrease seen in antiganglioside antibody reactivity in GBS and MFS caused by SM may be why antiganglioside antibody does not cause ubiquitous damage to the neurological system but damages only sites where gangliosides are densely localized.

Greater attention must be paid to the effects of the phospholipids that surround gangliosides on cell membranes of the human nervous system in order to determine the pathogenetic role of antiganglioside antibodies in autoimmune neuropathies. To better understand how phospholipids influence the interaction of antibodies and ganglioside in vivo, further investigation by the use of such system that mimic the myelin membrane as liposomes consisting predominantly of cholesterol, PE and PC is needed.

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Ganglioside Complexes as New Target Antigens in Guillain–Barré Syndrome

Ken-ichi Kaida, MD, PhD,[1] Daiji Morita, MD,[2] Mami Kanzaki, MD,[1] Keiko Kamakura, MD, PhD,[1] Kazuo Motoyoshi, MD, PhD,[1] Minako Hirakawa, MD,[2] and Susumu Kusunoki, MD, PhD[2]

Antibodies specific for a complex of gangliosides GD1a and GD1b (GD1a/GD1b) were found in sera from eight of 100 patients with Guillain–Barré syndrome (GBS) by the use of enzyme-linked immunosorbent assay and thin-layer chromatogram immunostaining. Those sera also had antibody activities to such ganglioside complexes as GD1a/GM1, GD1b/GT1b, and GM1/GT1b but had little or no reactivity to the each isolated antigen. Clustered epitopes of the ganglioside complex in the plasma membrane may be targeted by such an antibody, and interaction between the antibody and ganglioside complex may induce the neuropathy.

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Antibodies to gangliosides are present in the sera of approximately 60% of patients with acute immune-mediated polyradiculoneuropathy, Guillain–Barré syndrome (GBS).[1] Antiganglioside antibody may function in the development of certain neurological signs and in the pathogenesis of GBS through its binding to ganglioside antigens in peripheral nerves.[2,3] Although various antiganglioside antibodies in GBS patients' sera have been investigated, no antibodies to the ganglioside complex with clustered glycoepitopes have been reported. We found antibodies to a mixture of two ganglioside antigens in the sera of some GBS patients.

Materials and Methods

Representative Serum and the Antiganglioside Antibody Assay

After several days of flu-like symptoms, a 31-year-old man (Patient A) developed acute flaccid tetraparesis. GBS was di-

From the [1]Third Department of Internal Medicine, National Defense Medical College, Saitama; and [2]Department of Neurology, Kinki University School of Medicine, Osaka, Japan.

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Address correspondence to Dr Kusunoki, Department of Neurology, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osaka-Sayama, Osaka, 589-8511 Japan.
E-mail: kusunoki-tky@umin.ac.jp

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agnosed based on Asbury and Cornblath criteria.[4] His acute phase serum was checked for antiganglioside antibodies by an enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatogram (TLC) immunostaining.

TLC immunostaining was conducted on a high-performance TLC plate on gangliosides extracted from whole bovine brain gangliosides through an anion-exchange resin column, DEAE Sephadex A-25 (Amersham Biosciences, Uppsala, Sweden), as described elsewhere.[5] The gradients used were 0.05, 0.1, 0.2, and 0.4M ammonium acetate in methanol. TLC immunostaining also was done for GD1a, GD1b, and a mixture of GD1a and GD1b. The immunostained TLC plate was assayed in an Image analyzer (Luminescent Image Analyzer, LAS-1000plus; Fujifilm, Tokyo, Japan) to evaluate staining in the lane with the GD1a–GD1b mixture.

The ELISA was performed for antibodies to the gangliosides GalNAc-GD1a, GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, and GQ1b, as described elsewhere.[6] Serum diluted 1:40 with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) was added to wells coated with 0.2μg of antigen. Optical density (OD) values were corrected by subtracting the OD of a control well that had been similarly processed. When the corrected OD was more than 0.1, the serum was considered positive. Serum antibody to GD1a/GD1b, a mixture of 0.1μg each GD1a and GD1b, was investigated by the same methods. Responses of his serum to a 0.2μg mixture of GD1a and GD1b in various ratios (GD1a:GD1b = 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8) also were investigated with an ELISA. ELISAs were repeated twice in the same way, and the mean OD of the two experiments was calculated.

Sera from 100 Consecutive Patients with Guillain–Barré Syndrome and the Enzyme-Linked Immunosorbent Assays for Anti-GD1a/GD1b Antibodies

To investigate the frequency of anti-GD1a/GD1b antibody in GBS patients, between February and December 2002 acute phase sera were collected from 100 consecutive GBS patients who were diagnosed at various general and teaching hospitals throughout Japan, and their clinical data were sent to us at that time. All met the diagnostic criteria of Asbury and Cornblath.[4] Serum antiganglioside antibodies were investigated by ELISAs as described above. Anti-GD1a– or anti-GD1b–positive sera, in which the corrected anti-GD1a/GD1b antibody OD was 0.2 higher than the corrected anti-GD1a or anti-GD1b antibody OD, were considered anti-GD1a/GD1b antibody–positive. The cutoff value (0.2) for the anti-GD1a/GD1b antibody was decided arbitrarily. Anti-GD1a/GD1b antibody–positive sera were overlaid for TLC immunostaining, as described elsewhere.[5]

Anti-GD1a/GD1b antibody was surveyed in ELISAs of sera from16 normal subjects (normal control) and from 119 patients with neurological disorders other than GBS (disease control): cerebrovascular disease, 17; multiple sclerosis, 10; Parkinson's disease, 8; amyotrophic lateral sclerosis, 7; spinocerebellar degeneration, 7; myasthenia gravis, 6; chronic inflammatory demyelinating polyradiculoneuropathy, 5; other neuropathies, 3; and other neurological diseases, 56.

Antibody activities against a mixture of 0.1μg each of other gangliosides such as GM1/GD1a, GM1/GT1b, GD1a/GT1b, and GD1b/GT1b were investigated on the eight anti-GD1a/GD1b antibody–positive sera in the same way as used for GD1a/GD1b described above.

Results

Anti-GD1a/GD1b Antibody in Serum of Patient A

TLC immunostaining showed strong staining just below the position of GD1a in the lane with the ganglioside fraction extracted with 0.1M ammonium acetate (Fig 1A). Positive staining also was present in the lane for GD1a–GD1b mixture, but not in the lanes for GD1a and GD1b (see Fig 1A). In another developing solvent (C/M/0.2% $CaCl_2$ = 30/65/10) that separated the positions of GD1a and GD1b, the immunostaining in the lane of GD1a–GD1b mixture disappeared (see Fig 1B). The Image Analyzer assay showed specific immunostaining in the overlapping portion of the GD1a and GD1b antigens (see Fig 1C). ELISA results were negative for each of the test gangliosides but positive in the well coated with the mixture of GD1a and GD1b antigens (GD1a/GD1b). IgG anti-GD1a/GD1b antibody titer was 1:640 (see Fig 1D). ELISAs done with various combinations of the GD1a and GD1b (GD1a:GD1b) mixture showed that the corrected OD values were the highest at the ratios of 6 to 4 and 5 to 5 (see Fig 1E).

Anti-GD1a/GD1b Antibody Assay for the Consecutive GBS Patients and Controls

ELISAs showed that 8 of 100 consecutive patients (8%) with GBS had IgG anti-GD1a/GD1b antibodies, whereas none of the disease or normal control group patients did. Three of the eight GBS patients with IgG anti-GD1a/GD1b antibodies had neither the IgG anti-GD1a nor anti-GD1b antibody. Of 92 GBS patients without IgG anti-GD1a/GD1b antibody, 4 patients had IgG anti-GD1a antibody, and 11 had IgG anti-GD1b antibody.

TLC immunostaining of the anti-GD1a/GD1b antibody–positive sera from five patients with GBS gave the same results as for Patient A's serum (Fig 2). The quantities of sera obtained from the remaining two patients with anti-GD1a/GD1b antibody were insufficient to overlay for TLC immunostaining. The clinical features and antiganglioside antibodies of the eight GBS patients are given in the Table. Four anti-GD1a/GD1b antibody–positive patients required artificial ventilation. Electrophysiological studies showed "primary axonal" in Patients 2 and 8 and "equivocal" in Patient 3, which were classified as described elsewhere.[5] Five other patients had no available electrophysiological data.

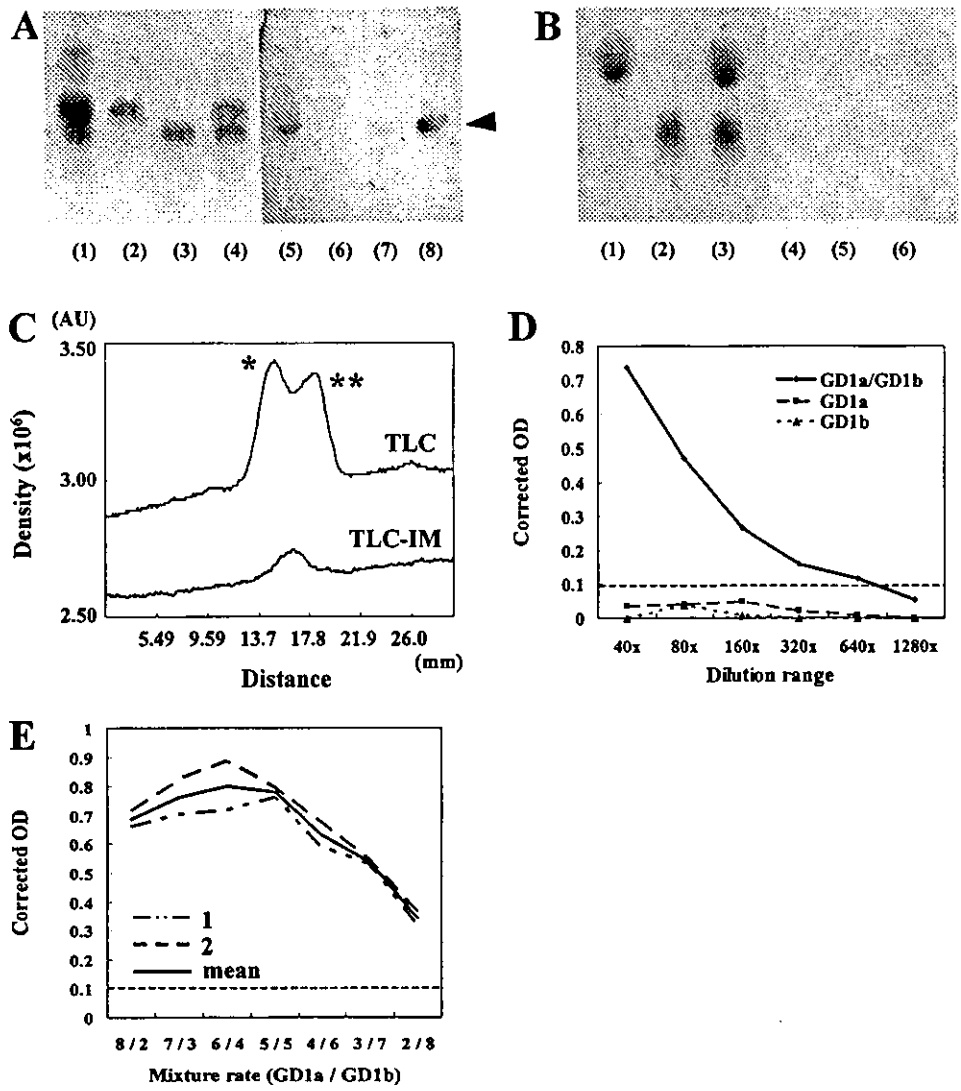

Fig 1. Enzyme-linked immunosorbent assay (ELISA) and thin-layer chromatogram (TLC) studies of Patient A's serum. (A) Left panel (lanes 1–4*): TLC results made visible by orcinol reagent, right panel (lanes* 5–8*): TLC immunostaining of his serum. The developing solvent consisted of chloroform, methanol, and 0.2%$CaCl_2$-$2H_2O$ (50:45:10, vol/vol). Serum was diluted 1:100. Peroxidase-conjugated goat anti-human immunoglobulin G Fc antibody (diluted 1:200; ICN Biomedicals Inc., Aurora, OH) was the second antibody. Immunoreactants were made visible with phosphate-buffered saline containing 0.01% H_2O_2 and 50mg/dl 3,3'-diaminobenzidine tetra-hydrochloride. Bovine brain gangliosides obtained by extraction with 0.1M ammonium acetate were applied to lanes 1 and 5, GD1a (3μg) to lanes 2 and 6, GD1b (3μg) to lanes 3 and 7, and both GD1a and GD1b (3μg each) to lanes 4 and 8. The arrowhead indicates the immunostaining on the overlapping portion of GD1a and GD1b. (B) These panels show TLC results using a solvent system, C/M/0.2%$CaCl_2$ = $2H_2O$ (30:65:10, vol/vol). Left panel (lanes 1–3): TLC results made visible by orcinol reagent, right panel (lanes* 4–6*): TLC immunostaining of his serum. GD1a (3μg) was applied to lanes 1 and 4, GD1b (3μg) to lanes 2 and 5, and both GD1a and GD1b (3μg each) to lanes 3 and 6. The immunostaining disappeared in the solvent system that allowed clear separation of GD1a and GD1b. (C) Analysis of TLC immunostaining with the Image Analyzer. The top line (TLC) indicates the densities of GD1a and GD1b on the HPTLC plate; the bottom line (TLC-IM) shows the density of the TLC immunostaining of Patient A's serum. The abscissa gives the distance from the base line. The single asterisk shows the GD1b density peak; the double asterisks the GD1a density peak. The peak in the lower line nearly corresponds to the bottom of the trough between the GD1a and GD1b peaks in the upper line. AU = arbitrary unit. (D) ELISA results. Serum was diluted serially from 1:40 to 1:1,280. IgG anti-GD1a and GD1b antibodies are negative. (E) ELISA results for various mixtures of GD1a and GD1b antigens. (line* 1*) first study results; (line* 2*) second study results;* (solid line) *the mean of 1 and 2.*

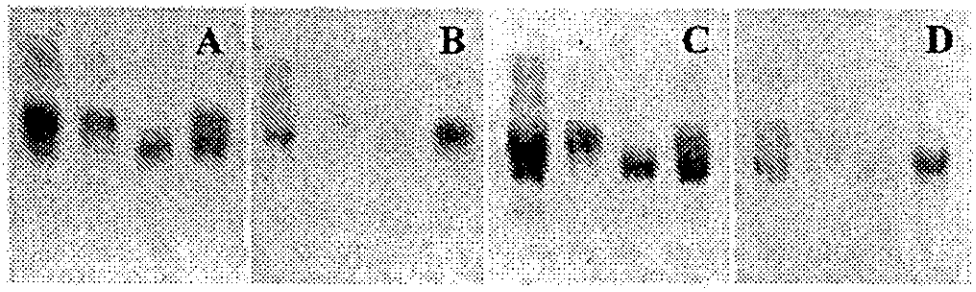

Fig 2. Thin-layer chromatogram (TLC) immunostaining of sera from Patients 1 and 3. TLC results in plates A and C are made visible by the orcinol reagent. Bovine brain gangliosides extracted by 0.1M ammonium acetate are in lane (1), GD1a (3μg) in lane (2), GD1b (3μg) in lane (3), and both GD1a and GD1b (3μg each) in lane (4). Plate B shows the TLC immunostaining of the serum from Patient 1, and plate D that of the serum from Patient 3. Compared with the slight immunostaining present in lanes (2) and (3), the overlapping portion of GD1a and GD1b in lane 4 is strongly immunostained.

The anti–GD1a/GD1b IgG antibody–positive patients also had antibody activities against a mixture of other gangliosides as shown in the Table.

Discussion

We first found an unidentified immunoreactive band in the position just below GD1a on TLC of a crude ganglioside fraction obtained through DEAE Sephadex A-25 column by extraction with 0.1M ammonium acetate. That led to the discovery of the antibody specific for the GD1a/GD1b ganglioside complex. We therefore focused our attention on the antibody to GD1a/GD1b in this article. The anti–GD1a/GD1b antibody–positive sera also had antibody activities to two

Table. Clinical Features and Antiganglioside Antibodies of GBS Patients with IgG Anti–GD1a/GD1b Antibody

Patient No.	Age (yr)	Sex	Antecedent Infection	F-score at Peak	Involved Cranial Nerves	Sensory Signs[a]	Corrected OD[b]			Antibodies to Other Ganglioside Complexes	Other Antiganglioside Antibodies
							Anti-GD1a/GD1b	Anti-GD1a	Anti-GD1b		
1	77	F	GI	5	9, 10	—[c]	1.24	0.15	0.23	GD1a/GM1, GM1/GT1b GD1b/GT1b	IgG: GT1b
2[d]	31	M	R	4	6	(++)	0.87	(−)	(−)	GD1a/GM1, GM1/GT1b GD1b/GT1b	(−)
3	30	M	GI	2	(−)	(−)	1.14	0.4	0.31	GD1a/GM1, GM1/GT1b GD1b/GT1b	IgG: GT1b IgM: GM1
4	70	M	R	5	3, 4, 5, 6, 7, 9, 10, 11, 12	(++)	0.98	0.14	0.15	GD1a/GM1, GM1/GT1b GD1b/GT1b	IgG: GQ1b
5	59	M	GI	4	7, 9, 10, 11, 12	(−)	1.16	0.18	0.83	GD1a/GM1, GM1/GT1b	(−)
6	47	F	GI	4	3, 4, 6, 7, 9, 10	(+)	0.92	(−)	(−)	GD1a/GM1, GM1/GT1b GD1b/GT1b	(−)
7	29	F	R	5	7, 9, 10, 12	(+)	0.61	(−)	(−)	GD1a/GM1	IgG: GM1
8	52	F	GI	5	5, 8, 9, 10, 11	(−)	0.95	(−)	0.15	GD1a/GM1, GM1/GT1b GD1b/GT1b	IgG: GM1 IgM: GM1

[a]Criteria for sensory signs: (−), no sensory signs or symptoms; (+), only paresthesia or dysesthesia; (++), sensory deficits
[b]Corrected optical density (OD) values of anti-ganglioside antibodies: corrected by subtracting the OD of a control (uncoated) well. (−) = negative result. F-score at peak: patient disabilities were graded on the Hughes functional grading scale [14]; 0 = no symptoms, 1 = minor signs or symptoms, 2 = able to walk 5m without support but incapable of manual work, 3 = able to walk 5m only with a cane, appliance, or support, 4 = bed- or chair-bound, 5 = requiring assisted ventilation, 6 = dead.
[c]No available data.
[d]Patient A

GI = gastrointestinal infection; R = respiratory tract infection.