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（総合）究報告書

Fisher 症候群および Guillain-Barré 症候群における外眼筋麻痺発症に対する血清抗 GQ1b IgG 抗体の病因的意義に関する研究

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研究要旨：Fisher 症候群（FS）および外眼筋を伴う Guillain-Barré 症候群（GBS-OP）において検出される血清抗 GQ1b IgG 抗体の外眼筋麻痺発症に対する病因的意義に関して次の２つの観点から検討した。

(1). 発症初期における抗 GQ1b IgG 抗体価の経時的変化と臨床症状の推移との詳細な対比検討：発症後からの検討を行った８例のうち７例で、抗体価は初回採取血清で最も高く、経時的に低下した。４例では外眼筋麻痺増悪期に抗体価の推移を観察出来たが３例では、外眼筋麻痺が増悪する時期に抗体価は既に低下していく段階にあった。発症前より検討を開始しえた FS の１例では外眼筋麻痺出現前に既に抗体価の上昇を認め、外眼筋麻痺発症の頃に抗体価はピークに達し、その後症状が増悪していく過程では抗体価は漸時低下していった。以上の結果からは、抗 GQ1b IgG 抗体は組織障害の結果として二次的に産生されるのではなく、先行感染に関連して上昇し外眼筋麻痺の発症初期のプロセスに関与している可能性があると考えられた。また症状の増悪期に既に抗体価は低下の段階に入っているということからは、臨床症状の重症度は抗体価のみによって規定されるものではなく、抗原抗体反応に続くステップが重症度を規定している可能性がある。そのプロセスの解明が今後の新たな治療開発の上でのターゲットを提供する可能性がある。

(2). 外眼筋支配脳神経における GQ1b 糖鎖抗原の微細構造レベルでの局在の検討：ヒト動眼神経または外転神経の剖検材料の凍結切片を、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。また各種糖脂質に対するモノクローナル抗体との二重染色を行い、GQ1b 糖鎖抗原と他の糖鎖抗原の局在の相互関係についても検討した。ヒトの外眼筋を支配する脳神経において GQ1b 糖鎖は Schwann 細胞の形質膜上に lipid raft 様の高密度部位を形成して斑状に分布し、特に傍絞輪部ではその高密度部位が特に高度に集積していると考えられた。抗 GD1b 抗体との二重染色において、GQ1b 糖鎖と GD1b 糖鎖との colocalization を認めた。抗 GM3 抗体による染色においても形態的に傍絞輪部と思われる部位に強い染色を認めた。しかし GQ1b 糖鎖とは colocalization していなかった。ヒトの動眼神経の傍絞輪部には局在する糖鎖の観点からは GQ1b 糖鎖と GD1b 糖鎖の集積しているものと GM3 糖鎖が集積しているものの２種類のものが存在すると考えられた。

A．研究目的

Guillain-Barré 症候群（GBS）および Fisher 症候群（FS）などの GBS の臨床亜型と考えられている病態の約３分の２の症例において、急性期血清中に何らかのガングリオシドと反応する抗体が検出され、今日その補助診断法として広く利用されるに至っている。中でもガングリオシド GQ1b を認識する IgG 抗体は、FS ならびに外眼筋麻痺を伴う GBS（GBS-OP）をはじめとして、何らかの先行感染後に生ずる経過良好な急性外眼筋麻痺の症例において極めて高率かつ特異的に出現することから、その診断的意義は極めて高い。認識される抗原である GQ1b 糖鎖抗原は、ヒトの外眼筋を支配する脳神経の傍絞輪部に特異的に高密度に局在していることが免疫組織化学的に示されており、これは抗 GQ1b IgG 抗体と外眼筋麻痺との臨床的関連によく相応しており、このことは本抗体が外眼筋麻痺発症と何らかの関わりを持つことを強く示唆するものである。

抗ガングリオシド抗体の病因的意義については、これまで *in vitro* あるいは *ex vivo* での患者血清やモノクローナル抗体による伝導ブロックの報告、あるいはガングリオシドを免疫することにより作成された動物モデルの検討から、その病因的意義を積極的に示唆するデータが集積されてきてはいるが、依然としてある「組織障害に関連した二次的な抗体の産生」という議論に対して、その積極的な反証となるような臨床データは殆ど示されてはいない。

自己抗体によって認識される抗原分子の局在の詳細と、さらにそれがその周囲のいかなる分子と相互作用をし生体内における機能を果たしているかを明らかにすることは、検出された自己抗体の病因的意義とそれによる自己免疫性疾病の成立機序を考える上で極めて重要な課題である。糖鎖抗原に関

しては、近年２つのガングリオシドが複合することにより新たな抗原エピトープを形成することも報告されている。また、このような抗体の作用機序の解明は、より効果的かつ合理的治療戦略を考える上で必須の事項である。

以上の様な観点から、我々は次の２つの事項を目的として今回の研究を行った：(1). 発症初期における抗 GQ1b IgG 抗体価の経時的変化と臨床症状の推移との詳細な対比検討から、FS・GBS の外眼筋麻痺発症と症状増悪のプロセスにおける本抗体の位置づけを考察する。(2). 標的となる GQ1b 糖鎖抗原の外眼筋支配脳神経における微細構造レベルでの局在と他の糖鎖分子との局在関係を明らかとする。

B．研究方法

患者血清：外眼筋麻痺発症後 6 日以内に初回の、また遅くとも 14 日以内に２回目の血清を採取しえた患者で、少なくとも２回目の血清採取前には副腎皮質ステロイド療法・血漿浄化療法・免疫グロブリン大量静注療法（IVIg）などの免疫系に影響を与える可能性がある治療が行われなかった、FS 患者７例および GBS-OP 患者２例の計９例。初回血清採取のポイントは、外眼筋麻痺発症５日前：１例、発症２日目：３例、発症３日目：３例、発症５日目：１例、発症６日目：１例であった。この内４例では、外眼筋麻痺増悪期に血清抗 GQ1b IgG 抗体価の推移と臨床症状の変化を詳細に検討した。

血清抗 GQ1b 抗体価の測定：既報告の方法に従い ELISA 法で測定した。抗体価の経時変化の検討に当たっては、同一患者からの一連の検体は全て同じプレート上で反応を行った。

免疫組織染色・免疫電顕：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、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。ミエリンとの相互関係を検討するために抗 CD57 (HNK-1)抗体と、また各種ガングリオシドとの局在関係を検討するために各種マウスモノクローナル抗体での二重免疫染色による検討を行った。用いた各種ガングリオシドに対する抗体とクローン名は次の通りである：抗 GM3 抗体 (GMR6)，抗 GM1 抗体 (GMB16)，抗 asialo-GM1 抗体 (AG-1)，抗 GD3 抗体(GMR19)，抗 GD2 抗体(GMR7)，抗 GD1a 抗体(GMR17)，抗 GalNAc-GD1a 抗体 (2A3D2)，抗 GD1b 抗体 (GGR12)，抗 GT1a 抗体 (GMR11)，抗 GT1b 抗体 (GMR5)。

（倫理面への配慮）

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C．研究結果

1．抗 GQ1b IgG 抗体価の経時的変化と臨床症状の推移との詳細な対比検討

発症後からの検討を行った８例のうち７例で、抗体価は初回採取血清で最も高く、経時的に低下した。

外眼筋麻痺増悪期に血清抗 GQ1b IgG 抗体価の推移を観察した４例のうち３例では、外眼筋麻痺が増悪する時期には抗体価は既に低下していく段階にあった。これら３症例はいずれも典型的な FS の患者であった。1 例では症状増悪期に抗体価の上昇を認めたが、この症例は FS として発症後、強い筋力低下と呼吸筋麻痺が出現し、GBS-OP に移行した症例であった。

発症前より検討を開始しえた FS の１例では外眼筋麻痺出現前に既に抗体価の上昇を認め、外眼筋麻痺発症の頃に抗体価はピークに達し、その後症状が増悪していく過程では、血清抗 GQ1b IgG 抗体価は徐々に低下していった。またこの患者では IgG class に比して抗体価は低かったが IgM class の抗 GQ1b 抗体も検出され、その抗体価の推移は IgG class のものと同様であった。

2．外眼筋支配脳神経における GQ1b 糖鎖抗原の微細構造レベルでの局在と他の糖鎖分子との局在関係

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全体としては淡い染色性ながら局在していることが認められた。しかしその部分での染色性は均一ではなく、傍絞輪部程ではないが斑状に比較的強く染まる部分が散在する分布を示していた。

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 colocalization をしない分布を認めた。

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。

抗 GD1b 抗体による染色において、7F5 と極めて類似した染色像を認めた。すなわち傍絞輪部と思われる中央のくびれた円筒形の強い染色部位とそれに続く神経線維の外表面に沿った斑状の染色部位の散在を認めた。7F5 での染色との重ね合わせ像で、２つの抗体による染色部位はほぼ一致していた。

抗 GM3 抗体による染色においても、切片内における染色部位の密度は7F5の場合よりも低いものの同様の中央のくびれた円筒形の強い染色部位を認めた。蛍光重ね合わせ画像では、7F5 による染色部位とは一致していなかった。

抗 GD3 抗体による染色では Schwann 細胞の核周囲の胞体と思われる部分に比較的均一に広がる染色を認めた。7F5 でも Schwann 細胞の核周辺部が極淡くではあるが染色され、その部分では GD3 抗原との colocalization を認めた。

D．考察

今回我々は GBS, FS 発症前からの抗ガングリオシド抗体価の推移を詳細に検討する非常に貴重な機会を得た。今回の結果からは、血清抗 GQ1b IgG 抗体価は外眼筋麻痺発症前に上昇しており、その発症の頃に抗体価はピークに達し、更に増悪する時期には既に低下していく段階にあるということが示唆された。このことは本抗体を組織障害の結果として二次的に産生されたという可能性に対する強い反証となるものであり、また GBS における抗ガングリオシド抗体は先行感染因子が抗体産生の刺激となっているという考えと合致するものである。

Kusunoki らはウサギに GD1b を免疫し感覚失調性ニューロパチーの動物モデルを、また結城らはウサギに GM1 を免疫することにより急性軸索型ニューロパチーの動物モデルを作成したが、いずれのモデルにおいても対応する血清抗体が発症前より上昇し、抗体価がピークに達した後に神経症状の出現をみている。この経過は今回の我々の臨床症例と一致しており、これらの動物モデルと実際の GBS における抗ガングリオシド抗体の病態に果たす役割の共通性を示唆するものである。このことは、動物モデルの発症プロセスの詳細な検討が GBS 発症機序の解明および治療法の開発につながるという考えを、支持するものである。

臨床症状増悪期に抗体価の推移を検討した４例の内３例は外眼筋麻痺増悪期にもすでに抗体価は低下傾向を示していた。このことからは、臨床症状の重症度は抗体価の高さのみによって規定されているのではなく、抗原抗体反応後のプロセスによって規定されていることが考えられる。そのプロセスの解明が今後の新たな治療開発の上でのターゲットを提供する可能性がある。

臨床症状増悪期に抗体価の上昇を示した１例は FS として発症したがその後重度の四肢筋力低下が出現し GBS-OP へと進展した症例であった。臨床的には FS として発症した症例が GBS-OP へと移行することは稀ならず経験されるが、FS のまま経過する症例と GBS-OP へと進展する群の間の免疫学的差異を検討した報告はこれまでにない。今回 GBS-OP へ進展した症例で抗体価の推移を詳細に検討し得た症例は１例のみで、さらに同様な症例での検討の蓄積を要するが、発症初期の抗体価の推移を詳細に検討することにより、FS として発症した症例に四肢筋力低下が加わり GBS として重症化するのか、それとも FS のまま推移するのかを予測できる可能性があるとすれば、このことは抗 GQ1b IgG 抗体の測定が FS として発症した症例の治療法を選択する上での重要な指標ともなりうることを意味していると考えられる。

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。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 細胞の形質膜と考えられ

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斑状に散在する染色性が認められている結果と一致している。またその斑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。ガングリオシド等のスフィンゴ糖脂質は一般に形質膜上で lipid raft と呼ばれる集積部位を形成して分布していることからは、今回認められた斑状の染色がそのような膜上での局在様式を反映したものであり、傍絞輪部ではその lipid raft が高度に集積している可能性が考えられる。

ヒトの動眼神経において抗GQ1b抗体と抗GD1b抗体の染色が colocalization していることを見いだした。今回用いた抗糖脂質抗体について、抗 GQ1b 抗体の 7F5 は GQ1b 以外に GT1a と交叉反応することが知られているが、その他の抗体は主要な糖脂質については monospecific な抗体であることから、抗 GQ1b 抗体と抗 GD1b 抗体の染色性が同一エピトープに対する交叉反応により生じているものとは考えにくい。これらの染色性は切片をクロロフォルム-メタノール処理することにより容易に消失することからは、抗原エピトープは有機溶媒への可溶性の高い分子上に存在すると考えられる。また我々が報告したヒトの各種末梢神経組織のガングリオシド組成の生化学的分析結果では、7F5 で特異的に傍絞輪部の染色を認める外眼筋支配脳神経においてはガングリオシド GQ1b の含有比率が他の末梢神経組織より有意に高いこととも考え合わせると、今回見いだされた染色性の colocalization はガングリオシド GQ1b, GD1b 分子の colicalization を反映しているものである可能性が十分に考えられる。抗 GQ1b 抗体と抗 GD1b 抗体の染色の colocalization は傍絞輪部のみではなく神経線維外表面上に斑在する染色部位においても認められており、２つの分子が lipid raft 上に共存している可能性が示唆される。最近２つのガングリオシド分子が複合することにより患者血清中の抗体の標的となる新たな抗原エピトープが形成されることが報告されているが、今回見いだした GQ1b 糖鎖と GD1b 糖鎖の colocalization により臨床的に意味を持つ新たな抗原エピトープが形成されるかどうかは今後の検討課題である。

抗GM3抗体により形態的に傍絞輪部と思われる部位の染色が認められた。この部位においてその染色は抗GQ1b抗体の染色と colocalization していなかった。このことはすなわちヒトの動眼神経の傍絞輪部には局在する糖鎖の観点からは、GQ1bおよびGD1b糖鎖の colocalization するものと、GM3 糖鎖の局在するものの２種類のものが存在する可能性を示唆している。このような傍絞輪部に局在する糖鎖の差異とその機能的意義については今後の検討課題である。

E．結論

1．FS および外眼筋麻痺を伴う GBS の急性期血清中に上昇する抗 GQ1b IgG 抗体は、組織障害の結果として二次的に産生されるのではなく、先行感染に関連して上昇し、外眼筋麻痺の発症初期のプロセスに関与している可能性が高い。

2．臨床症状の重症度は抗体価のみによって規定されるものではなく、抗原抗体反応に続くステップが重症度を規定している可能性がある。そのプロセスの解明は今後の新たな治療開発の上でのターゲットを提供する可能性がある。

2．GQ1b 糖鎖抗原は形態的には lipid raft 様の集積部位を形成してヒトの外眼筋を支配する脳神経の Schwann 細胞の形質膜上に斑状に分布し、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では特にその集積が高度であると考えられる。

3．ヒトの動眼神経において傍絞輪部および神経線維上で GQ1b 糖鎖が GD1b 糖鎖と colocalization している。

4．GM3 糖鎖が GQ1b 糖鎖の局在が見られない傍絞輪部に局在している。ヒトの動眼神経の傍絞輪部には局在する糖鎖の観点からは２種類のものが存在することが示唆される。

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G．知的所有権の取得状況

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ABSTRACT: Anti-GM1 immunoglobulin G (IgG) antibodies are frequently present in sera from patients with Guillain–Barré syndrome (GBS). A previous report on a patient who had a neuropathy with immunoglobulin M (IgM) M-protein binding to a conformational epitope formed by phosphatidic acid (PA) and gangliosides prompted us to investigate the binding of IgG antibodies in GBS sera to a mixture of GM1 and PA (GM1/PA). Of 121 GBS patients, 32 had anti-GM1 IgG antibodies. All 32 also had antibody activity against GM1/PA. Twenty-five (78%) of 32 patients had greater activity against GM1/PA than against GM1 alone. Twelve patients who had no anti-GM1 IgG antibodies had IgG antibody activity against GM1/PA. No GBS patient had IgG antibody against PA alone. In contrast, two rabbit anti-GM1 antisera had greater activity against GM1 alone than against GM1/PA. IgG antibody with greater binding activity against a mixture of GM1 and a phospholipid than against GM1 alone may have an important role in the pathogenesis of GBS and has implications for diagnosis.
Muscle Nerve **27**: 302–306, 2003

BINDING OF IMMUNOGLOBULIN G ANTIBODIES IN GUILLAIN–BARRÉ SYNDROME SERA TO A MIXTURE OF GM1 AND A PHOSPHOLIPID: POSSIBLE CLINICAL IMPLICATIONS

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Guillain–Barré syndrome (GBS) is an acute, self-limited, motor-dominant neuropathy frequently preceded by an infection. Its pathogenesis has yet to be clarified, but both cell-mediated and humoral immunities have been implicated.[8,10] The frequent presence of antiganglioside antibodies in sera from GBS patients has recently been reported, with titers being highest in the acute phase and decreasing with clinical recovery.[12] Such antibodies should therefore be good diagnostic markers of acute-phase GBS. Some also may be pathogenetic factors. Anti-GM1 immunoglobulin G (IgG) antibody, one of the most frequently detected,[19] has been associated with the pure motor variant of GBS[20] and acute motor axonal neuropathy.[6] Sensitization with GM1 has been reported to cause motor axonal neuropathy in rabbits.[21]

A patient who had a neuropathy with immunoglobulin M (IgM) M-protein that bound to a conformational epitope formed by phosphatidic acid (PA) and gangliosides has been reported.[5] This prompted us to investigate the binding of IgG antibodies in GBS sera to a mixture of GM1 and PA.

MATERIALS AND METHODS

Serum samples from 121 patients fulfilling the criteria for GBS proposed by Asbury and Cornblath[2] were obtained from several hospitals throughout Japan. They were all in the acute phase. We also investigated samples from 30 normal and 65 disease controls: 16 patients had chronic inflammatory demyelinating polyradiculoneuropathy; 5 had multifocal motor neuropathy; 20 had multiple sclerosis; 9 had a collagen disease; and 15 had other neurodegenerative diseases.

An enzyme-linked immunosorbent assay (ELISA) was performed as described previously.[14] Wells of a microtiter plate were coated with 0.2 μg of GM1, 0.1

Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; GBS, Guillain–Barré syndrome; GM1/PA, a mixture of GM1 and phosphatidic acid; Ig, immunoglobulin; OD, optical density; PA, phosphatidic acid; PBS, phosphate-buffered saline

Key words: autoimmune; ganglioside; Guillain–Barré syndrome; neuropathy; phospholipid

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μg of GM1 mixed with 0.1 μg of PA (GM1/PA), or with 0.2 μg of PA. An uncoated well served as control. After incubation with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), serum diluted 1:40 with 1% BSA in PBS was added to each well and the plate was incubated at room temperature for 1.5 h. Sera from two rabbits sensitized with GM1, diluted 1:1600 with the same solution, also were assayed. After each well had been washed with 0.1% BSA in PBS, peroxidase-conjugated goat anti-human IgG antibody (Cappel, West Chester, PA), diluted 1:500 with 1% BSA in PBS, or peroxidase-conjugated goat anti-rabbit IgG antibody (Southern Biotechnology Associates, Birmingham, AL), diluted 1:2000 with the same solution, was added to each well and the plate was incubated at room temperature for 1.5 h. The wells then were washed with 0.1% BSA in PBS and a color reaction was obtained by incubating the wells with 200 μl orthophenylenediamine dihydrochloride (40 mg/dl of phosphate-citrate buffer, pH 5.0) at room temperature for 2 min. The reaction was stopped by the addition of 8N H_2SO_4, after which the optical density (OD, at 492 nm) was read with an ELISA reader (Bio-Rad, Hercules, CA). Each OD value was corrected by subtracting the OD of the control well that had been similarly processed. Serum with corrected OD >0.1 was considered positive. For each patient, anti-GM1 and anti-GM1/PA assays were performed on neighboring wells of the same microtiter plate. Antibody activity of the patient or of the rabbit was expressed as the mean of corrected ODs of two independent assays.

Wells of a microtiter plate were coated with a total of 0.2 μg of a mixture containing varying ratios of GM1 and PA, and the binding activities of IgGs in sera from six representative patients in group C (see later) were determined by ELISA at serum dilutions of 1:40. The activity was expressed as the mean of corrected ODs of three independent assays.

The IgG reactivities against GM1 mixed with a phospholipid other than PA were investigated by ELISA for sera from three selected GBS patients, one from each of groups A, B, and C.

RESULTS

Of the 121 patients with GBS, IgG anti-GM1 antibodies were positive in 32. All those positive for anti-GM1 also were positive for anti-GM1/PA. Antibody activities against GM1/PA (mean ± SD: 0.788 ± 0.226) were significantly greater ($P < 0.00001$, Student's *t*-test) than those against GM1 (0.511 ± 0.284) (Fig. 1). Only 7 of those 32 patients (22%) had greater antibody activity against GM1 than GM1/PA

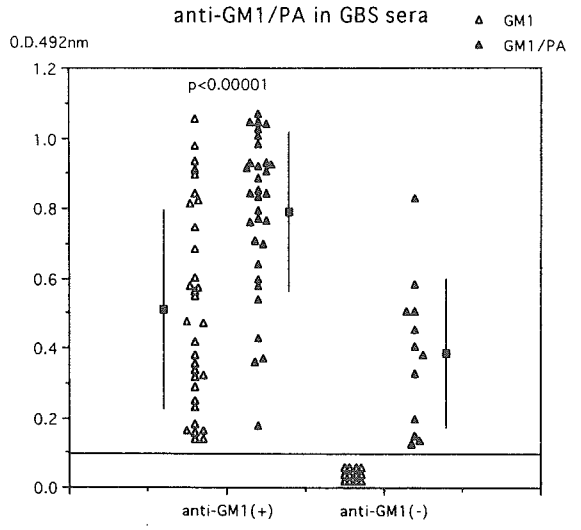

FIGURE 1. Anti-GM1/PA antibody activities compared with those against GM1 alone. Left lane: sera of anti-GM1 IgG-positive patients; right lane: sera of 12 patients with anti-GM1/PA IgG but no IgG activity against GM1 alone. Open triangles (left side of each lane): anti-GM1 activities; filled triangles (right side of each lane): anti-GM1/PA activities. Bar indicates mean ± SD.

(group A), whereas 25 (78%) had greater activity against GM1/PA than GM1 (group B). Twelve patients with GBS who were negative for anti-GM1 IgG antibody were positive for anti-GM1/PA IgG antibody. We classified them as group C (Fig. 1). No patient with GBS had IgG antibody activity against PA alone. None of the normal or disease controls had IgG antibodies against GM1 or GM1/PA.

The binding activities of serum IgGs at varying dilution factors were investigated by ELISA for sera from three selected GBS patients, one from each of groups A, B, and C. Their data are shown in Figure 2.

Antibody activities of the two rabbits were greater against GM1 than against GM1/PA (Fig. 3).

Maximal binding activities were observed at a GM1:PA ratio of 1:1 in three sera and of 1:4 in three sera from group C patients (Fig. 4). Phospholipids such as phosphatidyl inositol and phosphatidyl serine as well as PA had an enhancing effect in the anti-GM1 IgG antibody assay in group B and C patients (Fig. 5).

DISCUSSION

The GM1 antigen content per well was 0.1 μg in the anti-GM1/PA assay. Because this is half the amount used in the anti-GM1 assay, the activity against GM1/PA should be less than that against GM1 alone if the binding of the antibody is not affected by presence of PA. Rabbit anti-GM1 antisera actually showed such reactivity. IgG antibody activities

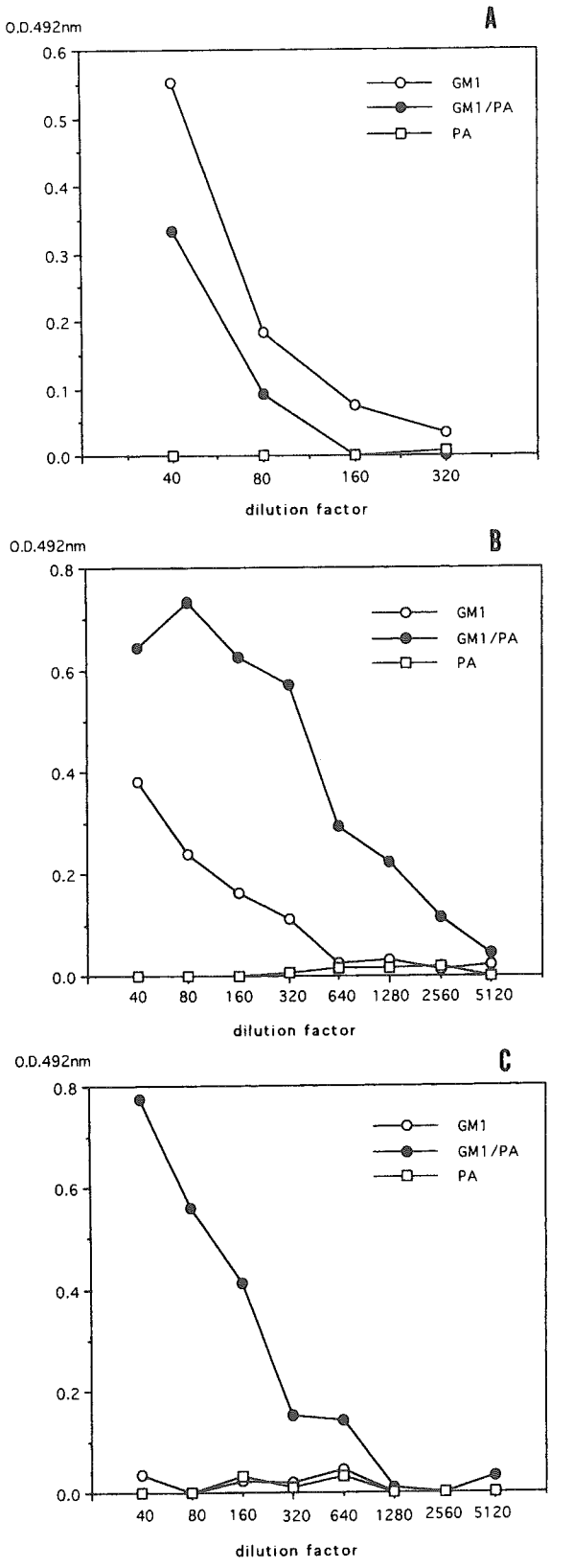

FIGURE 2. The binding activities of serum IgGs from a representative patient from group A (upper panel), group B (middle panel), and group C (lower panel) at varying dilution factors. Open circles: anti-GM1 activities; filled circles: anti-GM1/PA activities; open squares: anti-PA activities.

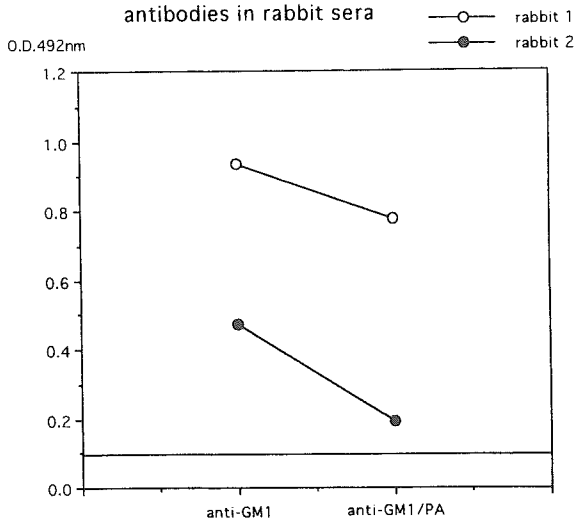

FIGURE 3. Antibody activities against GM1 and GM1/PA in sera from two rabbits sensitized with GM1. Anti-GM1/PA antibody activity is decreased compared to activity against GM1 alone.

against GM1/PA, however, were greater than those against GM1 alone in most of the anti-GM1 IgG-positive GBS patients. In addition, sera of group C patients had anti-GM1/PA activity, but no activity against GM1 alone. These findings were not due to reactivity against PA because no serum from GBS patients had reactivity against PA alone. ELISA on six sera of group C patients using a varying ratio of GM1:PA showed that maximal binding activities were obtained at a GM1:PA ratio of 1:1 in three sera and of 1:4 in three sera.

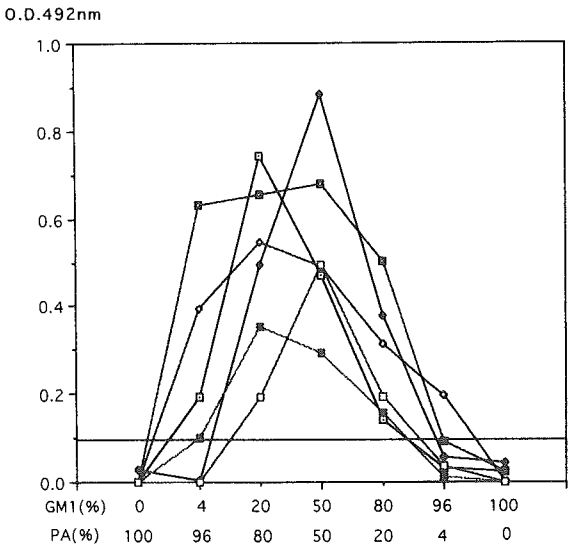

FIGURE 4. The binding activities of serum IgGs from six patients of group C using ELISA with a mixture containing various ratios of GM1 and PA as antigens.

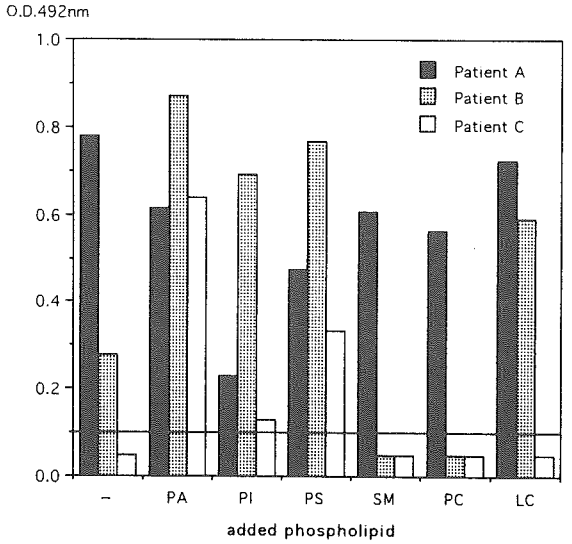

FIGURE 5. IgG antibody activities in sera from three GBS patients against GM1 mixed with varied phospholipids. Patients A, B, and C are from groups A, B, and C, respectively. On the horizontal axis, dash indicates no phospholipid (GM1 alone); PA, phosphatidic acid; PI, phosphatidyl inositol; PS, phosphatidyl serine; SM, sphingomyelin; PC, phosphatidyl choline; LC, lysophosphatidyl choline.

In addition to the report mentioned earlier,[5] greater binding activity of serum IgM against a mixture of GM1 and other lipids, such as lecithin/cholesterol[15] and galactocerebroside/cholesterol,[17] than against GM1 alone has been reported in patients with amyotrophic lateral sclerosis[15] and multifocal motor neuropathy.[17] Ours is the first report, to our knowledge, on IgG antibody in GBS sera.

There are two possible interpretations. One is that a conformational epitope formed by GM1 and PA is recognized by IgG antibodies in GBS patients in groups B and C. The other is that GM1 is presented in a more effective way for serum IgG of these GBS patients when it is mixed with phospholipids. We focused on PA because it was the antigen used in a previous report.[5] PA actually had stronger enhancement in the anti-GM1 antibody assay than the other phospholipids tested. However, phospholipids such as phosphatidyl inositol and phosphatidyl serine also had an enhancing effect in the anti-GM1 IgG antibody assay in our group B and C patients. This suggests that the latter possibility is more likely. Gangliosides present on the cell membrane are surrounded by phospholipids. Serum antibodies with greater binding activity against a mixture of GM1 and a phospholipid than against GM1 alone may have a stronger neuropathological effect on the cell membrane.

Anti-GM1 IgG antibody has been associated with antecedent *Campylobacter* infection[6,19,20] and the pure motor variant of GBS.[20] Its association with acute motor axonal neuropathy[6] has been reported, and the clinical features of classic acute inflammatory demyelinating polyradiculoneuropathy are also frequent in anti-GM1 IgG-positive GBS patients.[19] The clinical features of the patients in the three different groups (A, B, and C) that we described require further investigations in the future.

GM1 is the only ganglioside we investigated in this study. There are, however, various ganglioside antigens that are targets for serum antibodies in GBS. Anti-GQ1b IgG antibodies are specifically associated with ophthalmoplegia and ataxia in the Miller Fisher variant of GBS.[3,4,13] Anti-GalNAc-GD1a IgG antibodies are associated with the pure motor variant of GBS,[1,7,11] and anti-GD1a IgG antibodies with acute motor axonal neuropathy.[9] GBS patients with monospecific anti-GD1b IgG antibodies have the classic acute inflammatory demyelinating polyradiculoneuropathy type of neuropathy, with sensory as well as motor disturbance.[16] Whether these antibody activities are enhanced by the addition of certain phospholipids into the antigen requires further study.

Plasmapheresis and intravenous immunoglobulin therapy are the standard treatments for GBS.[18] Early diagnosis and an early start of therapy are the most important factors for successful treatment of GBS. An assay of antiganglioside antibodies in patients' sera is useful for early diagnosis. We showed that a positive ratio increases by the addition of PA to the antigen. This method should therefore make the antiganglioside antibody assay more useful for the diagnosis of GBS.

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□ CASE REPORT □

Amyotrophic Lateral Sclerosis with IgM Antibody against Gangliosides GM2 and GD2

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Abstract

We report a case of amyotrophic lateral sclerosis (ALS) with IgM antibody against gangliosides GM2 and GD2. A 57-year-old woman presented with slowly progressive muscular weakness of the upper extremities and dysarthria. She fulfilled the clinical and electrophysiological criteria of ALS, and died from sudden suffocation about 3 years after the onset of illness. The patient's serum IgM antibody was shown to recognize the structure shared by GM2 and GD2. Since anti-GM2 antibodies have been implicated in motor neuropathy or motor neuron syndrome, this rare case might contribute to the understanding of the immunological aspects of ALS.
(Internal Medicine 42: 277–280, 2003)

Key words: anti-GM2 antibodies, antiganglioside antibodies, motor neuron disease

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by loss of motor neurons in the central nervous system. The etiology of sporadic ALS remains unclear, although abnormalities of the Cu/Zn superoxide dismutase gene have been detected in some familial cases (1, 2). Several reports have shown that immunoinflammatory processes are involved in the pathology of ALS (3–5). In addition, a variety of immunological abnormalities have been reported in some patients with ALS (6–9). Gangliosides (sialic acid-containing glycosphingolipids) are abundant in the nervous system, and serum antiganglioside antibodies are occasionally detected in some immune-mediated neurological disorders (10–13). We encountered a sporadic ALS patient with serum IgM antibody against gangliosides GM2 and GD2.

For editorial comment, see p 220.

Case Report

Patient

The patient was a 57-year-old woman who was hospitalized with a 5-month history of slowly progressive muscular weakness of the upper extremities and dysarthria. On examination, weakness was prominent in the right forearm and small hand muscles (2–3/5 on the Medical Research Council scale), but lower extremity strength was preserved. Muscular atrophy was noted in the right and left thenars. Deep tendon reflexes were exaggerated in the extremities with bilateral Babinski sign. Fasciculations were observed in the tongue. There were no sensory deficits. She had no skin lesions suggesting malignant melanoma, which is occasionally associated with chronic inflammatory demyelinating polyneuropathy and IgM anti-GM2 antibodies (14).

The level of fasting blood sugar was within the normal range. Thyroid function was normal. M-protein was not detected by serum immunoelectrophoresis. Hepatitis B surface antigen was positive with mild liver dysfunction. IgM anti-cytomegalovirus antibody was negative. The antibody test for human T-lymphotropic virus type I (HTLV-I) was negative, which ruled out the possibility of a pseudo-ALS form of HTLV-I-associated myelopathy (15). Examination of cerebrospinal fluid revealed a cell count of 1/μl and protein level of 36 mg/dl. Motor nerve conduction studies showed mark-

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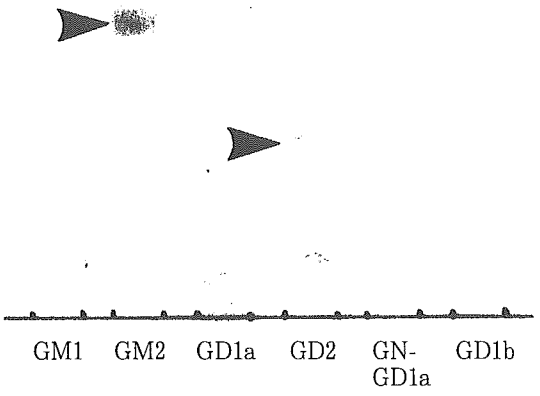

Figure 1. TLC-immunostaining with IgM in the patient's serum, diluted 1:80. The binding activity of the IgM antibody of the patient's serum to GM2 and GD2 is demonstrated (arrowheads). GN-GD1a: GalNAc-GD1a.

edly reduced compound muscle action potential amplitudes in the left median and the right median and ulnar nerves without slowed conduction velocity or conduction block. Needle electrode examination revealed evidence of active denervation in all extremities (first dorsal interosseous, extensor digitorum communis, and gastrocnemius muscles).

The patient did not respond to high-dose intravenous immunoglobulin treatment, which was administered considering the possibility of a treatable neuroimmunological disorder (13, 16, 17). She was clinically and electrophysiologically diagnosed as having ALS. After discharge, she developed dysphagia and muscular weakness of the lower extremities, and then became wheelchair bound. She died from sudden suffocation about 3 years after the onset of illness.

Methods

Serum antiganglioside antibodies (IgM and IgG) were investigated by enzyme-linked immunosorbent assay (ELISA) as described previously (12). Tested ganglioside antigens were GM1, GM2, GM3, GD1a, GD1b, GD3, GT1b, and GQ1b obtained from Funakoshi (Tokyo, Japan) and *N*-acetylgalactosaminyl GD1a (GalNAc-GD1a) obtained as described previously (12). Antibody titer was expressed as the maximal dilution factor which gave a corrected optical density of more than 0.1 (normal, less than 1:40). The results of ELISA were confirmed using thin-layer chromatogram (TLC)-immunostaining performed as described previously (12). ELISA using peroxidase-conjugated rabbit anti-human kappa or lambda light chain antibodies (DAKO, Glostrup, Denmark) as second antibodies was also performed to assess polyclonality or monoclonality of the patient's serum antibody.

The absorption test was performed as follows. Each well of a 96-well microtiter plate was coated with 0.4 μg of each ganglioside. The wells were filled with 1% bovine serum albumin in phosphate-buffered saline for 30 minutes and emptied. The plate was incubated with the patient's serum diluted 1:200 for 2 hours at room temperature then left overnight at 4°C. After absorption, the antiganglioside antibody titer of each sample was measured by ELISA.

Results

The patient's serum IgM was reactive with GM2 (antibody titer of 1:640), but not with GM1, GM3, GD1a, GD1b, GD3, GalNAc-GD1a, GT1b, or GQ1b (less than 1:40). The binding activity of the antibody to GM2 was detected with both anti-human kappa and lambda light chain antibodies as second antibodies, suggesting that the antibody was polyclonal (data not shown). There were no IgG antibodies against any of the antigens tested. The anti-GM2 antibody titer remained high throughout the course of the illness. Since the IgM anti-GM2 antibody of the patient was not cross-reactive with GM1 or GalNAc-GD1a, cross-reactivity with GD2 was additionally examined. The patient's serum IgM was also reactive with GD2 (1:320). Reactivities with GM2 and GD2 were confirmed by TLC-immunostaining (Fig. 1). Absorption with GM2 or GD2, but not with GM1 or GalNAc-GD1a, reduced the antibody activities (Table 1). Thirty other patients with ALS were also tested for serum anti-GM2 and anti-GD2 antibodies (IgM and IgG), and the

Table 1. Absorption Test

	IgM anti-GM2 antibody	IgM anti-GD2 antibody
Not absorbed	0.464	0.21
Absorbed with GM2	<0.1	<0.1
Absorbed with GM1	0.385	0.259
Absorbed with GalNAc-GD1a	0.366	0.218
Absorbed with GD2	<0.1	<0.1

Absorption of the patient's serum was performed with 0.4 μg of each ganglioside. Data are optical density values.

results were all negative (data not shown). In addition, we present previous cases with IgM anti-GM2 antibodies in our laboratory: one patient with multifocal motor neuropathy (antibody titer of 1:2,560) (13) and five with Guillain-Barré syndrome (GBS) subsequent to cytomegalovirus infection (1:40 to 1:640).

Discussion

Cavanna et al (18) measured IgM anti-GM2 antibodies in the sera of 224 patients with different neuropathies and motor neuron disease (MND), and found high titers of the antibodies in eight patients with dysimmune neuropathies. All of those patients had a concomitant IgM reactivity with either GM1 or GalNAc-GD1a, the latter sharing the terminal trisaccharide with GM2 (Fig. 2). However, the present patient's serum IgM was not reactive with GM1 or GalNAc-GD1a, but with GD2. The absorption test revealed that the IgM antibody mainly recognizes the moiety shared by GM2 and GD2 (Fig. 2). Similar reactivities with GM2 and GD2 have been observed in the serum antiganglioside antibodies of patients with GBS subsequent to cytomegalovirus infection (19, 20). Yuki et al (21) measured IgM and IgG anti-GD2 antibodies in the sera of 257 patients with various neurological disorders, and found high titers of the antibodies in seven patients including five with GBS. One of these GBS patients had IgM antibody against GD2, GM2, and GalNAc-GD1a.

GM2 may be an immunologically unique ganglioside. Tai et al (22) reported that GM2 appears to be the most immunogenic among gangliosides found on human melanoma cells. We recently reported that GM2 markedly enhances the production of tumor necrosis factor-α in peripheral blood mononuclear cell culture (23). In addition, GM2 is thought to be a possible major component of motor neuron gangliosides (24, 25).

Antibodies against several kinds of gangliosides other than GM2 have been detected in MND (26–28). O'Hanlon et al (29) recently reported three MND patients with IgM anti-GM2 antibodies whose cross-reactivity with GD2 was not described. Interestingly, high-titer IgM antibody against GM2 was detected in a patient who developed an ALS-like disorder after intramuscular treatment with bovine brain gangliosides for diabetic neuropathy (30). The IgM anti-GM2 antibody in that patient was cross-reactive with GalNAc-GD1a (31), but not with GD2 (30). In addition, Nakao et al (32) found two novel GM2-epitope containing gangliosides in bovine brain with that patient's serum IgM. Similarly, there might be unknown gangliosides in the central nervous system that react with our patient's serum IgM.

As one of the autoimmune hypotheses in ALS, autoreactive antibodies might be taken up at the nerve ending and transported within the axons (33). It was reported that immunoglobulins from ALS patients enhance spontaneous transmitter release from motor nerve terminals (7). IgM monoclonal antibody against terminal moiety of GM2 and

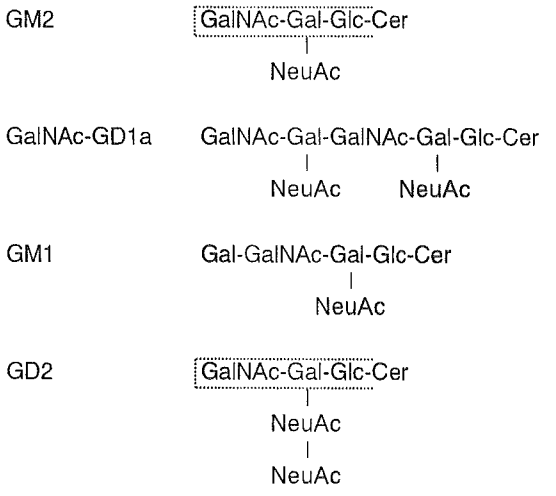

Figure 2. Structures of gangliosides GM2, GalNAc-GD1a, GM1 and GD2. GalNAc: *N*-acetylgalactosamine, Gal: galactose, Glc: glucose, Cer: ceramide, NeuAc: *N*-acetylneuraminic acid. GM2 and GD2 have a GalNAc-Gal-Glc-moiety in common (dotted line).

GalNAc-GD1a has recently been shown to have effects on neurotransmitter release (34). These pathophysiological mechanisms involving motor nerve terminals may account for selective damage to motor nerves or motor neurons. Although the pathogenic significance of IgM anti-GM2 antibodies in MND is still undetermined (29), the present rare case might contribute to the understanding of the immunological aspects of ALS.

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A family with *Campylobacter* enteritis: Anti-GD1a antibody with/without Guillain–Barré syndrome

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Guillain–Barré syndrome (GBS) is characterized by acute, motor-predominant neuropathy frequently preceded by infection. *Campylobacter jejuni* enteritis is involved in about one-third of patients.[1] Molecular mimicry between *C. jejuni* and gangliosides can lead to the production of serum anti-ganglioside antibodies, which may cause neuropathies.

A literature search revealed only one report describing a family with *C. jejuni* enteritis, in which GBS developed in one of three affected members. That report implicated anti-ganglioside antibody as the cause of GBS.[2] We now describe a second such family in which additional factors as well as anti-ganglioside antibodies may have contributed to the GBS onset.

Two brothers, 16 and 19 years old, had diarrhea of 3-days' duration. A week after the onset, the younger brother had severe tetraparesis. Neurologic and electrophysiologic findings were consistent with a diagnosis of axonal GBS. Blood specimens were obtained from the two brothers on the fourth day after the GBS onset. The brothers were sero-positive for anti–*C. jejuni* antibody. Anti-ganglioside antibodies were examined as described previously, using GM1, GM2, GM3, GD1a, GD1b, GalNAc-GD1a, GD3, GT1b, and GQ1b as antigens.[3] The brother with GBS had anti-GM1 IgM (1:320) and IgG (1:160) and anti-GD1a IgG (1:80) antibodies (normal <1:40 for both IgM and IgG). IV immunoglobulin therapy produced prompt marked improvement in the patient's condition. The elder brother had no signs or symptoms suggestive of GBS. The serum was strongly positive for anti-GD1a IgG antibody (1:320), confirmed to react with GD1a on thin-layer chromatography (figure). Anti-ganglioside antibody titers for both brothers decreased to <1:40 3 months after the disease onset.

Our study showed that GBS developed in only one of the two siblings with anti-ganglioside antibodies. One explanation is that anti-GM1 antibody was involved in the pathogenesis of the disease, whereas anti-GD1a antibody was not. The pathogenicity of anti-GM1 antibody is experimentally evidenced in GM1-immunized animals. However, anti-GD1a IgG antibody is more closely associated with acute motor axonal neuropathy than anti-GM1 IgG antibody.[4] Among 600 patients with definite or probable GBS, 4 were positive for only anti-GD1a IgG antibody, with titers ranging from 1:160 to 1:320 (S. Kusunoki, unpublished observation). The antibody titer in the patient without GBS was therefore comparable with that in patients with GBS. In addition, the