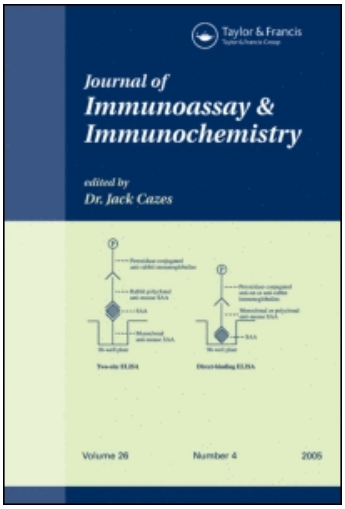

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Detection of Anti-Gm1 Ganglioside Antibodies in Patients with Neuropathy by a Novel Latex Agglutination Assay

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DETECTION OF ANTI-GM1 GANGLIOSIDE ANTIBODIES IN PATIENTS WITH NEUROPATHY BY A NOVEL LATEX AGGLUTINATION ASSAY

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ABSTRACT

Highly elevated titers of serum anti-GM1 ganglioside antibodies are closely associated with multifocal motor neuropathy, but low titers are commonly present in normal individuals or other diseases. Current systems for measuring anti-GM1 antibodies utilize the enzyme-linked immunosorbent assay (ELISA), in which serum dilutions are tested for binding to excess antigen immobilized on the surface of microwells. The ELISA system, however, is relatively time consuming, labor intensive, and costly, in addition to being prone to methodological variability. We have developed a novel agglutination assay for the detection of anti-GM1 antibodies, utilizing GM1 ganglioside-coated latex beads. In contrast to the ELISA system, antibody titers may be quantified by testing for agglutination using latex beads coated with decreasing amounts of antigen. The agglutination assay compares favorably to the ELISA system in sensitivity and specificity, but is considerably less costly and takes only a few minutes to perform.

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INTRODUCTION

Highly elevated titers of IgM anti-GM1 ganglioside antibodies are reported to occur in 20% to 85% of patients with multifocal motor neuropathy or reversible lower motor neuron disease, while lower titers are present in normal subjects and in patients with other diseases (1-7, 10). Assays for the detection of anti-GM1 antibodies are therefore increasingly used in clinical practice to aid in the evaluation and diagnosis of patients suspected of having motor neuropathy or motor neuron disease.

Titers of antibodies directed against GM1 ganglioside are commonly measured by the enzyme-linked immunosorbent assay (ELISA), in which increasing serum dilutions are tested for binding to GM1 ganglioside-saturated microwells. The ELISA system, however, is relatively time consuming and costly, and its use is clouded by issues of methodology such as antigen concentration, blocking medium, incubation time and temperature, and secondary antibody concentration, resulting in inter-laboratory variability (8, 9, 12, 13). A more rapid, economical, and reliable test for detecting anti-GM1 antibodies is needed.

Low titers of anti-GM1 ganglioside antibodies occur non-specifically in a variety of other neurological disorders, as well as in normal subjects (5, 7, 10). Therefore, in our laboratory, titers of 800 or below are not considered to be clinically significant and are reported as negative. Hence, in developing a new immunoassay for detection of these antibodies, it is imperative that the assay be designed such that it can differentiate between sera of clinically significant titer (>800) and those of low titer which have little clinical significance.

We describe a novel, economical, and rapid method for the detection of serum anti-GM1 ganglioside antibodies. The assay employs polystyrene microparticles coated with GM1 ganglioside. When combined with patient serum, agglutination of the particles signals the presence of significant amounts of anti-GM1 antibodies. The assay compares favorably with the ELISA system with respect to sensitivity, specificity, and reproducibility.

MATERIALS AND METHODS

Serum Samples

Serum samples were obtained from 29 patients; eight with multifocal motor neuropathy (MMN), ten with chronic inflammatory demyelinating polyneuropathy (CIDP), six with amyotrophic lateral sclerosis (ALS), four with demyelinating neuropathy associated with anti-myelin-associated glycoprotein (anti-MAG) antibodies, and one with Miller Fisher syndrome (MFS). In addition, sera from five normal subjects were evaluated as controls. All patient sera were prepared, aliquoted, and stored at -20 °C.

Preparation of Latex Particles

Latex beads were coated with GM1 ganglioside by passive adsorption. A 400 μg/mL solution of GM1 ganglioside (Sigma Chemicals, St. Louis, MO) was prepared by combining 40 μL of a 5 mg/mL stock solution of GM1 in methanol with 210 μL of H_2O and 250 μL of 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 6.1). A 1% suspension of 0.3 μ blue polystyrene latex particles (Seradyn Particle Technology, Indianapolis, IN) was prepared from the 2.5% stock suspension by adding H_2O. Adsorption of GM1 to the beads was initiated by addition of microparticle suspension to the ganglioside solution, followed by gentle stirring for 4 hours at room temperature. The suspension was then incubated for 72 hours at 4 °C. The particles were washed twice with a solution of 1% BSA in 25 mM MES buffer (pH 6.1) by centrifugation at 9,800 x *g* and 4 °C, and resuspended in the same solution. The coated beads were incubated for 48 hours at 4 °C before use. Control latex particles were prepared by coating them with GD1a ganglioside (Sigma Chemicals, St. Louis, MO) in place of GM1, following the same procedure.

To determine whether titers of anti-GM1 antibodies could be quantified by testing for reactivity with beads containing decreasing concentrations of GM1, sera were tested for agglutination using beads that were coated with varying concentrations of GM1 and GD1a. Preparation of the latex particles was the same as described for GM1, with the difference that increasing quantities of GD1a were

used to replace GM1, effectively lowering the concentration of GM1 coated. The following concentrations of GM1 were examined: 100% GM1, 50% GM1, 12% GM1, 6% GM1, 1.5% GM1, 0.75% GM1, and 0% GM1.

Agglutination Reaction

On a 3-ring glass slide (Cel-Line, Newfield, NJ), 4.5 μL aliquots of serum were placed. To each ring, 4.5 μL of the coated latex particles was added and mixed thoroughly with a plastic applicator. The slide was rocked gently for 30 to 40 seconds. Positive agglutination, characterized by blue clumps of beads, indicated the presence of anti-GM1 antibodies. We found that particle agglutination was more easily visualized when using colored latex beads instead of white beads. Strong results were clearly visible with the naked eye. Weak results could be visualized by holding the slide to a light source and observing for agglutination from underneath. To minimize inter-operator variability, all results were confirmed using a microscope (x 40 magnification). In the absence of agglutination, the reaction was considered to be negative. If agglutination were present, it was scored from 1 to 3 according to the degree of agglutination, where 1 denotes weak agglutination and 3 strong agglutination.

Enzyme-Linked Immunosorbent Assay (ELISA)

The presence of anti-GM1 IgM in sera was also measured by the commonly used enzyme-linked immunosorbent assay, following previously described procedure (11), with minor modification. Wells in 96-well round-bottom polystyrene microtiter plates (Becton Dickinson, Franklin Lakes, NJ) were coated with 0.5 μg of GM1 in 100 μL of methanol. After evaporation of the methanol, the wells were blocked by incubation with 300 μL of 1% bovine serum albumin (BSA) in 10 mM phosphate-buffered saline (154 mM NaCl, pH 7.4) (PBS) for 4 hours at 4 °C, and 100 μL of BSA/PBS-diluted patient or control serum was added to the wells. Wells coated with BSA instead of serum served as control. The plates were incubated overnight at 4 °C and then washed with the BSA/PBS solution. Antibody binding was detected by the addition of 100 μL peroxidase-

conjugated goat anti-human IgM secondary antibody (ICN Biomedicals, Costa Mesa, CA) after 1:1000 dilution in BSA/PBS solution (a final concentration of 2.14 μg/mL) to each well, and incubation for 2 hours at 4 °C. Plates were then washed and 100 μL of developing solution comprised of 27 mM citric acid, 50 mM Na_2HPO_4, 5.5 mM *o*-phenylenediamine, and 0.01% H_2O_2 (pH 5-5.5) was added to each well. The plates were incubated at room temperature for 30 minutes before measuring absorbance at 450 nm. The titer for each specimen was assigned as the highest dilution in which the absorbance reading was 0.1 units greater than in the corresponding BSA-coated wells. Sera with titers of 800 or lower were considered to be negative for the presence of clinically significant amounts of anti-GM1 antibodies, as such titers are also seen in normal subjects (10).

RESULTS

Sera from a total of 34 individuals were examined for anti-GM1 antibodies by both the agglutination assay and ELISA. Of the eight sera examined from MMN patients, six tested positive for anti-GM1 antibodies by the latex agglutination assay. All sera from patients with CIDP, ALS, demyelinating neuropathy associated with anti-MAG antibodies, and MFS, as well as those from normal subjects were found to be negative (Table 1). All specimens were tested on at least three different occasions. The assay proved to have a high reproducibility as repeated tests on each serum gave identical results, with the rankings remaining the same.

In developing the agglutination assay, we found that unlike the ELISA system, antibody titers could not be measured using serum dilutions, as these either caused non-specific agglutination of the lipid-coated microparticles or led to inhibition of the agglutination reaction. However, altering the concentration of coated GM1 antigen led to differences in reactivity with each serum. Undiluted sera with higher titers of anti-GM1 antibodies, as determined by ELISA, caused agglutination of microparticles coated with lower concentrations of antigen. The new agglutination assay was designed in such a manner as to give positive results

TABLE 1

Analysis of Patient Sera with Latex Agglutination Assay and ELSIA

Group	Number of serum samples	Number positive by latex agglutination assay	Number positive by ELISA
MMN	8	6	5
CIDP	10	0	0
ALS	6	0	0
Anti-MAG Neuropathy	4	0	0
MFS	1	0	0
Normal	5	0	0

only when testing sera with clinically significant titers of anti-GM1 antibodies. The sensitivity of the assay system was mainly dependent on the antigen concentration, that is the concentration of the coated GM1 ganglioside. That concentration was therefore adjusted to yield positive agglutination results with patient sera exhibiting anti-GM1 antibody titers of 800 or above, as measured in the ELISA system. Optimal results were obtained with incubation of a 1% suspension of 0.3 μ latex beads with a 400 μg/mL solution of GM1.

The agglutination assay exhibited equally good or better sensitivity when compared to the ELISA system. It gave positive results in all 5 of the 8 patients with MMN and elevated anti-GM1 antibodies as determined by ELISA, with titers ranging between 1,600 and 100,000 (Table 2). One other patient with MMN was positive by the agglutination assay but negative by ELISA, with a titer of 800. The two remaining patients with MMN were negative for anti-GM1 antibodies by both the agglutination and ELISA systems.

The agglutination assay appeared to be highly specific for patients with MMN, with none of the control patients or normal subjects exhibiting positive results. Four specimens with elevated levels of serum IgM and increased titers of anti-MAG antibodies, as well as a specimen from a patient with Miller Fisher syndrome (MFS) and antibodies against GQ1b ganglioside, tested negative for reactivity to GM1 with the agglutination assay.

TABLE 2

Comparison of ELSIA and Latex Agglutination Assay in Detection of Anti GM1 Antibodies in Sera of Patients with MMN

Patient No.	Anti-GM1 IgM Titer (ELISA)[1]	Latex Agglutination Assay[2]
1	100,000	3
2	3,200	3
3	50,000	3
4	<800	Negative
5	800	1
6	1,600	2
7	<800	Negative
8	6,400	3

[1]Titer for each specimen was assigned as the highest dilution in which the absorbance reading was 0.1 units greater than in the corresponding BSA coated wells.
[2]Results were scored from 1 to 3 according to the degree of agglutination.

TABLE 3

Latex Agglutination Assay in Detection of Anti GM1 Antibodies in Sera of Patients with MMN Using Latex Particles Coated with Different Ratios of GM1 to GD1a

Patient No.	Anti-GM1 IgM Titer (ELISA)[1]	Latex Agglutination Assay[2]						
		A	B	C	D	E	F	G
1	100,000	3	2	2	2	1	Neg.	Neg.
3	50,000	3	2	1	Neg.	Neg.	Neg.	Neg.
6	1,600	2	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
8	6,400	3	1	Neg.	Neg.	Neg.	Neg.	Neg.

[1]Titer for each specimen was assigned as the highest dilution in which the absorbance reading was 0.1 units greater than in the corresponding BSA coated wells.
[2]A: 100% GM1, 0% GD1a; B: 50% GM1, 50% GD1a; C: 12% GM1, 88% GD1a; D: 6% GM1, 94% GD1a; E: 1.5% GM1, 98.5% GD1a; F: 0.75% GM1, 99.25% GD1a; G: 0% GM1, 100% GD1a.

Four of the samples that exhibited reactivity to GM1 ganglioside in the agglutination assay were also tested for reactivity with latex particles coated with decreasing concentrations of GM1, in which GD1a was substituted (Table 3). None of the sera caused agglutination with particles coated with 100% GD1a, thus confirming the specificity of the GM1 reaction. On the other hand, all four sera yielded positive results with particles coated with less than 100% GM1; the higher the titer of anti-GM1 antibodies, the lower the concentration of the GM1 antigen that was required to produce agglutination. The serum with the highest concentration of anti-GM1 antibodies, having a titer of 100,000 by ELISA, reacted with beads that were coated with as little as 1.5% GM1.

DISCUSSION

A novel latex agglutination assay was developed for detection of serum anti-GM1 antibodies. The assay detects a functional antibody-antigen interaction that results in agglutination and compares favorably to the ELISA system in sensitivity and specificity. Additional advantages of the new assay include substantial reduction in the cost and time required for performing the test. Unlike the ELISA, which takes two days to perform and requires a plate reader, the agglutination assay is completed in minutes and requires no special instruments.

The agglutination assay can be readily used to rapidly screen sera for the presence of anti-GM1 antibodies. In light of the fact that a large number of sera are negative for the presence of anti-GM1 antibodies, the assay aids in screening out negative serum samples. If information on antibody titer is desired, reactive sera can then be tested using the ELISA system, which measures antibody binding at increasing serum dilutions, or by the agglutination assay, which tests for reactivity using microparticles coated with decreasing antigen concentrations.

In addition to testing for antibodies to isolated glycolipids such as GM1, the agglutination assay could be useful in detecting antibody reactivities to one or more antigens in a mixture of glycolipids coated onto the latex particles. This could be used in the form of sensitive assays for detection of antibodies that react with shared epitopes on two or more glycolipids (14), or that recognize

conformational epitopes that result from the interaction of two or more neighboring glycolipids (15). It could also be particularly useful in testing for the presence of antibodies directed against previously unrecognized antigenic glycolipids in other immune-mediated disorders.

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