ELISA-Type Titertray Assay of IgM Anti-GM1 Autoantibodies

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We report an ELISA-type titer assay for autoantibodies against ganglioside GM1. Trays were coated with ganglioside GM1 and reacted with patients' sera; bound IgM was detected with rabbit antibody to human IgM. High-titer serum from a patient was used as calibrator, another patient's serum as the positive control, and the GM1-specific cholera toxin as the control for GM1 coating. Regression curves of serum titers obtained from different patients were linear and parallel. Intra- and interassay CVs were 4.0–7.8% and 5.5–16%, respectively. We detected antibodies at a titer of 1:250 in normal subjects. Analytical specificity of the calibrator serum against GM1 was demonstrated by immune thin-layer chromatography. Anti-GM1 antibodies were increased in patients with chronic inflammatory demyelinating polyradiculoneuropathy (P < 0.002) or multiple sclerosis (P < 0.01). In Guillain–Barré syndrome, preliminary longitudinal studies showed a decrease in anti-GM1 titer that was related to clinical recovery.

Indexing Terms: autoantibodies/gangliosides/neuropathy/multiple sclerosis/Guillain–Barré syndrome

Circulating IgM antibodies against ganglioside GM1 are possible causative agents in neurological diseases (1–5), including multiple sclerosis (MS) (6–8), Alzheimer disease (9), motor neuron disease (MND) (5, 10–12), Guillain–Barré syndrome (GBS) (13, 14), and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) (12, 15). A high antibody concentration seems especially to be associated with diseases in which motor symptoms predominate (16). Quantification of circulating anti-GM1 autoantibodies has been suggested as being useful for diagnosis, as a predictive marker, and as a response analyte for monitoring therapy (1–4, 11, 13, 17, 18). At the time of diagnosis, a high anti-GM1 autoantibody concentration can be used for classification of motor or sensimotor neuropathies, as well as for MND (1–5). In GBS, high concentration predicts a severe course of disease and a poor prognosis (13, 14). In patients with multifocal motor neuropathy, a therapeutic reduction in anti-GM1 concentration has been associated with clinical improvement (17).

Several different methods, including ELISA (3–5, 11–15, 19), thin-layer chromatography (TLC) (11), and complement-dependent liposome lysis (6–8), have been used to measure circulating autoantibodies against GM1. Developers of these assays have tried various approaches to standardization: correlating titer values with those of a reference serum (3, 4, 10, 11, 14), stopping the enzyme reaction when the absorbance of a reference serum reaches a target value (5, 15), and calibrating against a patient's serum (13). However, there has been no thorough validation of the reproducibility of these assays. Here we describe an ELISA in which we use a high-titer patient's serum as calibrator, another patient's serum as a positive control, and the GM1-specific cholera toxin as a control for the GM1 well-coating.

Materials and Methods

Samples. Sera were obtained from 83 blood donors, 10 HIV-positive subjects (HIV), 29 patients with small cell lung cancer (SCLC), and 73 neurological patients: GBS (5), CIDP (11), MS (36), MND (15), and peripheral polyneuropathy (PPN; 6). The protocol was approved by the local science ethics committee.

Materials. Purified bovine GM1 ganglioside, bovine serum albumin (BSA), cholera toxin, and o-phenylene-diamine (OPD) were from Sigma Chemical Co. (St. Louis, MO); ganglioside mix and GM1 (purity checked by mass spectrometry) from Biocarb (Lund, Sweden); Polysorp titertrays from Nunc (Næstved, Denmark); skimmed milk powder and gelatin (Gelatine G) from Grinsted Products (Brabrand, Denmark); rabbit anti-human IgM and normal rabbit serum (NRS) from Dako (Copenhagen, Denmark); and "high-performance" TLC (HPTLC) plates from Merck (Darmstadt, Germany).

Titertray procedure. The final optimized procedure was as follows: Add purified bovine GM1 ganglioside (150 ng in 50 μL of ethanol) to microwell plates and let this evaporate to dryness at room temperature; store overnight at 4°C. Block the nonspecific binding sites with 10 g/L BSA in phosphate-buffered saline (PBS), pH 7.4, for 5 h at room temperature. Wash the wells three times in PBS containing 10 g/L BSA. Dilute sera and cholera toxin to various titers in the washing buffer, deliver 100 μL to each well, and incubate at 4°C for 24 h. After washing the wells three times in washing buffer, add peroxidase-conjugated rabbit anti-human IgM (diluted 1000-fold in washing buffer) and incubate for 1 h at room temperature. Again, wash the wells three times, and add 100 μL of OPD solution in 33.3 mg/L citric acid buffer; after 30 min stop the reaction.
with 150 μL of 1 mol/L sulfuric acid. Measure the absorbance of the reaction product at 492 nm, and subtract the corresponding absorbance at 690 nm.

Calibration and statistics. We used a high-titer patient's serum (PB) as calibrator. The patient, a 50-year-old man with CIDP, had no paraproteins in his serum and normal concentrations of IgM, IgG, and IgA. Cholera toxin completely inhibited the binding of PB IgM to GM1. Linear regression of the results for PB serum in serial dilutions (range 1:10 000 to 1:100 000) yielded a linear curve against which all test results were calibrated. Consequently, results are expressed as PB units. We used cholera toxin to control coating (PB = 100), and serum from another patient as a positive control (PB = 90).

Gaussian distribution was evaluated by probit plots and estimated by the Lilliefors test. Nonparametric (5% significance level) tests were used to compare medians (Wilcoxon nonpaired test) and distribution (Kolmogorov–Smirnoff) of serum anti-GM1 concentration.

Immune TLC. Immune TLC was performed as previously described (20). In short, a ganglioside mixture in chloroform:methanol (2:1 by vol) was spotted onto a HPTLC plate and allowed to dry. The plate was then developed in solvent, covered with poly(isobutyl)metacrylate solution, and incubated with the patient's serum. After a wash, the plate was incubated with rabbit anti-human immunoglobulin conjugated with peroxidase, and subjected to a color reaction with 3-amino-9-ethylcarbazole. For identification of gangliosides, a lane containing a ganglioside mix was stained with orcinol spray.

Results

Analytical Performance

Assay optimization. Hexane was a poor solvent for gangliosides; ethanol and methanol were better, giving very similar results (Fig. 1A). We chose ethanol because it gave the most reproducible results (data not shown) and is less toxic than methanol. The optimal coating concentration was 150 ng of GM1 in 50 μL of ethanol (Fig. 1B). Various PBS-based buffers were examined as blocking, washing, and diluting solutions: 10 mL/L skimmed milk, 10 g/L Gelatine G, 10 g/L BSA in 10 mL/L normal rabbit serum, 10 g/L BSA of high analytical grade, and 10 g/L BSA of low purity. Gelatine G and skimmed milk were not usable. The 10 g/L BSA of high analytical grade was chosen as blocking reagent and serum diluent; 10 g/L BSA of low purity was chosen as washing solution (Fig. 1C). A titer of 1:1000 for the second antibody was optimal (Fig. 1D). Immune TLC of a ganglioside mixture showed that the PB serum reacted with ganglioside GM1 and to a lesser extent with ganglioside GD1b (data not shown).

Linearity and recovery. The calibration curve (based on PB dilutions) was linear in the titer range 1:10 000 to 1:100 000, as estimated from 15 separate runs (r > 0.997 in all runs). Dilution curves for high-titer patients' sera, normal sera, and cholera toxin were approximately parallel and linear within the measured range. Two low-titer sera were each mixed with a high-titer serum, from which we recovered 99% and 98.8% of the expected calculated value.

Intra- and interassay variation. Intraassay CV in the detection range (> 5 PB) was 4–7.8% (Table 1) We determined this variation at different titers of anti-GM1 antibodies, analyzing in 32 replicates. Interassay variation was between 5.5% and 16% (Table 1). The CV for the reaction of cholera toxin against GM1 ranged from 12.9% to 21.1% (data not shown).

Storage. PB serum and a pool of five normal sera were stored at various temperatures, from −150°C to room temperature. Also, a PB sample was freeze-dried in aliquots and kept at −20°C. Samples from each storage arrangement were assayed in parallel every 2 weeks for 5 months; the various storage conditions showed no effect on the anti-GM1 concentrations measured.

Interference. IgG (Sandoglobin 3%; Sandoz Pharma, Basel, Switzerland) at concentrations similar to the

Fig. 1. Optimization of the assay: results for dilutions of PB serum under different conditions.

(A) Effects of solvents: △, hexane; ◇, ethanol; ◆, methanol. (B) Effects of GM1 concentration in coating solution: □, 200 ng; □, 100 ng; ◇, 25 ng; ◆, 2.5 ng. (C) Effects of blocking solutions: △, BSA (10 g/L) of low purity; ◇, BSA (10 g/L) + NSR (10 mL/L); ◆, BSA (10 g/L) of high purity; □, Gelatine G; ◆, skimmed milk (10 g/L). (D) Effects of dilution of rabbit anti-human IgG: □, 1:1000; □, 1:2000; ◇, 1:4000. Ordinate scale: absorbance × 1000.
Table 1. Precision of measurements of IgM and anti-GM1 autoantibodies

<table>
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<th>Antibody conc, PB units</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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<td></td>
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<td>200</td>
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<tr>
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physiological and pharmacological concentrations in serum (IgG 7.5–200 g/L serum) showed no IgG interference with IgM anti-GM1 antibody determinations.

Clinical Studies

Screening. Anti-GM1 concentrations were significantly different between 83 blood donors (median 18.5 PB) and patients with CIDP (median 115 PB; P < 0.0002) or MS (median 32.3 PB; P = 0.0067) (Fig. 2). Concentrations did not significantly differ (P > 0.05) between blood donors and patients with MND (median 33 PB), axonal PPN (median 10 PB), HIV infection (median 26.5 PB), or SCLC (median 17 PB) (Fig. 2).

Follow-up. Five patients with GBS were followed longitudinally (Fig. 3). In week 2, one was treated with plasmapheresis, four with intravenous immunoglobulin. In the early phase of treatments the concentration increased; with clinical improvement, there was a corresponding decrease in anti-GM1 concentration.

Discussion

Circulating antibodies against ganglioside GM1 have been demonstrated in many neurological diseases, such as MS, MND, GBS, and CIDP, with the highest antibody titers apparently in diseases involving motor neurons. It is not known whether the circulating anti-GM1 antibodies are causing the disease, play a role in its pathogenesis, or are an epiphhenomenon; however, high-titer anti-GM1 sera are able to block conduction and bind specifically to motor neurons (21, 22).

For grouping neurological patients into disease subgroups that will respond differently to various treatment modalities, a single reliable determination of IgM anti-GM1 antibodies in a patient before therapy has been suggested (1–5, 17, 18). Previous methods for this have included ELISA (3–5, 8–15), immune TLC (11), and liposome lysis (6, 7). Reports of these studies have included different standardization approaches; however, the data published for the various patients' groups have been ambiguous, and no data have been available on reproducibility. A previous multicenter study showed considerable disagreement on the classification of the distributed samples of high-, moderate-, and low-titer sera (19). To improve comparability of results, we established an ELISA-type assay with a high-titer patient's serum as internal calibrator and another patient's serum as control. Homogeneous coating of the titertray with ganglioside was controlled with cholera toxin. Validation of the assay showed intraassay CVs of 4–8%, interassay CVs of 5.5–16%, and recovery >98%.

Our calibrator serum has been collected in large quantities. However, for the future, a better solution would be the production of human GM1-specific monoclonal IgM antibodies. International standardization might require distributing a serum with an assigned titer value, in the same way the World Health Organization is distributing calibrators for IgM rheumatoid factor.

The immune system is challenged by many different microorganisms that may present gangliosides to the
immune system, which in turn produces ganglioside antibodies. This is well described concerning intestinal bacterial glycolipid and blood group antigens belonging to the carbohydrate structures. On the basis of this mechanism, we therefore anticipated that some normal individuals would carry increased amounts of specific or cross-reacting antibodies reacting against GM1. As our results show, this could be the case for ~10% of the blood donors.

According to the present study, application of a standardized anti-GM1 assay demonstrates an increased titer of anti-GM1 antibodies in CIDP and MS. Another intriguing observation was that anti-GM1 antibodies follow the clinical course in GBS, with high concentrations during disease progression and decreasing concentrations during recovery. This observation emphasizes the importance of longitudinal studies and raises the possibility that anti-GM1 antibodies can be used as markers of disease activity.

References