Published in final edited form as:

J Immunol Methods. 2014 June ; 408: 52–63. doi:10.1016/j.jim.2014.05.005.

A new approach to ELISA-based anti-glycolipid antibody evaluation of highly adhesive serum samples

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Abstract

The enzyme-linked immunosorbent assay (ELISA) is a standard immunoassay used in measuring antibody reactivity (expressed as titers) for glycosphingolipids (GSLs) such as gangliosides and sulfoglycolipids in the sera of patients with Guillain-Barré syndrome (GBS), variants of GBS, and chronic inflammatory demyelinating polyneuropathy (CIPD). In the present study, anti-GSL antibodies were evaluated using a new formula of affinity parametric complex (APC), calculated from limiting-dilution serum assay data, followed by affinity parametric complex criterion (APCC). Using assay results based on APCC, we analyzed serum samples categorized into acute inflammatory demyelinating polyneuropathy (AIDP), acute motor-sensory axonal neuropathy (AMSAN), CIPD, CIPD with Myasthenia Gravis (MG), and Amyotrophic Lateral Sclerosis (ALS). We were able to determine the affinity strength of antibodies otherwise hidden in the non-specific background activity in highly adhesive serum samples. The thin-layer chromatography (TLC)-immuno-overlay method assured us that this new method is an accurate and reliable way for evaluating anti-GSL antibodies using ELISA serum sample data.

Keywords

Anti-glycolipid antibody; GBS; ALS; CIPD; ELISA; Non-specific adhesions; False positive and negative

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Competing interests

The authors declare no conflict of interest.

1. Introduction

Glycosphingolipids (GSLs) are a type of glycolipids located primarily, albeit not exclusively, in the plasma membrane. GSLs can be isolated from tissues and cells, particularly those of the nervous system, that are rich in acidic glycolipids such as sialic acid-containing GSLs (gangliosides) and sulfate-containing GSLs (sulfoglycolipids) (Yu, 1994). Gangliosides are integral components of surface microdomains on nerve cells and participate in cell-cell recognition, adhesion, and signal transduction (Iwabuchi et al., 1998; Hakomori, 2000; Simons and Toomre, 2000; Yu et al., 2010). Anti-GSL antibodies are frequently encountered in patients with a variety of neurological disorders, such as Guillain-Barré syndrome (GBS), Alzheimer's disease (AD), and Amyotrophic Lateral Sclerosis (ALS) (Chapman et al., 1988; Niebroj-Dobosz et al., 1999; Mizutani et al., 2003; Yamazaki et al., 2008). While the details of pathogenesis for these diseases are still unknown, an autoimmune mechanism has been associated with disease development in such illnesses as acute inflammatory demyelinating polyneuropathy (AIDP), chronic inflammatory demyelinating polyneuropathy (CIDP), ALS, and other immune-mediated neurodegenerative disorders (Pestronk et al., 1988; Pestronk et al., 1989; Santoro et al., 1990; Hughes et al., 2007; Shimizu et al., 2011; Willison, 2011). Autoantibodies have been normally tested by enzyme-linked immunosorbent assay (ELISA), and were found to be elevated in sera of diseased patients, as well as correlated with the development and severity of these diseases. Such immune-reacting antigens are demonstrated to mostly be molecular species of GSLs: gangliosides such as GM1, GM2, GD1a, GD1b, GD3, GQ1b, and sulfoglycolipids such as sulfoglucuronosyl paragloboside (SGPG) and sulfatide (cerebroside sulfate ester, CSE) (Pestronk et al., 1988; Pestronk et al., 1989; Santoro et al., 1990; Ariga et al., 2001; Willison and Yuki, 2002; Dalakas, 2010).

Anti-ganglioside antibodies are often found in the serum of patients with motor neuropathies, such as GBS and ALS variants (Santoro et al., 1990; Niebroj-Dobosz et al., 2004; Nobile-Orazio et al., 2008; De Sousa et al., 2009). GBS is recognized as several disorders, all characterized by an immune-mediated attack on the peripheral nerves, and its subtypes have been characterized based on their clinical manifestations. The most common form, AIDP, is a multifocal demyelinating disorder caused by damage to the myelin sheath of peripheral nerves. AIDP is often accompanied by mild sensory symptoms (Willison and Yuki, 2002; Yu et al., 2006). Still other cases of GBS are associated predominantly with degeneration of the motor axonal processes; these are called acute motor axonal neuropathy (AMAN) (McKhann et al., 1993). Although these alternative variations of GBS do exist, more than 90% of GBS patients in Europe and North America are of the AIDP type (Yu et al., 2006). Acute motor-sensory axonal neuropathy (AMSAN) is also a subtype of GBS, characterized by reduction or absence of both motor and sensory nerve conduction velocities (Uncini and Yuki, 2009). The chronic type of demyelinating neuropathy is called CIDP and is often accompanied by more severe sensory dysfunction. There are also several variants of CIDP that have no sensory involvement; such a case of chronic acquired demyelinating neuropathy with purely motor dysfunction is termed multifocal motor neuropathy (MMN) (Ueda and Kusunoki, 2011). Similar motor neuropathies such as CIDP also have been found and classified as ALS variants. These cases generally show predominantly lower motor

neuron signs and axonal changes. High titers of IgM anti-ganglioside antibodies also occur frequently in the ALS variants and MNN (Pestronk et al., 2010).

The presence of anti-ganglioside or anti-sulfoglycolipid antibodies has been reported in cases of patients with GBS (Ariga et al., 2001). To diagnose disease subtypes and evaluate the effectiveness of treatments in clinical trials, an accurate measurement of antibody titers in sera of patients is one of the most important quantitative tests. However, there is as yet no generally accepted way of expressing ELISA results. The endpoint titer of a full dilution curve (e.g., the highest dilution that gives an optical density (OD) absorbance of 0.1 or 0.2 above the negative control) is currently one of the most common ways to express these results. Alternatively, data obtained from samples tested at a single dilution are also commonly used (e.g., direct absorbance values, rating (+/-, +1, +2, +3) according to absorbance values of the tested samples). However, there are potential problems with both methods of reporting ELISA data. When the assay shows low antigen specificity, it may result in a false positive; likewise, overrating absorbance values leads to lower antigen specificity, which can be misleading when highly non-specific adhesive sera are also included in tested samples. Using the current method of analyzing ELISA assays simultaneously on same ELISA plate with GSL-coated and non-coated wells, we have no way to determine with reliability whether the results for such serum testing may be positive or negative. In the study described here, we have developed a new method of evaluating ELISA data obtained from the influence of highly adhesive serum samples. This way of evaluating the data gives reliable and reproducible results for anti-GSL antibodies in the specialized serum samples of GBS, CIDP, and ALS patients.

2. Materials and Methods

2.1. Patients and serum samples

Using ELISA, we tested serum samples of 38 patients with motor neuropathy for antibody activity against gangliosides and sulfoglycolipids. Our patient population was composed of the following groups: ALS (28), AIDP (1), AMAN (1), AMSAN (2), CIDP (4), CIDP with MG (1), and CIDP with axonal involvement (1); and healthy controls (5). They are presented by serial sample ID# in Table 1. Cases of ALS and AIDP/CIDP were diagnosed by standard clinical criteria. Forty-five serum samples were collected at the Neurology Clinic, Georgia Regents University Health Center, including ID# 1–40 from patients, #32 and 33 from same subject with AIDP, #41–45 from healthy controls. Of particular interest were serum samples #32 and #33 which were obtained from same subject with AIDP before and after treatment with intravenous immunoglobulin (IVIg). All serum samples were stored in aliquots at –20°C until analysis.

2.2. ELISA assay

Forty-four serum samples were tested for the presence or absence of anti-GSL antibodies against the following antigens: GM1, GM2, GD1a, GD1b, GT1b, GD3, GQ1b, SGPG, and CSE. Gangliosides, SGPG and CSE were purified in our laboratory using previously established methods (Ledeen and Yu, 1982). Serum samples were initially tested at a single dilution (1:400) with 1% bovine serum albumin/Dulbecco's phosphate buffered-saline (1%

BSA/PBS). Sera testing positive were identified preliminarily by their increased absorbance values. All positive sera were then further tested by limiting-antibody dilution (1:400, 1:800, 1:1,200, 1:1,600, 1:2,000, 1:3,000, 1:4,000, 1:5,000, 1:6,000, and 1:10,000). The serially diluted serum samples were compared using same ELISA plate with GSL-coated and GSL-non-coated wells.

The single dilution and the limiting dilution tests were performed according to the following procedure. Each well of a 96-well flat-bottom polystyrene microtiter plate (Immunlon 1B, Lab System, Franklin, MA) was coated with 25 ng of a GSL dissolved in absolute ethanol solution. After the solvent was evaporated in an incubator, nonspecific binding sites in the wells were blocked using 100 μ L of 1% BSA/PBS, and the plate was incubated for 30 min at room temperature. After the blocking solution was decanted, the plate was washed 5 times with 300 μ L of a washing buffer (0.1 % BSA/PBS) using the ELx50 microplate strip washer (BioTek Instruments, Inc., Winooski, VT). After washing, the plate was filled with 100 μ L of patient serum at a single dilution (1:400 in 1% BSA/PBS) or serial dilutions, and the plate was incubated for 15 to 16 hours at 4°C. The plate was then washed again as described above and subjected to addition of an appropriate secondary antibody. The secondary antibodies were purchased from Sigma (St. Louis, MO, USA) and used as follows: human IgG (horseradish peroxidase-conjugated goat anti-human IgG, 1:100,000 dilution in 1% BSA/PBS), and human IgM (horseradish peroxidase-conjugated goat anti-human IgM, 1:50,000 dilution in 1% BSA/PBS). After incubation with secondary antibodies for 2 hours, the plates were washed again, followed by addition of a coloring reagent (100 μ L of OPD Peroxidase Substrate in PBS [Sigma]). The ELISA plate was incubated for 2 min in the dark at room temperature, and the reaction stopped by addition of 50 μ L of 3 N sulfuric acid. The absorbance of each well was measured at 490 nm with a microplate spectrophotometer (BioRad, Hemel Hempstead, UK). Anti-GSL antibody titers were assigned as the highest dilution at which the absorbance was 0.1 or less.

2.3. Purification of IgM and TLC-immuno-overlay

Using a HiTrap IgM Purification HP column chromatography (column volume, 1 mL; GE Healthcare, Uppsala, Sweden), we purified human IgM from patients similar to the procedure we used for sera of rabbits immunized with GA1 (rabbit-serum; #103L) (Taguchi et al., 2004). Briefly, the column was equilibrated with 20 mM sodium phosphate and 0.8 M ammonium sulfate/water (pH 7.5) binding buffer. Twenty-five μ L of serum samples were diluted to 1:100 in the binding buffer and applied onto the column. The unbound sample was washed out with 5 mL of the binding buffer. The IgM was eluted with 12 mL of 20 mM sodium phosphate buffered (pH 7.5) elution buffer and concentrated to 0.5 mL with an Amicon Ultra Centrifuge Device (Membrane MWCO 30,000, Millipore Corporation, Bedford, MA, USA). The concentrate was adjusted to 2.5 mL of 1% BSA solution and used for TLC-immuno-overlay (Usuki et al., 2005). Briefly, authentic GSLs were applied onto a high-performance TLC (HPTLC) plate. The plate was developed with the solvent system of chloroform:methanol:0.2 % CaCl_2 in water (55:45:10, v/v). After drying, the plate was soaked with 0.1% polyisobutylmethacrylate in n-hexane for 1 min. The plate was overlaid with 1% BSA/PBS buffer for 20 min at room temperature for 1 h and then with the patient IgM at 4°C overnight. After washing with PBS buffer containing 0.05% Tween 20, the plate

was then incubated with a secondary antibody solution (horseradish peroxidase-conjugated anti-human IgM, 1:1,000 dilution in 1% BSA/PBS). The plate was incubated at room temperature for 2 h and then washed as described above, followed by detection with OPD Peroxidase Substrate. Bands were recorded by an imaging scanner.

2.4. Model simulations

The data points from the limiting-dilution test were simulated by the following mathematical formula, given as a Hill-type equation (Hill, 1910). The dose-response relationship is shown as a sigmoidal curve, given by the Hill equation (Wagner, 1968). The Hill equation can be used to derive an EC₅₀. This is represented by the following equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + (\text{EC}_{50}/X)^{\text{Hill Slope}}) \quad (1)$$

where Y is the observed value; Bottom is the lowest observed value; Top is the highest extrapolating value from observed single; and the Hill Slope is the trend of the slope, described as steep or shallow.

On the other hand, a response of competitive inhibition is defined by negative Hill Slope values, with IC₅₀ given as:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + (\text{IC}_{50}/X)^{-\text{Hill Slope}}) \quad (2)$$

The serum logarithmic dilution equation is defined by the same sigmoidal curve as is competitive inhibition. Instead of IC₅₀, an inhibitor concentration corresponding to 50% of the spread (Top - Bottom) is expressed as serum dilution factor (D50) to give half of the primitive absorbance value at a single dilution of 1:400. Therefore, we used the following serum logarithmic dilution equation (Fig. 1):

$$Y1 = B1 + (T1 - B1) / (1 + (X1/D1)^{H1}) \quad (3)$$

where T1 is the top absorbance value; B1, the bottom absorbance value; X1, logarithmic dilution; D1, dilution factor indicating half value of (T1-B1); and H1, slope factor as Hill slope. According to this equation, graphical properties were observed via computerized simulation to make use of parametric properties for probabilistic estimation of antibody avidity.

2.5. Fitting for dilution curve and estimation of parameters

Since the data points from the ELISA were rather scattered, they were curve-fitted using the serum logarithmic dilution equation to obtain best-fitted parameters by nonlinear regression analysis without weighting. This was accomplished by software (Graphpad PRISM 5.0) using either the Gauss-Newton algorithm or the Marquardt's compromise estimation algorithm.

Initial values T1 and B1 were derived from the single dilution values of 1:10,000 through 1:400. When the computation of nonlinear regression failed to result in convergence, we

tried to obtain suitable parameters for convergence via several repetitions of nonlinear regression after selection of initial values. This method gave a curve close to the ELISA data points using the constraint function.

2.6. Affinity parametric complex criterion (APCC)

Considering the connection between the serum-dilution equation and antibody binding affinity, we introduced a criterion of antibody affinity to remove an influence of non-specific adhesion that is an affinity parametric complex criterion (APCC) into our evaluation of non-specific adhesion in tested samples. Fig. 2a shows 3 simulations of limiting-dilution equations using the following parameters: $H1 = 1.0, 2.0$, and 3.0 ; $T1 = 1.0$; $B1 = 0.1$; and $D1 = 1000$. As $H1$ increases, the antibody loses binding activity more than 1000 of $D1$. $H1$ shows a trend of antibody dissociation. In contrast, $1/H1$ shows a trend of antibody adhesion consisting of antibody binding activity and non-specific adhesion. In addition, the antibody dilution curve is characterized by two factors. One is a net value of Top absorbance ($T1-B1$), and another factor is the $D1$ value. In order to eliminate the influence of non-specific adhesion involved in these two values, the two factors must be corrected using a suitable formula that can reduce an attribution of nonspecific adhesion in any extent of change of $D1$ and $(T1-B1)$.

To correct for a better decision of positive antibody, we introduced a correction of values of exponentiation of $(T1-B1)$ and $D1$. As shown in Fig. 2b, the corrected D ; $D1^{1/H1}$ is remarkably lowered at $H1$ values more than 1.5 (in the right graph), and raised less than 1.5 (in the left graph). Such a correction by $1/H1$ exponentiation is also applicable for $(T1-B1)$ values. Thus, we reached an APC formula that such that APC is assumed to become a criterion of antibody adhesion, if APC is composed of the product of $(T1-B1)^{1/H1}$ and $D1^{1/H1}$.

APC1 is calculated using diluted serum data by GSL-coated wells of an ELISA plate, and is given as a formula composed of Hill slope ($H1$), absorbance value at an extrapolation number ($T1-B1$), and diluting factor ($D1$), where 50 % of the antibody exhibits the response ($D50$):

$$APC1 = (T1 - B1)^{1/H1} D1^{1/H1} \quad (4)$$

APC2 is calculated using the slope factor $H2$, absorbance value at an extrapolation number ($T2-B2$), and diluting factor $D2$ calculated using ELISA data from a non-coated well in the same plate. APC2 is given by the following formula:

$$APC2 = (T2 - B2)^{1/H2} D2^{1/H2} \quad (5)$$

Specific antibody binding activity is quantified by an equation termed the affinity parametric complex criterion (APCC):

$$APCC = \frac{(T1 - B1)^{1/H1} D1^{1/H1}}{(T2 - B2)^{1/H2} D2^{1/H2}} \quad (6)$$

The APCC with correction by involvement of H1 and H1 (Formula 6) was compared with APCC with no consideration of H1 and H2 (c-APCC). c-APCC was defined as $[(T1 - B1)D1] / [(T2 - B2)D2]$. The behaviors of APCC and c-APCC were calculated and compared by using theoretical parametric alteration, as shown in Table 2. In consideration of the actual extension and fluctuation of any ELISA-observed absorbance values, we should be careful of the empirical finding to which APCC is subjected mostly under limitation by real values of H1 and H2 greater than 1.0. Additionally, the D2 value is mostly smaller than D1. When the H2 value becomes more than 1.5, APCC is especially corrected with a suitable strong amplification as compared with c-APCC (Fig. 3a). In consideration of any extension and fluctuation of possible values in T2 and D2, APCC indicated a remarkable suppression when D2 is less than 1000 and T2 is less than 0.5, as shown in Figs. 3b and c. When c-APCC is taken, amplification of nonspecific binding is caused by non-specific binding parameters, D2, and T2. This disadvantage is able to be overcome by the introduction of 1/H1 exponentiation into APCC.

In addition, we concluded that it is necessary to introduce a cut-off value for APCC, using parametric constraint in our evaluation of antibody. The principle for using the APCC cut-off value is discussed below. We validated the values generated using the APCC formula as being representative of true antibody affinity, and the validation results are described below.

3. Results

As shown in Methods, ELISA was used to test patient serum samples at a single serum dilution (1:400) for anti-GSL antibodies against authentic gangliosides: GM1, GM2, GD1a, GD1b, GT1b, GQ1b, GD3, SGPG, and CSE. There were no IgG-positive serum anti-GSL antibody samples in 38 patients (data not shown). On the other hand, sera with IgM-positive anti-GSL antibody titers were found in ALS (2/28), and in AIDP (1/1), CIDP (0/4), CIDP with axonal (1/1) or CIDP with MG (0/1) as shown in preliminary positive sera (Table 1). The healthy controls were negative for IgG- or IgM-type anti-GSL antibody activities (0/5).

3.1. Model simulations

It was presumed that serum samples that were highly adhesive on the ELISA plate wells exhibit non-specific binding ($T2 = 0.5$) and antibody-specific binding ($T1 = 1.0$). Alterations of parameters H2 and D2 were applied to the serum dilution equation Y2, and theoretical APCCs were calculated as shown in Table 2. Considering how variations of H2 and D2 may influence APCC values, APC1 and APC2 should be limited by the following conditions: If the APCC is less than or equal to 1.0 ($APC1 \leq APC2$), the specific antibody reaction is most likely negligible, due to very weak or no antibody activity; and if the APCC is greater than 1.0 ($APC1 > APC2$), the specific antibody reaction is not negligible due to the unequivocal presence of antibody activity.

3.2. APCC cut-off value

To examine how the APCC cut-off value is related to parametric variations, a bivariate response surface model was established for T1-T2 (Fig. 4a). Cross-sectional layering of the surface model resulted in a division of bivariate combinations into two zones. These two zones on each layer corresponded to antibody-negative reactions (grey zone) and antibody-positive reactions (white zone) as shown in the graphs of Fig. 4a. As the APCC cut-off value increases, the percentage of space occupied by the antibody-negative zone in a cross-sectional layer increases (Fig. 4b).

Next, in order to calculate an appropriate APCC cut-off value, surface models were built using 9 combinations of bivariate responses for T1, H1, D1 vs. T2, H2, D2 (Fig. 5). B1 and B2 were excluded from the combinations for the reason that APCC was minimally influenced by contributions from the bottom absorbance levels. Table 3 shows the percentage of the antibody-negative zone formed in each of the 9 cross-sectional layers at APCC 1.0 and 1.5. As mentioned above, an APCC of 1.0 implies very weak or no antibody activity. However, only 33.90% of cross-sectional layers was assigned as antibody-negative. The average percent of space occupied by the white zone (APCC = 1.0, 66.10%) is too high to adopt 1.0 as antibody positive. An APCC of 1.0 implicates there is no difference between APC1 and APC2. When we see that a percentage of adoption equals to abandonment, we need to reset the APCC cut-off value. Fortunately, we found an APCC of 1.5 indicated a 50% for the average antibody-negative zone (Table 3). We are not sure of the exact probability of selecting true and false decisions implicated in our evaluation method of APCC. However, we should set up a restraint of 50% in our APCC-ELISA method. The reason for 50% is that the APCC-ELISA method is needed to keep at least 50% accuracy for true or false decision. This value was validated by the TLC-overlay method as described below.

3.3. Best-curve fit and antibody evaluation

ELISA dilution data points from the preliminarily positive serum samples were fitted to a curve using serum dilution equations (3) (Figs. 6). Convergent parameters for the equations were obtained from the best-fit curves in the presence (●) and absence (■) of GSL-coating of the ELISA plate. APC1, APC2, and APCC were also calculated and are shown in Table 4. Antibody-positive serum samples were ranked according to APCC values (+1 to +3).

Serum #32 (Fig. 6) is an unusual case with highly adhesive behavior, even without the GSL coating on the ELISA plate wells. The blocking solution of 1% BSA might be a factor to cause a possible strong adhesion to this serum. This was determined by repeating the experiment on the same ELISA plate using blocking solutions of 1% rabbit serum albumin (RSA) and 1% chicken serum albumin (CSA) instead of BSA solution. APC_{BSA} , APC_{RSA} , and APC_{CSA} were measured using serial serum dilutions on non-coated ELISA plates. One-way ANOVA showed no difference of APCs among 1% BSA, 1% RSA, and 1% CSA blocking solutions ($APC_{BSA} = 34.79 \pm 3.34$, $APC_{RSA} = 25.57 \pm 6.88$, $APC_{CSA} = 12.30 \pm 7.10$, mean + standard error; $N = 3$, $p = 0.05$). Thus, we demonstrated that the high adhesiveness of serum sample #32 is attributable to background levels, and is not affected by an interaction with any component in the BSA blocking solution. This finding led us to conclude that the

antibody affinity, as evaluated by APCC, is independent of the variety of blocking solution used, and that we can use APCC to reliably determine antibody activities to be either true-positive or true-negative.

The results of evaluating antibody reactivity are summarized in Table 5. Anti-GM1, anti-GD1a, anti-GD1b, and anti-GD3 activities of #32 serum and anti-GM2 activity of #33 serum were excluded as false-positive because the evaluation was negative (APCC is 1.2, which is less than 1.5).

3.4. TLC-immuno-overlay

The antibody activities in patient sera were examined before and after IgM purification (sample ID #1, #9, #24, #32, and #33) by the method of TLC-immuno-overlay. The results are shown in Fig 7. Before purification, these serum samples showed the same immuno-positive profile as that from the ELISA (Table 1). The TLC-immuno-overlay with IgM of #1, #9, and #24 showed same result as the ELISA method. On the other hand, before and after IgM purification, #32 and #33 showed a very good agreement of results obtained by the TLC-immuno-overlay and the ELISA-APCC method. Anti-GM1, -GD1a, -GD1b, and -GD3 activities of #32 were also demonstrated to be false positive by the TLC-immuno-overlay method. In addition, serum #33 was determined to be anti-GSL antibody negative serum because of the absence of anti-GM2. Thus, the reliability of the APCC-ELISA method was validated by TLC-immuno-overlay with IgM.

4. Discussion

Human sera, especially those from patients with autoimmune diseases, usually contain high levels of immunoreactive substances, causing high background levels of reactivity in an ELISA assay. These non-specific reactions are caused by adhesive immunoglobulins or other proteins contained in the human serum, which strongly adhere to the plastic plate surface via hydrophilic binding (Takahashi et al., 1989). For example, elevated levels of intercellular adhesion molecule-1 (ICAM-1) were often found in the sera of GBS and CIDP patients (Jander et al., 1993; Hadden et al., 2001; Drescher et al., 2002). ICAM-1 may enhance the ability of IgM antibodies to adhere to the plastic plate surface. These false positive results cannot be simply avoided by use of a blocking agent, such as BSA, or a washing agent, such as Tween 20. Despite avoidance trials for non-specific reactions, false positive reactions caused by the serum samples themselves have not been well-understood nor evaluated. Instead, such results are often mistaken for a true GSL-antibody interaction and cause serious confusions in the literature. Furthermore, a comparison of the antibody activities reported by different laboratories may also be rendered difficult due to non-specific antibody determinations and errors introduced from the “batch effect”.

In order to obtain a reliable estimation of GSL-antibody reactivities that minimizes the introduction of false positives and contribution of false negatives, it is critical to determine the non-specific background value of individual samples. This is often accomplished by comparing absorption values for GSL-coated wells versus non-coated wells, and then deducting the obtained background absorbance value from the absorbance value for GSL-coated wells. However, there is an additional problem related to comparing data from

different ELISA plates tested at different times. ELISA is an indirect assay of primary antibody activity in a sample, using the enzyme activity of a secondary antibody. Thus, any fluctuation in secondary antibody activity would also affect the reliability of the results, making it difficult to determine the true presence or absence of the primary antibody if non-specific reactions cause a high background activity level of the secondary antibody. For these reasons, it is difficult to estimate real antibody affinity by either the absorbance value at a single dilution (e.g., 1:400) or the endpoint titer in a full serial dilution. The APCC evaluation is preferable because it overcomes such a weak point of estimating sample reactivity based on the absorbance value of a single dilution or endpoint titer, and it allows us to determine the affinity-strength of each antibody hidden in the background reactivity of highly adhesive serum samples.

The present study showed that IgM anti-ganglioside antibodies were elevated in 2 out of 28 patients with ALS, and 2 out of 11 patients with AIDP/CIDP. Particularly noteworthy, ALS serum #1 contained a high-titer antibody (APCC evaluation +3), in addition to a low level of non-specific reactions on the ELISA plate (Fig. 6A). On the other hand, AIDP serum #32 showed high non-specific adhesion concealing any intrinsic antibody-specificity that the serum might have contained.

The non-specific adhesion of serum #32 was also confirmed by use of the TLC-immuno-overlay method (Fig. 7). The non-specific adhesion was removed by use of purified IgM samples to some extent to uncover and determine the true activities of GSL antibodies. We adopted an APCC cut-off value of 1.5 in the APCC-ELISA method and decided either positive or negative activity. This set-up value was validated by the finding that the TLC-overlay method coincided with the APCC-ELISA method.

Most interestingly, sera #32 and 33 were obtained from same subject before and after IVIg treatment. Before the treatment, the serum showed adhesion due to abnormality of its high viscosity. Serum #32 was characterized as a typical example of serum samples with highly adhesive behavior that could not be evaluated by a preliminary, single dilution ELISA assay. After APCC evaluation, however, anti-GM2, -GT1b, -GQ1b, -SGPG, and -CSE antibody activities were revealed as having produced true positives among all of the preliminarily positive GSLs. Using our present method, an APCC cut-off value of 1.5 is crucial for elucidating the true presence and affinity of sample antibodies, but serum #32 could not be titrated to an antibody-limiting dilution due to a high background reactivity with IgM. An absorbance value of higher than 0.1 still remained, even at 1:100,000 dilution (data not shown).

In serum #33, highly adhesive behavior was observed in preliminarily test, and might have misled to a false-positive activity of anti-GM2 antibody. The subtracted absorbance value ($T_1 - T_2$) was less than 0.1, implying that the serum's adhesion was due to more than just its interaction with GM2. When H_1 and H_2 were less than 1.5, the slope was very shallow, implying that the serum was highly adhesive. Although the serum did interact weakly with GM2, this was determined to be antibody-negative according to the APCC cut-off value. In addition, the decision of true-positive and false-positive antibodies by this APCC-ELISA method was coincident with those corroborated by TLC-immuno-overlay using the purified

IgM samples. From #33 serum, it may be safe to conclude that in the case of highly adhesive serum samples, no matter how effective and timely a patient is treated by IVIg administration, it is unreliable to judge the efficacy of treatment based on antibody titer measurement alone. This finding would cast some doubt as to the beneficial effect of IVIg treatment. This caveat should be lessened by the current APCC-evaluation, leading to a more precise and reliable assessment of serum autoantibody titers.

5. Conclusions

To summarize, the APCC-ELISA method, as determined by D50 and Hill Slope, provides a new, standardized evaluation to decide the presence or absence of antibody activity, offering improved evaluation of ELISA results with more accurate determination of the presence and affinity of a particular antibody in a given serum sample. The method also offers an opportunity to compare data obtained from different laboratories and techniques, which is critical for disease diagnosis and assessment of treatment efficacy.

Acknowledgments

This work was supported by a USPHS-NIH grant NS26994 and a contract from the Centers for Disease Control and Prevention to RKY. We wish to thank Ms. Quarles Brandy, research associate/coordinator for investigating clinical records of patients, and Dr. Rhea-Beth Markowitz for her editorial assistance.

Abbreviations

ELISA	enzyme-linked immunosorbent assay
GSLs	glycosphingolipids
GBS	Guillain-Barré syndrome
APC	affinity parametric complex
APCC	affinity parametric complex criterion
ALS	Amyotrophic Lateral Sclerosis
TLC	thin-layer chromatography
AIDP	acute inflammatory demyelinating polyneuropathy
CIDP	chronic inflammatory demyelinating polyneuropathy
AMSAN	acute motor-sensory axonal neuropathy
AMAN	acute motor axonal neuropathy
SGPG	sulfoglucuronosyl paragloboside
CSE	cerebroside sulfate ester
MG	Myasthenia Gravis
IgG	immunoglobulin G
IgM	immunoglobulin M
PBS	phosphate-buffered saline

BSA bovine serum albumin
IVIg intravenous immunoglobulin

GSLs are abbreviated using the IUPAC-IUB recommendations (1977) except gangliosides, which were abbreviated according to the nomenclatural symbols of Svennerholm (Svennerholm, 1964)

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Parameter
values
 $T1=1.0$
 $B1=0.1$
 $D1=1000$
 $H1=1.5$

Figure 1. Theoretical curve for serum dilution equation for ELISA assay

The serum logarithmic dilution equation was simulated using parameters ($T1=1.0$, $B1=0.1$, $D1=1000$, $H1=1.5$) by the following Hill equation:

$$Y1 = \frac{(T1 - B1)}{1 + \left(\frac{X1}{D1}\right)^{H1}} + B1$$

Figure 2. APC formula reflects (T1-B1), D1, and 1/H1

(a) The continuous-lined graphs show the increasing Hill Slope (H1 of 1.0, 2.0, and 3.0) by the equation Y1 (3), using parameters (T1=1.0, B1=0.1, D1=1000). The surrounding dotted-line rectangle was given as a constant value by the formula (T1-B1)D1.

(b) D1 was corrected by exponentiation of 1/H1 (0.5 to 1.5 of H1), and showed a remarkably faster alteration of more than 1000 of D1 in the left graph. In the contrast, H1 of 1.5 to 4.0 showed a slower alteration of more than 1000 of D1 in the right graph.

Figure 3. Effect of parametric change on APCC and c-APCC

When the equation of Y1 was given by fixed parameters ($T1=1.0$, $B1=0.1$, $D1=1000$, $H1=1.5$), APCC's behavior was compared with c-APCC for alterations of H2, D2, and T2. APCC and c-APCC were shown by % values of APC2 and c-APC2 ($T2=0.5$, $B2=0.1$, $D2=1000$, $H2=1.5$). The vertical lines of graphs a, b, and c show a percent of APCC (■) or c-APCC (●).

Figure 4. Surface model of bivariate response APCCs (using T1 and T2)

(a) Cross-sectional layers with increased APCC cut-off values of 0 to 2.0.

(b) Percentage area occupied by the grey zone is expressed using a graph of APCC cut-off values (b).

Bivariate APCCs were calculated using maximum absorbance values ($T1=0.2$ to 2.5 , $T2=0.2$ to 1.0) and expressed using a 3-dimensional surface model of APCC, defined by Formula (6). The following constant parameters were given to Formula (4): $B1=0.1$, $D1=1000$, $H1=2.0$; Formula (5): $B2=0.1$, $D2=1000$, $H2=2.0$.

Figure 5. Surface model of bivariate response APCCs (using 9 different combinations of parameters) shows cross-sectional layers with an increased APCC cut-off value of 1.5. The following formulas were used with the constant parameters, as given initially: Formula (4) used $B1=0.1$, $D1=1000$, and $H1=2.0$; and Formula (5) used $B2=0.1$, $D2=1000$, and $H2=2.0$. The grey zone of a given cross-sectional layer in a bivariate response surface model is expressed as an area with an APCC cut-off value of 1.5. The parameter values were then changed to test 9 different combinations, as follows: (a) $T1=0.2$ to 3.0 and $T2=0.2$ to 1.0 ; (b) $T1=0.2$ to 3.0 and $H2=1.0$ to 4.0 ; (c) $T1=0.2$ to 3.0 and $D2=250$ to 4000 ; (d) $H1=1.0$ to 4.0 and $T2=0.2$ to 2.0 ; (e) $H1=1.0$ to 4.0 and $H2=0.2$ to 2.0 ; (f) $H1=1.0$ to 4.0 and $D2=250$ to

4000; (g) D1=250 to 4000 and T2=0.2 to 2.0; (h) D1=250 to 4000 and H2=1.0 to 4.0; (i) D1=250 to 4000 and D2=250 to 4000.

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Figure 6. Best-fit curves for serum dilution equation, using ELISA data. (a) #32, (b) #33, (c) #1, (d) #9, and (e) #24.

ELISA data points obtained from diluted serum samples were fit to a curve using the serum dilution equation, Y1 (for ELISA assay with GSL [●]), and Y2 (for assay without GSL [■]), as given by the following formula:

$$Y1 = \frac{(T1 - B1)}{1 + \left(\frac{X1}{D1}\right)^{H1}} + B1$$

$$Y2 = \frac{(T2 - B2)}{1 + \left(\frac{X2}{D2}\right)^{H2}} + B2$$

Figure 7. TLC-immuno-overlay method for ALS patient sera #1(a), #9 (b), #24 (c), #32 (d), and #33 (e)

Each of the following GSL samples (100 ng), GM1, GM2, GD1a, GD1b, GT1b, GQ1b, GD3, SGPG, and CSE, was applied onto HPTLC plates (2 × 5 cm), developed with a solvent of chloroform:methanol:0.2% CaCl₂ (55:45:10, by volume) until the solvent front ascended to the top edge of the plate. After chromatography, the plates were treated with 0.1% polyisobutylmethacrylate in n-hexane for 1 min, and with 1% BSA/PBS solution for 20 min. Subsequently, the plates were treated at 4°C overnight with patient serum (1:100 dilution) or patient IgM. After washing with PBS buffer containing 0.05% Tween 20, the

plates were treated with a secondary antibody for 2 hours, and then washed again. The washed plates were treated with the coloring reagent (OPD Peroxidase Substrate).

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Table 1

Preliminary positive sera by ELISA assay

Patient ID#	GM1	GM2	GD1a	GD1b	GT1b	GQ1b	GD3	SGPG	CSE
1	-	+	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-
8	- ^a	-	-	-	-	-	-	-	-
9	+ ^b	+	-	-	-	-	-	+	+
10	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-	-
23	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	+
25	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-

Patient ID#		GM1	GM2	GD1a	GD1b	GT1b	GQ1b	GD3	SGPG	CSE
29	CIDP	-	-	-	-	-	-	-	-	-
30	CIDP (with MG)	-	-	-	-	-	-	-	-	-
31	AMAN	-	-	-	-	-	-	-	-	-
32 ^c	AIDP	+	+	+	+	+	+	+	+	+
33 ^d	AIDP	-	+	-	-	-	-	-	-	-
34	none									
35	ALS	-	-	-	-	-	-	-	-	-
36	ALS	-	-	-	-	-	-	-	-	-
37	ALS	-	-	-	-	-	-	-	-	-
38	ALS	-	-	-	-	-	-	-	-	-
39	ALS	-	-	-	-	-	-	-	-	-
40	ALS	-	-	-	-	-	-	-	-	-
41	Control	-	-	-	-	-	-	-	-	-
42	Control	-	-	-	-	-	-	-	-	-
43	Control	-	-	-	-	-	-	-	-	-
44	Control	-	-	-	-	-	-	-	-	-
45	Control	-	-	-	-	-	-	-	-	-

^a - : serum dilution (1:400) with absorbance less than 0.2

^b + : serum dilution (1:400) with absorbance more than 0.2

^c 32: serum from AIDP patient before IVIg treatment

^d 33: serum from same patient as #32 after IVIg treatment

Table 2
Comparison of affinity parametric complex criterion (APCC) with increasing parameter values in Equation Y2

Parameter	T1	B1	D1	H1	c-APC	APC1	T2	B2	D2	H2	c-APC2	APC2	c-APCC (%)	APCC (%)	Fig. 3
H2	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	1000	0.5	400	1600000	2.25 (100.0)	0.001 (0.033)	(a)
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	1000	1.0	400	400.000	2.25 (100.0)	0.233 (13.6)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	1000	1.5	400	54.2880	2.25 (100.0)	1.717 (100.0)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	1000	2.0	400	20.000	2.25 (100.0)	4.661 (271.4)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	1000	3.0	400	7.368	2.25 (100.0)	12.651 (736.8)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	1000	4.0	400	4.472	2.25 (100.0)	20.844 (1213.9)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	125	1.5	50.0	13.572	18 (800.0)	6.868 (400.0)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	250	1.5	100.0	21.544	9 (400.0)	4.327 (252.0)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	500	1.5	200.0	34.200	4.5 (200.0)	2.726 (159.0)	
D2	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	1000	1.5	400.0	54.300	2.25 (100.0)	1.717 (100)	(b)
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	2000	1.5	800.0	86.177	1.125(50.0)	1.082 (63.0)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	3000	1.5	1200.0	112.924	0.75 (33.3)	0.825 (48.1)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	4000	1.5	1600.0	136.798	0.563 (25.0)	0.681 (40.0)	
	1.0	0.1	1000	1.5	900.0	93.2	0.5	0.1	1000	1.5	400.0	0.543	2.25 (100.0)	171.707 (100.0)	
T2	1.0	0.1	1000	1.5	900.0	93.2	0.4	0.1	1000	1.5	300.0	0.448	3 (133.3)	208.008 (121.1)	(c)
	1.0	0.1	1000	1.5	900.0	93.2	0.3	0.1	1000	1.5	200.0	0.342	4.5 (200.0)	272.568 (158.7)	
	1.0	0.1	1000	1.5	900.0	93.2	0.2	0.1	1000	1.5	100.0	0.215	9 (400.0)	432.675 (252.0)	
	1.0	0.1	1000	1.5	900.0	93.2	0.2	0.1	1000	1.5	100.0	0.215	9 (400.0)	432.675 (252.0)	

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Table 3

Proportion of APCC grey zone that is formed by parametric changes

APCC	T1-T2	T1-H2	T1-D2	H1-T2	H1-H2	H1-D2	D1-T2	D1-H2	D1-D2	Average (%)
1.0	14.20	25.60	25.50	90.30	50.10	24.60	25.30	27.20	22.40	33.90
1.5	36.50	31.00	59.40	92.50	36.40	62.70	56.90	26.50	50.00	50.50

Table 4

Evaluation of antibody activity using affinity parametric complex criterion (APCC)

Patient ID#	GSLs	T1	B1	D1	H1	T2	B2	D2	H2	APC1	APC2	APCC	Evaluation ^d
1	GM2	0.675	0.161	1529	1.83	0.081	0.054	1057	2.69	38.2	3.5	10.0	+3
9	GM1	0.345	0.093	1685	1.63	0.306	0.097	1413	1.80	41.3	23.5	1.7	+1
9	GM2	0.330	0.093	1693	1.66	0.316	0.099	1160	2.07	36.8	14.4	2.5	+1
9	SGPG	0.436	0.077	1771	1.41	0.313	0.101	2454	1.86	98.0	28.7	3.4	+2
9	CSE	0.331	0.095	1536	2.40	0.243	0.087	1274	2.63	11.6	7.5	1.5	+1
24	CSE	0.289	0.100	2126	1.54	0.269	0.095	1035	2.21	49.6	10.5	4.7	+2
32	GM1	0.430	0.080	2412	1.63	0.430	0.088	2591	1.58	61.7	73.1	0.8	–
32	GM2	0.460	0.070	2079	1.45	0.297	0.069	3648	1.82	103.4	39.9	2.5	+1
32	GD1a	0.286	0.075	1300	1.72	0.305	0.078	1258	1.80	26.1	23.0	1.1	–
32	GD1b	0.348	0.077	1391	1.62	0.361	0.079	1481	1.69	38.6	35.5	1.0	–
32	GT1b	0.665	0.150	3035	2.07	0.553	0.149	3923	2.43	34.9	20.6	1.6	+1
32	GD3	0.456	0.104	2762	1.57	0.429	0.112	3141	1.72	80.3	55.2	1.4	–
32	GQ1b	0.599	0.125	2540	1.62	0.522	0.150	2550	1.92	78.9	35.3	2.2	+1
32	SGPG	0.420	0.089	1190	1.85	0.247	0.082	1702	2.93	25.3	6.8	3.6	+2
32	CSE	0.369	0.070	1676	1.33	0.342	0.074	1251	1.84	109.1	23.7	4.6	+2
33	GM2	0.282	0.092	3589	1.21	0.185	0.100	3496	1.11	218.7	169.0	1.2	–

^d Serum samples with APCC < 1.5 (–); 1.5 < APCC < 3.0 (+1); 3.0 < APCC < 5.0 (+2); and APCC > 5.0 (+3)

Table 5

Summary of antibody evaluation using APCC

Sample ID#		GM1	GM2	GD1a	GD1b	GT1b	GQ1b	GD3	SGPG	CSE
1	ALS	-	+3 ^a	-	-	-	-	-	-	-
9	ALS	+1	+1	-	-	-	-	-	+2	+1
24	CIDP (with MG)	-	-	-	-	-	-	-	-	+2
32 ^b	ADP	-	+1	-	-	+1	+1	-	+2	+2
33 ^c	ADP	-	-	-	-	-	-	-	-	-

^a Serum samples with APCC < 1.5 (-); 1.5 < APCC < 3.0 (+1); 3.0 < APCC < 5.0 (+2); and APCC > 5.0 (+3)^b 32: serum from AIDP patient before IVIg treatment^c 33: serum from same patient as #32 after IVIg treatment