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Comparison of GD2 Binding Capture ELISA Assays for Anti-GD2-Antibodies Using GD2-Coated Plates and a GD2-Expressing Cell-Based ELISA

Gopalan Soman^{*}, Xiaoyi Yang, Hengguang Jiang, Steve Giardina, and Gautam Mitra
Biopharmaceutical Development Program, SAIC-Frederick Inc., National Cancer Institute, MD 21702, United States

Abstract

Two assay methods for quantification of the disialoganglioside (GD2)-specific binding activities of anti-GD2 monoclonal antibodies and antibody immunofusion proteins, such as ch14.18 and hu14.18-IL2, were developed. The methods differed in the use of either microtiter plates coated with purified GD2 or plates seeded with GD2-expressing cell lines to bind the anti-GD2 molecules. The bound antibodies were subsequently detected using the reactivity of the antibodies to an HRP-labeled anti-IgG Fc or antibodies recognizing the conjugate IL-2 part of the Hu 14.18IL-2 fusion protein. The bound HRP was detected using reagents such as orthophenylene diamine, 2, 2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid] or tetramethylbenzidine. The capture ELISA using GD2-coated plates was developed earlier in assay development and used to demonstrate assay specificity and to compare lot-to-lot consistency and stability of ch14.18, and Hu14.18 IL-2 in clinical development. During this study, we found a number of issues related to plate-to-plate variability, GD2 lot variability, and variations due to GD2 storage stability, etc., that frequently lead to assay failure in plates coated with purified GD2. The cell-based ELISA (CbELISA) using the GD2 expressing melanoma cell line, M21/P6, was developed as an alternative to the GD2-coated plate ELISA. The results on the comparability of the capture ELISA on GD2-coated plates and the cell-based assay show that both assays give comparable results. However, the cell-based assay is more consistent and reproducible. Subsequently, the anti-GD2 capture ELISA using the GD2-coated plate was replaced with the CbELISA for product lot release testing and stability assessment.

Keywords

disialoganglioside; antibody; enzyme-linked immunosorbent assay; cell-based enzyme-linked immunosorbent assay; potency assay

1. Introduction

The vast majority of antibodies that react preferentially with tumors of neuroectodermal origin such as melanoma, neuroblastoma, and glioma, with little or minimal reactivity with

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^{*}Corresponding Author: Gopalan Soman, PhD, Development Manager III, Biopharmaceutical Development Program, SAIC-Frederick, Inc., NCI-Frederick, PO Box B, Frederick, MD 21702, (301) 846-5594, Fax # (301) 846-6886, somang@mail.nih.gov.

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normal cells are directed to gangliosides. Several murine, mouse–human chimeras and humanized anti-GD2 molecules have been produced and have gone through extensive clinical evaluation. Disialoganglioside (GD2) is a sialic acid containing glycosphingo lipid expressed primarily on the cell surface (Mujoo et al., 1987; Mujoo et al., 1989). GD2 expression is predominantly restricted to the central nervous system, peripheral nerves, and skin melanocytes (Mujoo et al., 1987; Mujoo et al., 1989; Schulz et al., 1984; Svennerholm et al., 1994; Zhang et al., 1997). Because of the high degree of specific tumor-selective expression of GD2 on the cell surface, anti-GD2 molecules are very attractive targets for tumor-specific antibody therapy. The effectiveness of an anti-GD2 strategy in the treatment of neuroblastoma has been recently reported (Navid et al., 2009; Raffaghello et al., 2003; Yang and Sondel, 2010). The different anti-GD2 molecules produced and studied so far are listed in the recent articles (Castel et al., 2010; Navid et al., 2010).

The murine monoclonal antibody (mAb), designated mAb 14.18, recognizes glycolipid GD2 on the surface of many neuroblastoma, melanoma, glioma, and small lung carcinoma cell lines and tissues (Mujoo et al., 1989). GD2 on tumor cells is the target antigen for the mAb 14.18-mediated cytotoxicity and suppression of tumor growth (Mujoo et al., 1987). The human/mouse chimeric anti-GD2 antibody, ch14.18, is a genetically engineered construct produced by combining the variable regions of the mAb 14.18 with the constant regions of human IgG1 light chains (Gillies et al., 1989). Ch14.18 has the GD2 binding specificity of the parent mAb 14.18 and reactivity towards Fc-specific anti-human IgG1. It is 50–100 times more efficient at mediating tumor ADCC in vitro than is the parent murine 14G2a (Mueller et al., 1990).

Ch14.18 has been used in different clinical evaluation studies (Simon et al., 2004; 2005; 2011). A recent study indicated that immunotherapy with ch14.18 may prevent late relapses in high-risk neuroblastoma patients (Simon et al., 2011). A number of combination therapies of ch14.18 with immune stimulatory cytokines such as interleukin-2 (IL-2) (Ozkaynak et al., 2000) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have also been reported (Gilman et al., 2009). The ch14.18 antibody has activity against neuroblastoma, and such activity is enhanced when ch14.18 is combined with the GM-CSF or IL-2 (Castel et al., 2010). A recent study showed that immunotherapy with ch14.18, GM-CSF, and IL-2 was associated with a significantly improved outcome, as compared with standard therapy in patients with high-risk neuroblastoma (Yu et al., 2010). Recombinant fusion molecules like humanized 14.18 interleukine-2 (hu14-18IL-2) (King et al., 2004; Osenga et al., 2006; Sondel and Hank, 1997; Yamane et al., 2009) and ch14.18-GM-CSF fusion protein (Batova et al., 1999) are also evaluated for clinical efficacy.

Potency assays are quantitative measures of a product-specific biological activity that is linked to a relevant biological property and, ideally, mimicking a product's in vivo mechanism of action. Both in vivo and in vitro assays can be used for potency testing. Functional and/or biological activity of antibodies and other protein products in initial clinical investigations are very often defined by quantitation of binding of the products to specific antigens or antigen receptors. ELISAs using purified antigen-coated plates to bind the product, or binding of the product to cells that express the antigen or receptor are very often used to quantitate the relative activities of product lots. We have developed a number of ELISA and CbELISA methods for a variety of products under clinical development. CbELISA methods for an anti-IL-2 α receptor antibody (7G7/B6) and anti-IL-2/IL-15 β receptor (Mik β 1) were published earlier (Yang et al., 2003; Yang et al., 2006). The utility of ELISA and CbELISA and other receptor binding assay methods for quantitative estimation of functionality or biological activity have been demonstrated and documented in literature (Gervay and McReynolds 1999; Kong et al., 2010; Meegan et al., 2010; Waerner et al., 2007).

Specificity and functionality of the ch14.18 product is defined in terms of GD2 binding activity. Frequent assay failure in the GD2 coated plate ELISA due to quality of the GD2 reagent, GD2 lot variations, GD2 storage stability, and/or plate-to-plate variations was noticed during the assay development and qualification. Subsequently, a CbELISA was developed using a GD2-expressing M21/P6 cell line. In this report, the performance of both assays is compared. The CbELISA is shown to give more consistent results compared to the GD2-coated ELISA.

2. Materials and methods

2.1 Reagents

Ch14.18 and hu14.18IL-2 used in this study were produced and purified within the Biopharmaceutical Development Program of SAIC-Frederick, Inc., National Cancer Institute, Frederick. The disialoganglioside (GD2) was obtained from two sources (American Research Products, Belmont, MA; and Sigma Aldrich, St. Louis, MO). Ninety-six-well ELISA plates were also obtained from two sources (Nunc-Immuno 96-well plate, catalog # 44204, 269787, NT 51089; Nunc-Immuno strips, catalog #469949, and Costar 96-well plate, catalog # 3590). The secondary (detection) antibody used was HRP-conjugated affinipure goat anti-human IgG from Jackson Immuno Research Lab Inc. (catalog # 109-035-098). The HRP-detection reagent used was orthophenylene diamine (OPD) or tetramethylebenzidine (TMB) from Sigma-Aldrich, MO.

2.2 Cells and cell culture

The melanoma cell line M21/P6, which expresses GD2 on the surfaces (Sen et al., 1997), was a generous gift from Jean Surfus of the University of Wisconsin, Madison, Wisconsin. NCI-H460 cells, a large lung cancer cell line, expressing none or a very low level of GD2 (Yoshida et al., 2001) was obtained from Dr. William Kopp of the Applied Development and Research Support Laboratory of SAIC-Frederick, Inc., NCI-Frederick.

M21/P6 cells were routinely cultured and maintained in RPMI 1640, containing 10% FBS, 10 mM HEPES buffer, 2 mM L-glutamine, 100U/ml penicillin and 100 µg/ml streptomycin. The NCI H460 cell line was cultured in RPMI 1640, 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.

2.3. Capture GD2 ELISA

The GD2 capture ELISA was performed by a direct GD2 capture binding ELISA, following a modification of the procedures described earlier (Gillies et al., 1989; and Sen et al., 1997) for determination of competitive binding of chimeric antibodies or direct GD2 binding of anti-GD2. GD2 samples from the supplier were reconstituted following the manufacturers recommendations and the GD2 concentration provided by the supplier was used in calculating the dilution. Briefly, a 96-well polyvinyl microtiter plate was coated with 50 µl of 2 µg/ml GD2 in carbonate-bicarbonate buffer (200 mM, pH 9.4) overnight at 2–8°C. The plate was washed with PBS-T buffer, and the antigen (GD2)-coated plate was blocked with 5% BSA-PBS for 120 minutes at 37°C. The plate was washed with PBS-T buffer three times and incubated with 50 µl of the reference standard and test samples in two-fold serial dilutions (approximately 5000 ng/ml – 40 ng/ml) for 2 hours at room temperature. The plate was then washed three times with PBS-T, using a plate washer (Bio-Tek instruments) and incubated with 50 µl of HRP-conjugated goat anti-human IgG Fc (200 ng/ml) in sample dilution buffer (1% BSA in PBS) for 1 hour at 37°C. The washed plate wells were then incubated with 50 µl/well of OPD at room temperature for 15 minutes. The reaction was stopped by adding 50 µl/well of 2N sulfuric acid. The absorbance at 490 nm was recorded, using a micro plate reader from Molecular Devices.

2.4. M21/P6 CbELISA

The human melanoma M21/P6 cells were seeded at 5×10^4 per well in 200 μ l cell growth medium in a 96-well microtiter plate and incubated at 37°C, 5% CO₂ overnight. On the second day, the plate was washed three times in Dulbecco's phosphate-buffered saline (DPBS) and the cells fixed with glutaraldehyde (1/100 V/V dilution with H₂O) for 10 minutes. After washing, the plate wells were blocked with 4% nonfat dry milk in PBS-T for 30 minutes at 37°C. The plate was then washed once in PBS-T, the reference standard and test samples (approximately 1000 ng/ml – 1.372 ng/ml) were added and incubated for 1 hour at 37°C. After washing three times in PBS-T, the plate was incubated with HRP-conjugated goat anti-human IgG Fc (40 ng/well) for 30 minutes at 37°C. At the end of the incubation period, plate wells were washed three times in washing buffer and incubated with 50 μ l/well of TMB substrate solution at room temperature. The reaction was stopped by adding 50 μ l/well of 2N sulfuric acid. The absorbance at 450 nm was measured using a micro plate reader.

2.5. Data analysis

After completion of the assay the plates were read using a micro plate reader from Molecular Devices (currently GE) and data analysis was performed by a 4-parameter curve fit mode using SoftMaxPro program provided by the instrument supplier. Standard and test sample dilutions are run in triplicate wells and the software program calculates the mean values of the triplicate well runs and standard deviation. For quantitative estimates of the GD2 binding activity in samples, test sample was run at different dilutions and treated as unknown dilutions. The optical density (OD) read out of the test sample was extrapolated to the standard curve generated using the reference standard of known concentration and the result obtained is multiplied with the sample dilution factor. The value so obtained is referred as the adjusted result or back calculated concentration. The adjusted results for all of the test sample dilutions with OD values that fall within the steep portion of the 4-parameter curve of the reference standard were averaged and reported as estimated value. Alternatively, the data could be filtered to cover best linear fit correlation and the relative activities could be calculated by comparing the slopes of the linear curve fit curve of reference standard (control) and test samples. The relative activity % is calculated using the formula: relative activity of test article = (estimated activity $\div$ protein concentration) $\times$ 100. The ED₅₀ values (half-maximal dose-response concentration) were determined by the C parameter of the 4-parameter curve-fit plot. Relative activity based on ED₅₀ ratio was calculated using the formula: relative activity of test article = (ED₅₀ of reference standard $\div$ ED₅₀ of test article) $\times$ 100%. Spike recovery with 50 ng/ml standard (P50) values was estimated by treating the spike as a test article with unknown concentration and extrapolating the OD read out of the spike wells to the standard curve. The value obtained is taken as the estimated value for the spiked wells. Spike recovery (%) = [estimated value (ng/ml) $\div$ 50] $\times$ 100%

3. Results

3.1. Capture ELISA on GD2-coated plates

GD2 binding activity of ch14.18 and other anti-GD2 antibodies or antibody cytokine conjugates such as hu14.18IL-2 was assayed by measuring binding to GD2-coated plate. GD2 from two sources (American Research Product, catalog # 05-52146; and Sigma, catalog # G0776) was tested. A GD2 binding ELISA titration curve is shown in Figure 1A. The assay was optimized and adapted for routine testing and stability monitoring of different lots of ch14.18 or Hu14.18IL-2 as part of the functional activity assay for initial phase clinical evaluation studies. The dose-response curve and the absorbance readings were dependent on the source of GD2 (not shown). The dose-response curves on different days

showed significant variations in the reactivity (absorbance readings) from day to day. The concentration range of ch14.18 from 40 to 5000 ng/ml did not always cover the full dose-response range for a four-parameter curve-fit; and therefore, precise estimate of the ED₅₀ values or ED₅₀ ratio for the reference standard and test articles were not feasible, as the dose-response curves did not always cover the lower and upper asymptotes (Figures 2, 3 and 5 C, D, E)

These experiment-to-experiment variations in the dose-response curves and the absorbance readout were noticed by different operators and also with GD2 from different sources (data not shown). However, an accurate estimate of relative activity of a product lot in reference to a selected reference standard lot was obtained by extrapolation of the test article readings in the linear part of the standard curve, to the standard curve. Alternatively, the data could have been filtered to cover a satisfactory linear curve-fit (Figure 1B) and the relative activities could have been estimated by comparing the slope ratio or extrapolation of the test article absorbance to the standard curve, adjusting the values for dilution and averaging the values at the different dilutions.

Table 1A shows the relative activity estimates of two lots of ch14.18 relative to the same reference standard at different times. Figure 2 shows the intra-day, inter-plate variations in the GD2 binding ELISA reactivity of three lots of ch14.18. Table 1B shows the intra-day lot-to-lot and plate-to-plate variations observed, as estimated from data filtered to cover the linear curve-fit range (Figure 2 B) and relative activity determined as the slope ratio of the linear fit curves. The observed plate-plate variations in the ED₅₀ values and other coefficient values in the four-parameter curve fit were significant between plate 3 and the other two plates, while there was very good consistency between plate 1 and plate 2. However, the relative activity estimates were comparable in all three plates.

3.2. Assay variations in the capture ELISA on GD2-coated plates

The effect of different lots of purified GD2 from the same source on the ch14.18 binding ELISA was compared. As shown in Figure 3A, the plates coated with different lots of GD2 from the same source showed significant variability. GD2 from different sources also showed significant differences in reactivity (not shown). The absorbance readouts, as well as the effective concentration ranges, were different. There was also a significant variability in day-to-day experiments (not shown). Different GD2 samples from the same source, but purchased at different times, were also tested. GD2 was dissolved in dichloromethane: methanol (V/V: 1/1). Aliquots (11 µL/each vial) were stored at -20°C. These aliquots were used at different time points from April 17 to May 21, 2003, to perform GD2-binding ELISA. The same reference standard and test articles were used in all experiments (Figure 3B). The results are summarized in Table 2. Our routine procedure followed 15 minutes incubation after addition of the detection reagent OPD. However, in these experiments, only the tests performed on day 1 and 6 showed acceptable results (Figure 3B and Table 2). With two experiments using GD2 stored for 20 and 34 days, the reactivity was much lower and the estimates were not feasible. We also tested 96-well plates from different sources and different lots of plates from the same source (data not shown). In all cases, significant plate-plate and day-day variations were detected (not shown). Based on the above experiments, it was concluded that reproducibility of the GD2-coated ELISA may be significantly affected by quality of GD2 reagents used for plate coating.

GD2 stability, adherence of GD2 to container vials, and plate-plate variations are only a few of the factors involved. From all the trouble-shooting investigational experiments, it became evident that the binding ELISA for anti-GD2 by the capture ELISA on a GD2-coated plate, performed as described here, has several limitations. The major anticipated factors contributing to these variations were the plate-matrix-glycolipid interaction or binding under

the conditions used in the assay, storage stability of dissolved GD2, loss of GD2, and degradation of GD2. Disialoganglioside is a glycolipid and the binding of this glycolipid to the polyvinylidene plate wells in aqueous buffers may be significantly affected by minor micro environmental variations on the plate, as well as on the individual well surfaces. The significant well-to-well variation is a reflection of the subtle microenvironment variations within the plate, and the differences in the binding reactivity between different plates are a reflection of the changes or variations in the surface characteristics of different plates. Besides these plate-matrix interactions, the source of GD2, storage of GD2 in solution, and changes in GD2 lots from the same source also contributed to the observed variation.

Although these limitations could probably be eliminated or minimized by working out an alternate procedure for uniform coating of GD2 to the plate wells and improving the quality and control of GD2, we have explored alternate binding ELISA modes.

3.3. Specificity of the M21/P6 CbELISA

Using the human melanoma cell line M21/P6, which expresses GD2 on the surfaces, a CbELISA was developed to test GD2 binding activity of anti-GD2 molecules. Both ch14.18 and hu14.18IL-2, showed dose-dependent titration curves (Figure 4A). The cytokine IL-2 and an isotype-matched HuIgG *k* were tested to define the anti-GD2 specificity, and these molecules did not show any reactivity in the CbELISA. Under similar conditions, the NCI H460 cell line showed very low reactivity with ch14.18 or Hu14.18IL-2 (Figures 4B and C), which is consistent with previous report showing little or no expression of GD2 on this cell line (Yoshida et al., 2001). The CbELISA conditions were optimized with respect to cell seed density, ch14.18, or Hu14.18IL-2 concentration, incubation time for different steps, etc., and a standard procedure was developed for routine assay and stability monitoring. The cell bound anti-GD2-cytokine conjugate (Hu14.18IL-2) could be detected using either HRP-anti-IgG or HRP-anti-IL-2 conjugates (not shown).

The assay performance was also assessed by using positive controls in the experiments (control samples of known ch14.18 or anti-GD2 cytokine conjugate concentration prepared from the standard). Results are shown in Table 3A. These results suggested that when comparing the experimental values to actual protein concentration, the M21/P6 cell-based assay showed accurate mass recovery.

3.4. Reproducibility of the M21/P6 CbELISA

Using a M21/P6 CbELISA, and following a standard procedure, GD2 binding activity of ch14.18 was tested on different days using cells at different passages. As shown in Table 3B and Figure 5A and B, M21/P6 CbELISAs showed reproducible results. The relative activity of the test articles lots in the three experiments were in the range of 94–102% of the reference standard. The absorbance readout and the C value (ED₅₀) of the four-parameter curve fit showed within plate, day-day, plate-plate variability (example: the ED₅₀ values plotted in Figures 6A–B), which could partly be due to changes in cell growth characteristics. Hence, a reference standard of known reactivity is required for quantitative estimation of relative binding activity. Table 3C shows the inter-day variation in the relative activity estimates of a test article compared to the reference standard. The relative activity of the test article in the four experiments performed over a 50-day interval is in the range of 93.84–103.25% of the reference standard activity.

3.5. Comparison of GD2 binding assay between GD2-coated plate and M21/P6 cell-coated plate

Three sets of experiments comparing the performance of the ELISA using a GD2-coated plate and M21/P6 CbELISA are shown in Figure 5. Results demonstrate the better

reproducibility and performance of the CbELISA (Figure 5 A and B) over the GD2 binding ELISA (Figures 5 C, D and E). When the assays worked satisfactorily, the results obtained from both methods from three independent sets of experiments were consistent and comparable, as evidenced from the results shown in Table 3B.

The GD2-coated binding ELISA frequently showed frequent assay failure. Quality of the GD2 reagent, GD2 lot-lot variability, and GD2 storage stability may contribute to this effect. However, the M21/P6 CbELISA (Figure 5A and B) displayed good binding dose-response curves. The use of M21/P6 cells eliminates the problems of GD2 degradation, insolubility of aliquot substrate due to solvent evaporation, and adherence of GD2 to container vials (attributable in general as the quality of GD2 reagent). Therefore, it was determined appropriate that we switch from the GD2-coated ELISA to the CbELISA to quantitate the GD2 binding activity and lot-lot comparison of the GD2 binding activity of ch14.18 or hu14.18-IL2.

3.6. Performance and application of M21/P6 CbELISA

We have opted to use the M21/P6 cell line for CbELISA. The assay worked more effectively with glutaraldehyde-fixed cells, and fixed cells were used in subsequent assay development and optimization. The assay conditions were optimized. The assay parameters selected for optimization are described (results not shown). Studies were performed with different cell seed densities (1×10^4 to 5×10^4 cells/well), and antibody dose response range starting concentration from 5000 ng/ml or 1000 ng/ml. Assay was also performed for comparing the dose response curves using different concentration of the secondary antibody (40 ng/ml to 200 ng/ml). Based on the optimization study results, the procedure described in Materials and methods section 2.4 was adapted. The assay performance on the passage number of cells after thawing of the cells from freezer was also evaluated. The assay performed satisfactorily with cells in continuous culture between passage 3 and thirty five after the thaw from freezer. We compared the performance of M21/P6-based CbELISA and GD2 capture ELISA under the optimized procedure. The M21/P6 CbELISA was used for comparing the binding activity of different lots of ch14.18, and hu14.18IL-2, and for monitoring the stability of different lots. The binding activities of different clinical lots of ch14.18 were comparable and all the lots were very stable with respect to the M21/P6 cell binding activity, proving the consistency and stability of the clinical lots (data not shown). The inter-day assay variations in the relative activity of a test article compared to the reference standard is shown in Table 3C. The variations in the ED₅₀ values for the reference standard and two lots of ch14.18 over a 4–5 year period are shown in Figure 6A and B. Though the absolute ED₅₀ values showed significant variations from day to day, the relative activities calculated using ED₅₀ ratio of standard: test article (Figure 7A and B) showed consistency and comparability with the estimates, using extrapolation of the test article absorbance to the standard curve. The average values for the relative activity for lot A in the 9 stability data points were $(110 \pm 11)\%$ of standard with CV% 9.9 from ED₅₀ ratio comparison and $(106 \pm 9)\%$ of standard and CV% 8.5, based on extrapolation of the absorbance readings to the standard curve, for lot A. For lot B, the average relative activity based on ED₅₀ ratio was $(99.5 \pm 14.2)\%$ of standard and CV% 14.3 and $(106 \pm 9)\%$ of standard with CV% 8.5, based on extrapolation of test article absorbance readings to the standard curve. The estimated values for the assay control (50 ng/ml reference standard) are also shown in Figure 7C. From 49 experiments performed over a 6–7 year period, the mean estimated value for the P50 control was $(97 \pm 8.8)\%$, with CV% of 9.4 relative to the expected value (100%). These ch14.18 lots were being tracked by physicochemical analysis over the same period and showed no degradation (data not shown). It supports the idea that the product itself is very stable in structure as well as function.

4. Discussion

Functional or biological activity or potency of the product need to be qualified to meet the requirement for the appropriate phases of clinical development and eventually validated for the intended use as the product enters the marketing stages. The ch14.18 and hu14.18IL-2 are in clinical development. Ch14.18 is in NCI supported phase 3 clinical trials. During the initial development stage, an ELISA-based assay based on specific binding of the antibody or conjugates to GD2 coated on a 96-well plate was used as an index of functional activity of the ch14.18. As elaborated in the Results section, the ELISA developed using direct coating of the plate with GD2, showed unacceptable assay variations and frequent assay failures. These variations could be attributed to the quality of the GD2 reagent and or limitations of the assay as developed. This necessitated, further development of the assay by changing the coating conditions, plate selection and or improving the quality of the reagent. Developing alternate methods for measuring functional activity was alternate option. This manuscript describes the approach we have used to replace the existing GD2-binding ELISA with CbELISA using a stable melanoma cell line expressing GD2.

Binding of anti-GD2 molecules to GD2-expressing cell lines has been demonstrated in several laboratories. By using cell lines that express GD2 and negative control cell lines that do not express GD2, we demonstrated dose-dependent binding of ch14.18 and hu14.18IL-2 binding specifically to a GD2-expressing cell line. The cell-binding ELISA was subsequently developed using the GD2-expressing melanoma cell line M21/P6. The CbELISA using the M21/P6 cell line gave comparable results and more reproducible data, compared to the direct ELISA method using a GD2-coated plate. Therefore, we have replaced the GD2-binding ELISA with the CbELISA using the M21/P6 cell line.

CbELISA is useful for comparing the relative activities of different lots of ch14.18 with a reference standard. The dose-response curves and the ED₅₀ values estimated showed significant variation from day to day and experiment to experiment. These types of variations in the ED₅₀ values are very common with the ELISA, CbELISA, and other cell-based bioassays, such as cell proliferation and cytolytic assays. The ED₅₀ ratio for the reference and test article is an alternate way to express the relative activity of a test article compared to standard. The relative activities based on ED₅₀ ratio, and back-calculated activity by extrapolation of test article dilution absorbance readings to standard curve showed comparable results with a similar trend (Figure 6A–E). The ED₅₀ estimates could be controlled better by further optimization of the dose-response curves to cover the entire sigmoidal curve with both the lower and upper asymptotes covered in the dose-response experimental curve. This would enable researchers to address the issue of variability in ED₅₀ values. Such a control of ED₅₀ values could be used as a better index of the product functional activity, and a satisfactory range for acceptable ED₅₀ range could be assigned.

FACS-based GD2-binding studies could be used to determine whether the variation in the CbELISA parameters has any correlation to GD2 expression level. Most of the tumor cell lines expressing GD2 are known to show stable GD2 expression. The M21/P6 melanoma cell line we used in this study is a cell line that could be maintained in continuous culture for more than 50 passages after thawing of the cell. Though the curve-fit parameters changed from experiment to experiment, excellent dose-response curves were obtained over a 7-year period, and the maximal absorbance readings in all cases were within 10–20% variation. These data suggest a fairly consistent stable surface expression of the GD2 molecules.

In conclusion, M21/P6 CbELISA method is more consistent and reproducible comparing to the GD2-coated ELISA. The M21/P6 CbELISA described here has been used for product testing and stability monitoring of different lots of ch14.18 and hu14.18IL-2 used in NCI-

sponsored clinical evaluation studies. Although there are significant variations in the absolute ED₅₀ values, the relative activities of different lots of clinical product referenced to a standard reference lot were comparable and maintained the same relative activity for more than a 5-year period in the CbELISA.

Highlights

- Two GD2-binding assays for determination of anti-GD2 antibody and conjugate fusion proteins were developed using GD2-coated plate ELISA and GD2-expressing M21/P6 cell based ELISA and their performances are compared and contrasted.
- The GD2 antigen coated plate ELISA has limitations due to intra and inter-day plate-plate variations caused by antigen instability and lot to lot differences.
- The M21/P6 cell based ELISA performed better in consistency, reproducibility and intra-and inter-day variations.
- The utility of M21/P6 cell based ELISA in the clinical products lot releases and stability monitoring is summarized.

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
CbELISA	cell-based ELISA
CDC	complement-dependent cytotoxicity
ch14.18	mouse-human chimera of mAb14.18
DPBS	Dulbecco's phosphate-buffered saline
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
GD2	disialoganglioside
HRP	horseradish peroxidase
hu14.18IL-2	humanized 14-18IL-2
IL-2	Interleukin-2
mAb	monoclonal antibody
OD	optical density
OPD	orthophenylene diamine
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Tween 20 0.05%
TMB	tetra methylene benzidine

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Fig. 1. Typical dose-response curves of GD2 binding ELISA

ELISA was performed as described in the Materials and methods section 2.3. Each sample dilution is run in triplicate wells and the SoftMaxPro software calculates the mean value of the triplicate wells and standard deviation. The error bars in the dose response curves refers to the standard deviation of the average of triplicate. (A) Data analyzed using four-parameter curve-fit. (B) Data was filtered to cover the best linear-fit range; linear fit curve is shown.

Fig. 2. Intra-day plate-plate variations in the dose-response curves of GD2 binding ELISA

Three lots of ch14.18 (L1, L2 and L3) were tested side by side in the same plates. The assay was performed in three plates (P1, P2 and P3) in the same day, using the same three lots of ch14.18. The average readings of each sample in the three plates were imported to a Softmax 96-well format and analyzed using 4-parameter curve-fit (A) and linear curve fit after filtering the data to cover the linear fit-range (B).

Fig. 3. GD2 lot-lot and storage effect on GD2 binding ELISA

A. GD2 lot-lot differences on the ch14.18 dose-response curves. The dissolved GD2 from different lots (L1, L2 and L3) was coated on a 96-well plate and incubated for 24 hours at 2–8°C. Assay was performed as described in Materials and methods section 2.3.

B. Effect of storage time of dissolved GD2 aliquot on the ch14.18 binding activity. One GD2 lot (1 mg) was dissolved in 1 ml dichloromethane:methanol (1:1), aliquoted, and stored at –20°C. The dissolved GD2 aliquots were thawed and coated on a 96-well plate on different days (1 day, 6 days, 20 days and 34 days after freezing), and incubated for 24 hours. Assay was performed as described in Materials and Methods section 2.3. Error bars corresponds to the standard deviation from the average (mean) of the triplicate well readouts.

Fig. 4. Specificity of M21/P6 CbELISA

A. M21/P6 cell binding of ch14.18 and Hu 14.18 IL-2 are GD2-specific. M21/P6 myeloma cell line was seeded into the wells of a 96-well plate (5×10^4 cells/well in 200 μ l of medium) and fixed on the plate wells by treatment with glutaraldehyde. The fixed, washed cells were incubated with serial dilutions of anti-GD (ch14-18), humanized anti-GD2 cytokine conjugate (hu14.18IL-2), a human IgG (HuIgG) control, and human cytokine (IL-2) control. After 1 h incubation, cells were washed, incubated with HRP-human IgG for 1 h, washed again, and incubated with TMB substrate. After 15 min incubation, 2N H_2SO_4 was added and the plate read at 450 nm. Error bars corresponds to the standard deviation from the average (mean) of the triplicate well readouts.

B. Ch14.18 binds effectively to the GD2-positive melanoma cell line, compared to the large lung cancer cell line NCI H460, with little or no GD2 expression. M21/P6 melanoma cell and NCI H460 large lung cancer cells were seeded at the same cell density at different regions of the same 96-well plate. CbELISA was performed as described in Materials and Methods, following the conditions described in the legend for Figure 4A, using ch14.18.

C. Hu14.18IL-2 binds effectively to the GD2-positive melanoma cell line, compared to the large lung cancer cell line NCI H460, with little or no GD2 expression. M21/P6 melanoma cell and NCI H460 large lung cancer cells were seeded at the same cell density at different regions of the same 96-well plate. CbELISA was performed as described in Materials and Methods, following the conditions described in the legend for Figure 4A, using hu14.18IL-2.

Fig. 5. Comparison of the reproducibility and consistency of the GD2 ELISA and M21/P6 CbELISA

Two sets of experiments (A and B) using M21/P6 CbELISA and three sets of experiments (C, D, and E) using GD2-coated plate ELISA were performed for comparing the dose-response curves of two lots of ch14.18. Assays were conducted following the procedures described in Materials and methods.

Fig. 6. Inter-day assay variations of ED₅₀ values over a 5-year period (stability monitoring and trend analysis)

A: Distribution of ED₅₀ values of reference standard and test article lot A. 1 (standard); 2 (lot A); 3 (average of standard); and 4 (average of lot A).

B: Distribution of ED₅₀ values of reference standard and test article lot B. 1 (standard); 2 (lot B); 3 (average of standard); and 4 (average of lot B).

Fig.7. Variations in the relative activities of two different lots of ch14.18 over five year storage at 2–8°C compared to a reference standard lot and the Positive control (P50) values

A. Distribution of % relative activity of lot A compared to reference standard lot. 1 (relative activities calculated from ED_{50} ratio); 2 (relative activities calculated from extrapolation of test article absorbance reading to standard curve); 3 (average value for ED_{50} ratio); and 4 (average value based on OD extrapolation).

B. Distribution of % relative activity of lot B compared to reference standard lot. 1 (relative activities calculated from ED_{50} ratio); 2 (relative activities calculated from extrapolation of test article absorbance reading to standard curve); 3 (average value for ED_{50} ratio); and 4 (average value based on OD extrapolation).

C. Estimated values of P50 control (1) and % estimated value to theoretical value (2) from 49 experiments.

Table 1

A. Variations in the estimated relative activities of test articles from the GD2 capture ELISA.

Experiment	Estimated activity			
Test article A		Test Article B		
	4-P plot	Filtered linear plot	4-P plot	Filtered linear plot
1	6.46 ± 1.32	6.34 ± 0.30	5.76 ± 1.21	5.75 ± 0.19
2	6.58 ± 0.43	6.86 ± 1.40	5.46 ± 0.20	5.28 ± 0.20
3	6.09 ± 0.55	6.53 ± 0.29	6.14 ± 0.37	5.67 ± 0.37
Average	6.4	6.6	5.8	5.6
Std Dev	0.3	0.3	0.3	0.3
CV (%)	4.0	4.0	5.9	4.5

B. Intra-day plate-plate and lot-lot variations.

Lot	Plate 1		Plate 2		Plate 3	
	Slope	Rel. Activity	Slope	Rel. Activity	Slope	Rel. Activity
Lot 1	1.88	1.0	1.88	1.0	2.82	1.0
Lot 2	2.53	1.3	2.53	1.3	3.47	1.2
Lot 3	2.93	1.6	2.93	1.6	3.22	1.1

The same reference standard and test articles were assayed on three days under the same conditions by the same analyst. Test article in the dilution range covering the steep part of the standard four-parameter curve-fits or the standard curve data was filtered to get the best linear curve-fit and test article dilutions with OD readout in the standard curve range was extrapolated to get the estimated activity of the test articles. The average and standard deviations of the individual experiments used in calculation are reported. The estimated values from three independent experiments and the average and standard deviation of those three independent estimates are also shown.

Three lots of ch14.18 were tested in three plates on the same day to assess the lot-lot and plate-plate variations. Data filtered to cover best linear fit. Relative activities are calculated using the slope ratio of test article vs. standard.

Table 2

Storage stability of GD2

	1 Day	6 Days	20 Days	34 Days
Protein Conc. (mg/ml)	5.84	5.84	5.84	5.84
*Binding activity (mg/ml)	5.95±0.32	5.64±0.34	No linear range	Not detectable
Relative activity	101.88%	96.58%	-	-

* The average and standard deviation from all the test article dilutions used in calculation are shown.

The GD2 binding activity of two lots of ch14.18 was tested on different days by GD2-coated plate ELISA using ch14.18. The same GD2 stock solution aliquots stored at -20°C for different days were used in the assay. The storage time of the GD2 aliquots at -20°C is shown.

Table 3

A. Estimated values and recovery of the positive control in the M21/P6 CbELISA.

Experiment Date	Expected value ng/mL	Estimated value* ng/mL	CY%	Recovery%
111502	50	52.3 ± 4.25	8.1	104.63
112502	50	50.1 ± 1.13	2.3	101.32
41703	50	51.5 ± 5.80	11.3	103.08
Average	50	51.31	7.2	102.61

B. Comparison of GD2-binding activity of two Ch14.18 lots by GD2-coated plate ELISA and M21/P6 CbELISA.

	GD2-coated Plate ELISA			M21/P6 CbELISA		
	Lot 1	Lot2 (E1)	Lot2 (E2)	Lot 1	Lot2 (E1)	Lot2 (E2)
Protein concentration (mg/ml)	5.84	4.89	4.89	5.84	4.89	4.89
GD2 binding activity (mg/ml)*	5.95±0.32	4.74±0.41	4.60±0.21	5.58±0.40	5.22±0.52	5.07±0.03
Relative activity (%)	101.88	96.93	94.07	95.55	104.40	103.68

C. The reproducibility of the GD2 binding activity of Ch14.18 determined by M21/P6 CbELISA (Inter-day variations)

	M21/P6 CbELISA			
	Day 1	Day 10	Day 30	Day 50
Protein concentration mg/ml	5.84	5.84	5.84	5.84
*Binding activity mg/ml	5.58 ± 0.40	5.48 ± 0.61	6.02 ± 0.57	6.03 ± 0.10
Relative activity (%)	95.55	93.84	103.08	103.25

* Estimated values are average for the triplicate wells ± standard deviation

Test sample of known concentration (50 ng/ml) prepared from the reference standard was used as a control in triplicate wells, and the GD2 binding relative activity was determined by extrapolation of the control absorbance reading to the standard curve..

* Average of all the individual dilutions used in the calculation ± standard deviation

GD2 binding activities of two Ch14.18 lots were tested by both GD2-coated plate ELISA and M21/P6 CbELISA. The same reference standards are run in each experiment, and the relative activities are determined. Lot 1 was run one time and Lot 2 was run in two experiments (E1 and E2), run on different days.

* Average estimated values for all the individual dilutions used in the calculation ± standard deviation.

The GD2 binding activity of Ch14.18 was tested on different days by M21/P6 ChELISA. The same test article lot and reference standard lot were used in all four assays. The initial experiment is dated Day 1 and subsequent experiments are dated following Day 1.