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Development of a novel radioimmunoassay to detect autoantibodies to amyloid beta peptides in the presence of a cross-reactive therapeutic antibody

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ABSTRACT

An increasing need in the development of biotherapeutic agents is the ability to monitor a potential autoimmune response to the therapeutic target of interest. Unfortunately, the presence of high concentrations of therapeutic antibody can hinder such detection, because there is competition for binding in cases where epitopes are not structurally distinct. This situation was encountered in the development of LY2062430, a therapeutic mid-domain monoclonal anti-amyloid beta peptide (Aβ) antibody undergoing clinical trials for the treatment of Alzheimer's disease. This communication reports the development and validation of a novel radioimmunoassay used to measure potential patient immune responses to Aβ in the presence of LY2062430. This assay employs a radioiodinated analog of the human amyloid beta 1–40 peptide (Aβ1–40) in which a single amino acid substitution of alanine for phenylalanine at position 19 (F19A) effectively eliminates binding by LY2062430. In contrast, F19A binding by monoclonal antibodies specific for the N- and C-termini of the human Aβ1–40 peptide was shown to be unaltered. Additional experiments involving a polyclonal rabbit antibody raised against the midregion of Aβ1–40 (residues 15–30) resulted in only a slight reduction in binding to the F19A tracer, suggesting that the modification does not affect distal epitopes in Aβ1–40 and supporting the notion that this conservative substitution produces only subtle change in the overall peptide structure. The assay is therefore believed to detect most, if not all, patient antibodies to native Aβ peptides. The assay was validated for use in clinical trials allowing detection of antibodies to Aβ in human serum in the presence of therapeutic concentrations of LY2062430.

1. Introduction

Today it is a regulatory expectation that any clinical program to develop a biotherapeutic drug will include immunogenicity testing to detect and characterize the potential generation of anti-drug antibodies. Several white papers and United States Food and Drug Administration draft guidance have been published to guide the development and validation of anti-drug antibody assays, and subsequent analyses of patient samples [1–3]. Therapeutic antibodies specific for soluble targets can, in some cases, significantly raise the levels of the target protein. Thus, it may be important to measure changes in the titer of potential autoantibodies to the target in the presence of a therapeutic antibody. Assays to monitor antibody formation to the therapeutic, as well as the soluble target may be necessary to properly assess the safety of a biotherapeutic drug candidate. Immunoassays have been developed previously to detect autoantibodies to citrullineated peptides in rheumatoid arthritis [4] and xxx peptides in multiple sclerosis [5]. This type of direct binding format would be subject to interference if a therapeutic monoclonal directed at the target protein was also present in samples.

While the existence of autoantibodies against amyloid beta peptide (Aβ) has been reported in both healthy individuals and patients with Alzheimer's disease, the significance of these antibodies is unknown [6]. Autoantibodies to Aβ are common among elderly individuals, and generally have very low titer [7]. Furthermore, neither the presence nor the level of autoantibodies to Aβ, in the absence of active immunization, correlates with the likelihood of developing Alzheimer's disease [8,9]. In a mouse model of Alzheimer's disease, active immunization with anti-Aβ antibody altered Aβ levels in plasma and the central nervous system [10–12].

Abbreviations: Aβ, amyloid beta peptide; Aβ1–40, human amyloid beta 1–40 peptide; F19A, single amino acid substitution of alanine for phenylalanine at position 19; RIA, radioimmunoassay.

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Table 1
Validation statistical summary. Accuracy and precision data from 6 assays with 3 reportable results at each control level in each assay. The assay exhibits acceptable accuracy and precision from 5 to 50 ng/mL.

Characteristic	Concentration (ng/mL)				
	5	12.5	50	100	150
N	18	18	18	18	18
Observed (ng/mL)	5.2	12.8	53.0	116.8	163.7
Mean recovery (%)	104.7	102.7	106.0	116.8	109.2
Mean bias (%RE)	4.7	2.7	6.0	16.8	9.2
Precision (%CV)					
Intra-assay	4.2	9.3	6.8	15.1	31.9
Inter-assay	7.6	11.8	13.9	29.7	33.7
Total error (|%RE| + %CV)	12.3	14.5	19.9	46.5	42.9

Abbreviations: %CV, percent coefficient of variation; *N*, number of results; %RE, percent relative error.

LY2062430, a fully humanized monoclonal antibody directed against the midregion of Aβ, is currently undergoing investigation as a treatment for Alzheimer's disease. During initial clinical testing, investigators needed an assay to detect and monitor potential changes in anti-Aβ autoantibodies following therapeutic antibody administration. However, the high serum levels of the therapeutic antibodies created a substantial analytical challenge for detecting host autoantibodies in the presence of LY2062430. Accordingly, we devised and validated a novel radioimmunoassay (RIA) to permit specific detection of anti-human Aβ autoantibodies in the presence of these candidate therapeutic antibodies. A unique feature of the RIA is that it employs a radioiodinated analog of human amyloid beta 1–40 peptide (Aβ1–40) in which a single alanine substitution was made at position 19 (F19A). This modified radiolabeled analog displayed negligible reactivity with LY2062430 at concentrations as high as 1000 μg/mL and, thus permitted detection of host anti-human Aβ autoantibodies in patients undergoing treatment with the candidate therapeutic antibodies. To confirm that the single amino acid substitution did not alter immunoreactivity of the peptide with other antibodies, we tested two monoclonal antibodies and a polyclonal antisera specific for the midregion of Aβ1–40 (Table 1). This RIA was validated for the quantification of human serum concentrations of anti-Aβ antibodies in the presence of LY2062430 and subsequently used to screen samples from a phase 2 clinical trial.

2. Materials and methods

2.1. Reagents

Radioiodinated Aβ and analog peptides were [^{125}I]-human Aβ1–40 F19A analog, Custom Iodination, Lot #0419, from Amersham/GE Healthcare Inc. (Piscataway, NJ). The peptides were iodinated to a specific activity of 2000–2170 Ci/mmol via chloramine T oxidation of the tyrosine residue. Iodinated peptide tracer was separated from unreacted (cold) peptide by reverse phase HPLC. Murine monoclonal anti-human Aβ antibodies, clone 3D6 and clone 2G3, were obtained from Harlan Laboratories, Inc. (Indianapolis, IN). The Aβ1–40, Reference Standard Material, Lot #RS0395, was provided from Lilly Research Labs (Indianapolis, IN). Pooled human sera, Product #HS-300, were purchased from SeraCare Life Sciences (Milford, MA). Individual human serum samples, Catalog #HMSRM, from normal healthy adults and patients with clinically diagnosed Alzheimer's disease were obtained from Bioreclamation, LLC (Hicksville, NY). Assay buffer (pH 7.5) contained 50 mM Na HEPES, 0.9% sodium chloride, 0.1% EDTA, 0.1% Tween-20 0.1% sodium azide and 0.2% bovine serum albumin, Catalog #A7888 from Sigma–Aldrich Corporation (St. Louis, MO).

2.2. Radioimmunoassay

A conventional (noncompetitive) RIA was developed and optimized to detect circulating anti-Aβ autoantibodies. The 400 μL reaction was initiated by sequential additions of 100 μL of assay buffer, 100 μL of standard (an equal mixture of murine monoclonal anti-human Aβ antibody clones 3D6 and 2G3 diluted in assay buffer), 100 μL of quality controls or human serum test samples, and 100 μL of radioiodinated tracer diluted to approximately 20,000 count per minute (CPM)/100 μL in assay buffer. The reaction was mixed and incubated overnight at 2–8 °C. After 16–24 h, the bound and free forms of tracer were separated by precipitation using 100 μL of 1% bovine gamma globulin diluted in assay buffer as carrier, followed by 1 mL of ice-cold 20% polyethylene glycol (PEG) dissolved in water (8000 mw), Catalog # P2139 from Sigma–Aldrich Corporation (St. Louis, MO). After vortexing, the samples were incubated at 2–8 °C for about 20–30 min and centrifuged at about 3000 × *g* for 30 min at 2–8 °C. The liquid phase was decanted, and the pellets were washed with 1 mL of ice-cold 12.5% PEG and centrifuged again. After decanting the liquid phase, the tubes were counted in a γ-counter for 2 min. The levels (ng/mL) of anti-human Aβ antibodies in test samples were estimated by interpolation using a weighted 4/5-PL algorithm against a standard curve consisting of a mixture of the two murine monoclonal antibodies. (A diagram of Aβ peptide sequence with antibody binding specificities is shown in Fig. 1.)

2.3. Assessment of assay performance

All RIA standard curve and calibration model assessments were performed and a finalized assay format was used to establish the accuracy and precision of the assay. Further, the upper limits of quantitation (ULOQ) and lower limits of quantitation (LLOQ) were determined and the assay was validated to screen for autoantibodies to Aβ [1].

Dilutional linearity was determined in samples of normal human serum supplemented with monoclonal anti-Aβ antibodies (equimolar mixtures of monoclonal antibodies 3D6 and 2G3) at 10 μg/mL that were diluted 1:100 and 1:150 in two separate assay runs. Interference from LY2062430 was determined in samples of normal human serum supplemented with the anti-human Aβ monoclonal antibody mixture at 12.5, 50, and 100 ng/mL by adding 0, 62.5, 125, and 250 μg/mL LY2062430 to generate three reportable results per concentration level in a single assay.

Sample stability was determined in samples of normal human serum supplemented with the anti-human Aβ monoclonal antibody mixture at 12.5, 50, and 100 ng/mL.

An assay cut point was established by analyzing samples from 50 different normal healthy adults never exposed to LY2062430. The same samples were also analyzed in the presence of excess (20 μg/mL final concentration) Aβ1–40. Each sample was analyzed ±Aβ1–40 to generate a single result over two assays by two analysts. Patient samples that have an antibody concentration above the assigned cut-point concentration were classified as 'putative positive.' Analysis of samples in the presence of excess Aβ1–40 (or analog) provided evidence regarding the specificity of a 'putative positive' response observed in the absence of excess Aβ1–40.

3. Results

3.1. Standard curve and calibration model assessment

The standards used in this validation were prepared from murine monoclonal anti-human Aβ antibodies (an equimolar mixture of antibody clones 3D6 and 2G3). A 10 μg/mL standard stock

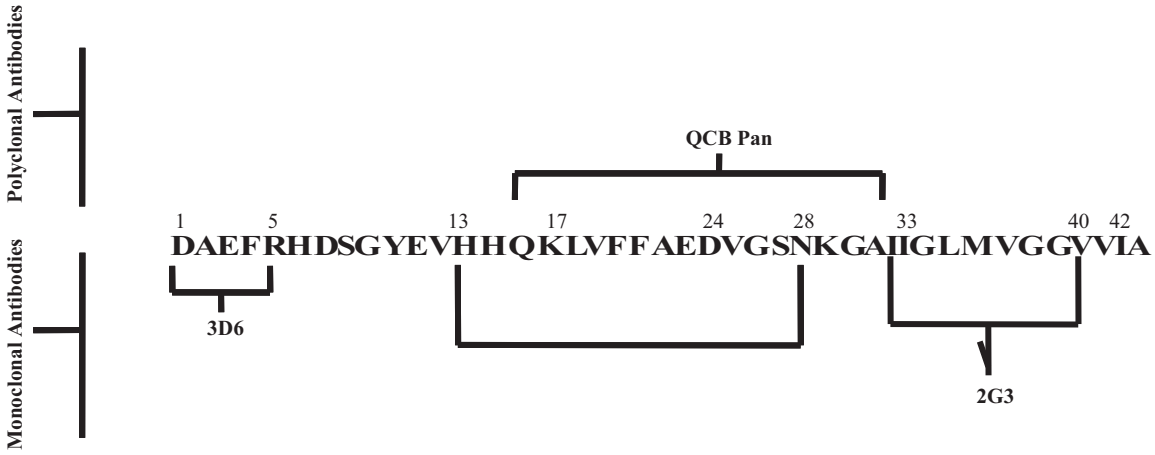

Fig. 1. Aβ 1–42 peptide sequence and antibody epitope map. Binding regions for the various antibodies are diagramed. As shown, the therapeutic antibody binds to the midregion whereas the monoclonal antibodies used as standards and controls bind to the termini of the Aβ peptide.

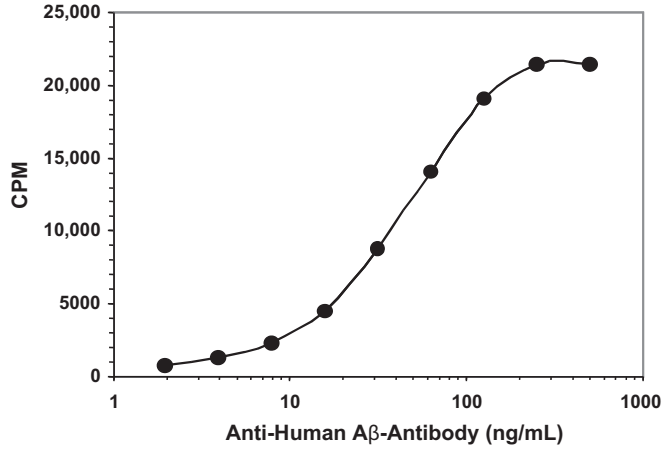

Fig. 2. Representative standard curve. An equimolar mixture of clones 3D6 and 2G3 were prepared at concentrations ranging from 500 to 1.96 ng/mL and incubated overnight with radiolabeled Aβ F19A analog tracer. Antibody bound tracer was precipitated with polyethylene glycol (PEG) and counted in a gamma counter to generate a standard curve. *Abbreviations*: Aβ, amyloid beta peptide; CPM, counts per minute; F19A, single amino acid substitution of alanine for phenylalanine at position 19.

solution (10×) was prepared, pipetted into aliquots, and then stored at approximately −70 °C. For each validation run, the stock standard was removed from the freezer, thawed at ambient temperature (20–25 °C), and serially diluted in assay buffer to 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91, and 1.96 ng/mL. The standard curve was supplemented with normal human serum at the time of assay. Each standard curve concentration was assayed in duplicate. The fitted standard curves were used to calculate the concentration of all validation samples by interpolation. Calibrators encompassing the validated range displayed −1.53% to 0.98% relative error (RE) and 2.49–5.14% coefficient of variation (CV). For calibrators within the validated range of 5–50 ng/mL, the percentage RE (%RE) and the percentage CV (%CV) of the back-fitted concentrations were within the acceptance criteria. A representative standard curve is presented in Fig. 2.

3.2. Cross-reactivity assessment

The markedly decreased affinity of LY2062430 for the radiolabeled form of the Aβ1–40 F19A analog was confirmed in experiments where tracer binding remained at or near background levels, even at antibody concentrations as high as 1 mg/mL (Fig. 3a). In contrast, as shown in Fig. 3b, Aβ1–40 F19A peptide binding by 3D6 and 2G3, murine monoclonal anti-human Aβ antibody clones specific for the N- and C-termini of the Aβ1–40 molecule, respectively, was not different from that seen with native Aβ1–40. These results suggested that the F19A modification did not to affect binding to distal epitopes in Aβ1–40. Moreover, a polyclonal rabbit antibody raised against peptides derived from the middle region of Aβ1–40 (residues 15–30) showed only a partial reduction in binding to the Aβ1–40 F19A peptide, as shown in Fig. 3c, suggesting that the F19A substitution causes relatively subtle changes in the overall structure and antigenicity of the peptide compared to native Aβ1–40, and that the assay will detect most, if not all, patient antibodies to native Aβ1–40 peptides.

3.3. Assay accuracy, precision, and working range (LLOQ/ULOQ)

Murine monoclonal anti-human Aβ antibody (an equimolar mixture of antibody from clones 3D6 and 2G3) were prepared in serum from normal healthy adults (negative serum base pool) to assess accuracy and precision as described in Section 2. Validation samples were prepared, pipetted into aliquots and then immediately frozen at approximately −70 °C. These samples were examined across six assay runs over several days, yielding 18 total reportable results (three per concentration). Accuracy (mean bias) was determined in serum by comparing the measured analyte concentration assayed to the nominal level. Precision (intra- and inter-assay) was determined by calculating the percentage coefficient of variation (%CV) between the reportable results generated for each validation sample concentration level within and between the six assays. A summary of the accuracy and precision data is shown in Table 1. At the concentrations of 5, 12.5, and 50 ng/mL, the mean bias expressed as percentage relative error (%RE) ranged from 2.7% to 6.0%. At these concentrations, the intra-assay precision (%CV) ranged from 4.2% to 9.3%, while the inter-assay precision (%CV) ranged from 7.6% to 13.9%. The total error ranges from 12.3% to 19.9%. These results were within the acceptance criteria. At the 100 and 150 ng/mL concentrations, the precision (%CV) and total error results were outside the acceptance criteria.

3.4. Dilutional linearity

Validation samples to evaluate dilutional linearity were prepared from a 10 μg/mL stock solution of murine monoclonal anti-human Aβ antibody (equimolar mixture of clones 3D6 and 2G3) prepared in assay buffer. The stock solution was pipetted

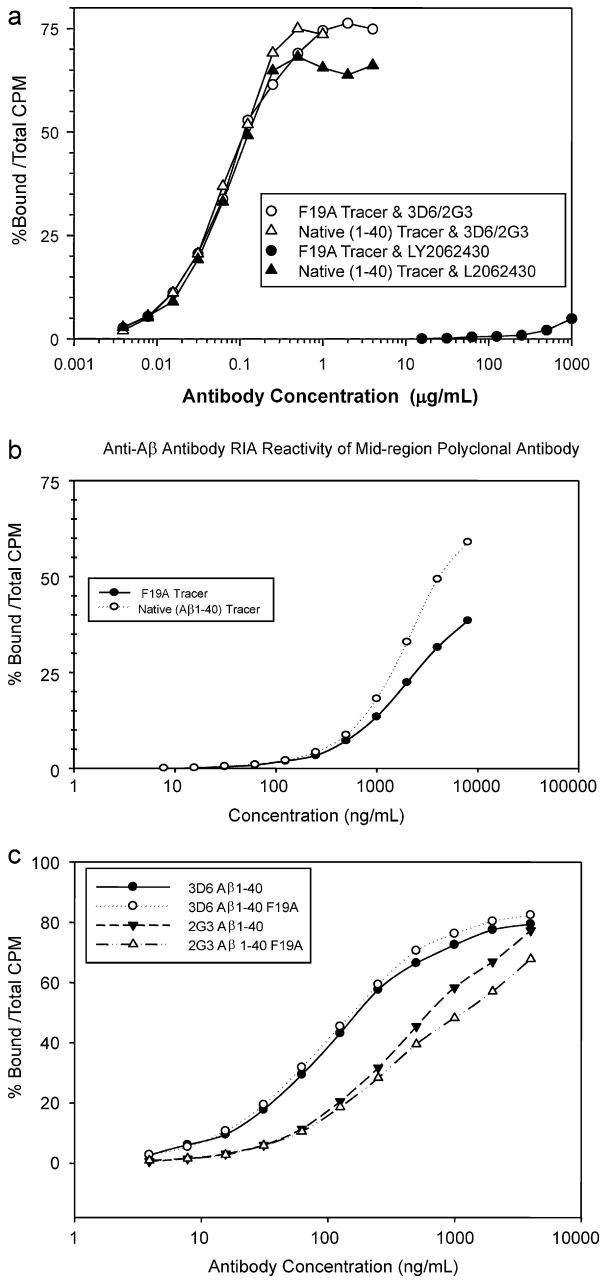

Fig. 3. Specificity assessment. (a) Anti-Aβ monoclonal antibodies were titered and tested for binding to the native Aβ1–40 and analog radioiodinated peptides. Data is percentage binding of total counts. The approximate 5–6 log shift in the EC50 for F19A tracer binding by the therapeutic antibody suggests that the RIA should permit detection of anti-human Aβ antibodies in the presence of pharmacological circulating concentrations of LY2062430. (b) Individual monoclonal anti-human Aβ antibody (clones 3D6 and 2G3) directed at the termini of the peptide display similar binding to the native and Aβ F19A tracers. (c) Anti-human Aβ antibody RIA reactivity of midregion polyclonal antibody. Aβ1–40 midregion-specific polyclonal binding to native and analog tracer. The Aβ F19A substitution causes a minor reduction in binding of these antisera. *Abbreviations*: F19A, single amino acid substitution of alanine for phenylalanine at position 19; Aβ1–40, human amyloid beta 1–40 peptide; CPM, counts per minute; F19A, single amino acid substitution of alanine for phenylalanine at position 19; RIA, radioimmunassay.

into aliquots and then immediately frozen at approximately −70 °C until used in the validation assay. For each validation run, the validation samples were removed from the freezer, thawed at ambient temperature (20–25 °C) and diluted appropriately in normal human serum. Diluted validation samples were analyzed three times in duplicate in two assays (i.e., six reportable results) performed by a single analyst over 2 days. After correcting for dilution,

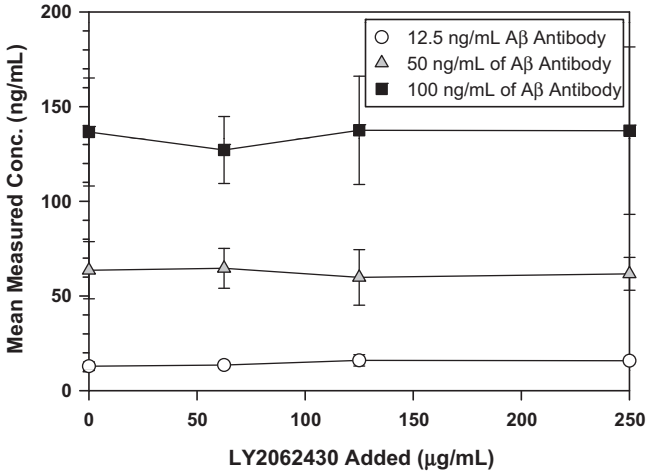

Fig. 4. Anti-therapeutic radioimmunoassay (RIA) interference assessment. Increasing concentrations of LY2062430 were added to the controls containing 12.5, 50, or 100 ng/mL of monoclonal control antibody. Graphed is the mean and standard deviation from three reportable results. The assay exhibits good tolerance to increasing concentrations of LY2062430. *Abbreviations*: Aβ, amyloid beta; Conc., concentration.

the %RE for each dilution series and the overall %CV of the cumulative back-calculated concentrations were determined. The %RE was −6.6% for the 1:100 dilution and 5.8% for the 1:150 dilution. The overall %CV of the cumulative back-calculated concentrations was 7.8%. These values are within the acceptance criteria. Although the measured concentrations are above the 50 ng/mL ULOQ, they are at or below the 100 ng/mL concentration of the next validation sample above the ULOQ. The %CV, which was above the acceptance criteria for the 100 ng/mL validation sample, was <6% for both dilution series. In spite of the fact that the measured concentrations are above the ULOQ, the recovery and precision data demonstrate that dilution of these samples is valid.

3.5. Interference from LY2062430

The presence of LY2062430 in patient samples has the potential to interfere with the detection of anti-human Aβ antibodies in the assay method. This was investigated by supplementing the validation samples (12.5, 50, and 100 ng/mL of murine monoclonal anti-human Aβ antibody) in assays with LY2062430 added to a concentration of 0, 62.5, 125, and 250 μg/mL. Each sample dilution was analyzed three times in duplicate (i.e., three reportable results) across each concentration level in a single assay. The degree of interference by LY2062430 was assessed relative to the concentration of the unspiked control sample. A graph of the data is presented in Fig. 4. As shown, concentrations of LY2062430 as high as 250 μg/mL do not interfere with the assay method.

3.6. Stability

3.6.1. Freeze–thaw stability

Freeze/thaw stability was determined using validation samples containing 12.5, 50, and 100 ng/mL of murine monoclonal anti-human Aβ antibody were freshly prepared and pipetted into aliquots and stored at approximately −70 °C. One group of validation samples was thawed and refrozen once, another group twice, and the third three times. The final thaw of each group was timed to occur on the same day. The samples from the three groups were assayed simultaneously in a single assay, along with a set of three reference aliquots which had not gone through a freeze/thaw cycling routine, to generate three reportable results per control per condition. Prior to thawing, samples had been stored frozen for at

least 12 h at ≤−70 °C. For each concentration, the percentage difference from reference was within the acceptance criteria for all three time points. This demonstrates sample stability through at least three freeze/thaw cycles.

3.6.2. Short-term stability

Short-term stability was determined using 12.5, 50, and 100 ng/mL of murine monoclonal anti-human Aβ antibody validation samples. Stability was assessed following the incubation of samples at ambient temperature (20–25 °C) and 2–8 °C for approximately 24, 48, and 72 h. All samples were assayed three times in duplicate in a single assay. The percentage difference from reference was calculated for each sample. The reference value is defined as the mean of nine reportable results from three separate analytical runs of each sample concentration, which have been removed from −70 °C and analyzed after 12 hours and within 1 week of preparation. At both 2–8 °C and 20–25 °C, the percentage difference from reference for all concentrations was within the acceptance criteria at all three time points. These data demonstrate stability of these samples at both 2–8 °C and 20–25 °C for at least 72 h.

3.7. Cut point

Establishment of the range of anti-human Aβ antibody concentrations in the serum of healthy individuals and assignment of the assay 'cut point' for reporting antibody positive results was determined by analyzing 48 normal human serum samples across four assay runs performed by two analysts over 4 days. Each sample was analyzed with and without excess Aβ1–40 to assess the specificity of positive results. The distribution of the anti-human Aβ antibody responses were evaluated statistically to estimate a cut point value for reporting antibody samples as "putative positives." Cut point was assessed by the analysis of 48 normal human serum samples and estimated statistically to be 1.092 percentage bound/total (B/T). This allowed assignment of a cut point of 5 ng/mL. Samples above the assigned cut point and demonstrate at least 50% inhibition in the presence of 1 μg/mL of Aβ1–40 were classified as 'true positives.'

4. Discussion

Several ELISA formats are currently being utilized to detect antibodies to therapeutic proteins. Some assay formats include an up-front acid disruption and/or an acid elution to enable detection of anti-therapeutic antibodies in the presence of the therapeutic antibody [13–16]. This RIA, which takes advantage of a single amino acid substituted analog peptide, was also adapted to an ELISA format. Synthesizing the F19A analog peptide with a biotin molecule added to the N- or C-terminal end of the peptide allowed this assay to be converted to a non-radioactive 96-well plate format.

The existence of pre-existing anti-human Aβ antibodies has been demonstrated in normal samples as well as in samples from patients with Alzheimer's disease [9]. Both anti- human Aβ antibodies and Aβ-reactive peripheral blood lymphocytes can be detected in healthy individuals, and this reactivity increases in individuals over 65 years of age. In contrast, patients with Alzheimer's disease show a marked decrease in Aβ-reactive peripheral blood lymphocytes which may suggest T-cell tolerance to Aβ [17]. Data have been published demonstrating both a reduction in both serum and cerebrospinal fluid anti-human Aβ titers in patients with Alzheimer's disease compared to healthy elderly controls [7]. Conflicting data support an increase in Aβ-reactive antibodies in patients with Alzheimer's disease [9,18]. Continued study is needed to understand this discrepancy and to determine if data generated in mouse models of Alzheimer's disease showing that certain epitope-specificities or isotypes of anti-human Aβ antibodies can be advantageous [19,20] and if beneficial Aβ-specific T-cell-mediated immune response can be elicited.

5. Conclusion

In summary, this assay tests for the presence of anti-peptide antibodies in the presence of a therapeutic monoclonal antibody directed at the peptide. Sensitivity, accuracy, precision, and dilutional linearity were found to be acceptable. The minimal single amino acid substitution will likely not decrease the reactivity of the analog to most autoantibodies. The data presented support the conclusion that this assay is valid for the quantification of antibodies against human Aβ1–40 in human serum with a range from 5 to 50 ng/mL. Although somewhat hindered by its use of a radiolabeled peptide that must be remade every 2 months, this solution-phase direct-binding RIA, provides a sensitive assay with low background that is relatively easy to perform. This approach of creating an amino acid substituted analog peptide could be utilized to detect autoantibodies to other target proteins in the presence of a therapeutic antibody directed at the particular target. It would be possible to apply this strategy in situations where the endogenous target is small enough for peptide synthesis (i.e., <50 aa) and there is specific information about the binding epitope of the therapeutic antibody.

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