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Development and validation of immunoassays to quantify the half-antibody exchange of an IgG4 antibody, natalizumab (Tysabri®) with endogenous IgG4

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ABSTRACT

Natalizumab is a humanized IgG4 monoclonal antibody which binds human α4 integrin and is approved for treatment of multiple sclerosis and Crohn's disease. Assessment of the in vivo disposition of natalizumab presents a unique assay development challenge due to the ability of human IgG4 antibodies to undergo half-antibody exchange in vivo. Such exchange generates IgG4 molecules of mixed specificity comprising a natalizumab heavy–light chain pair coupled to an IgG4 heavy–light chain pair of unknown specificity. Since exchanged and non-exchanged species cannot be quantified independently using a single enzyme linked immunosorbent assay (ELISA), a novel quantitation strategy was developed employing two ELISAs: one measuring total natalizumab including both intact and exchanged molecules, and the second measuring only intact natalizumab. The presence and amount of exchanged natalizumab in serum is calculated by the difference in values obtained in the two assays. To evaluate assay performance, a control reagent was created from natalizumab and an irrelevant humanized monoclonal IgG4 antibody. Subsequent validation demonstrated that both assays are specific, accurate, and precise within the working ranges of the assays (1.5–10 μg/mL for total and 0.5–12 μg/mL for intact natalizumab assays). The mean accuracy, intra- and inter-assay precision for both assays were 82–113%, ≤9% and ≤20%, respectively. Additionally, the limits of detection of intact and exchanged natalizumab were established using statistical methods. The utility of the two-assay strategy was confirmed by analyzing samples from a pharmacokinetic study in rats using different variants of natalizumab administered along with another human IgG4 antibody as an exchange partner.

1. Introduction

Natalizumab (Tysabri®) is a recombinant, humanized anti-human α4 integrin monoclonal antibody constructed utilizing a human IgG4 constant domain. The IgG4 subtype was originally chosen due to its reduced effector activity compared to the IgG1 antibody subtype, including lower binding to Fcγ receptors and C1q of the complement system [1]. It is now known that IgG4 antibodies undergo half-antibody exchange with other IgG4 antibodies in vivo, randomizing the Fab arms and making the population functionally monovalent toward a specific antigen [2–4]. Consequently these exchanged bispecific IgG4 antibodies are less able to create immune complexes, which can potentially reduce effector function activation and resultant tissue damage. This half antibody exchange is mediated by the unusual IgG4 hinge sequence rendering the disulfides linking the heavy chains especially susceptible to reduction [5,6]. Furthermore, the IgG4 CH3 domain forms less stable homodimers compared to IgG1 antibodies, also facilitating the exchange process [4]. One approach taken with therapeutic IgG4 antibodies to reduce the susceptibility to reduction and subsequent exchange is to mutate the hinge sequence (S228P), to more closely resemble the IgG1 hinge [7].

While IgG4-based antibody therapeutics are desirable in certain settings, they present an added challenge in the evaluation of their pharmacokinetics as the half-antibody exchange process creates a dynamically changing population of intact and exchanged molecules of different specificities. A number of methods have been utilized to investigate IgG4 half-antibody exchange. The initial methods exploited known IgG4 specificities of tested antibodies, such as response to two distinct allergens [8] and utilized a bridging radioimmune assay format. In this format one antigen was

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coupled to a solid phase and another antigen was radiolabeled, leading to detection of only antibodies bispecific toward these two antigens. In more recent work, in vivo exchange of selected monoclonal antibodies, including natalizumab, was demonstrated in human serum samples by capturing the antibody on cells expressing the target antigen and detecting associated lambda light chains that exchanged with the original natalizumab kappa light chain [3]. In the same study, liquid chromatography electrospray ionization mass spectrometry methods were used to demonstrate the existence of bispecific antibodies. These methods, although suitable for demonstrating the occurrence of half-antibody exchange, are not suited for quantitative measurements due to the qualitative set up of the assays and the absence of appropriate calibrators and controls.

Recently, Stubenrauch et al. [9] reported a strategy for measuring IgG4 exchange that employed two ELISAs, one that detected the total IgG4 antibody, and another that detected only bispecific antibodies. These assays, although presenting a valuable approach for detection of IgG4 half-antibody exchange, were developed as a research tool and were not optimized to assure accurate quantitative measurements of both intact and exchanged natalizumab species. Specifically, it was not demonstrated that the assay that measures total natalizumab was equally sensitive to both intact and exchanged species, which, as described in this article, is a crucial component of a two-assay strategy. Additionally, the assays were not validated using an appropriate bispecific antibody control to assure accuracy of measurement of exchanged IgG4 species, nor shown to be suitable for evaluating clinical serum samples.

In this report, we describe a validated method that uses two assays to quantify half-antibody exchange of natalizumab in serum. Essential elements of the assay development such as an appropriate bispecific antibody control and a natalizumab half-antibody control (without heavy chain disulfide bonds), as well as a statistical approach to determine working ranges of the assays are described.

2. Materials and methods

2.1. Reagents

Natalizumab reference standard and control IgG4 monoclonal antibody (mAb) referred to as mAbX were produced at Biogen Idec. Murine anti-natalizumab idiotype mAb 12C4 was generated at Elan Pharmaceuticals. The Fab of 12C4 was produced at Biogen Idec by papain digestion. The panel of natalizumab anti-idiotype Fabs was generated in collaboration with MorphoSys AG (Martinsried, Germany). Normal human serum and serum from multiple sclerosis patients were obtained from Bioreclamation, Inc. (Hicksville, NY). The remaining reagents were from commercial suppliers as described in subsequent sections or from Thermo Fisher Scientific (Rochester, NY).

2.2. Preparation of bispecific natalizumab/mAbX

Natalizumab and mAbX were mixed in a 10:1 molar ratio at a total concentration of 5 mg/mL in phosphate buffered saline (PBS, pH 7.4, 10 mM NaH_2PO_4, 150 mM NaCl) and the interchain disulfides were reduced by addition of DL-dithiothreitol (10 mM) for 1 h at ambient temperature. At the end of the incubation the pH of the solution was lowered by dilution with 5 volumes of sodium citrate (pH 3.5, 0.5 M), with NaCl (0.15 M) to facilitate half-antibody exchange. After 20 min at ambient temperature, the sample was neutralized by the addition of a 0.1 volume of HEPES buffer (pH 8.0, 1 M), and dialyzed into PBS. After dialysis, the antibody was re-oxidized by the addition of a mixture comprised of oxidized glutathione (2 mM) and reduced glutathione (0.1 mM), with incubation at room temperature for 24 h [10]. Subsequently, the sample was dialyzed into PBS and purified on Affi-Gel 10 (Biorad, Hercules, CA) coupled with the antigen ligand specific for mAbX. The column was washed with 11 column volumes of PBS and the bispecific antibody was eluted with ethylene glycol (50%) in CAPS buffer (pH 11.5, 0.1 M) into tubes containing 1/6 volume of MES buffer (pH 6.0, 1 M) for immediate neutralization, and then dialyzed into PBS. The bispecific antibody was further purified using size exclusion chromatography (SEC) on a Superdex 200 GL column (GE Healthcare, Piscataway, NJ) in HEPES buffer (pH 7.2, 0.05 M) with NaCl (0.15 M). The bispecific natalizumab/mAbX antibody was characterized by SDS-PAGE, analytical SEC on a BioSep S3000 column (Phenomenex, Torance, CA), and mass spectrometry (MS). For MS analysis, samples were treated with PNGase F under non-reducing conditions and analyzed by liquid chromatography (LC)–MS (LCT mass spectrometer, Waters, Milford, MA). The masses were deconvoluted using MaxEnt1 software (Waters, Milford, MA).

2.3. Preparation of natalizumab non-disulfide linked bivalent antibody

To prepare the non-disulfide linked bivalent form of natalizumab, in which the hinge cysteines are in intrachain disulfide linkage rather than the normal heavy to heavy interchain linkage, natalizumab was reduced with dithiothrietol (10 mM) for 1 h at ambient temperature and then dialyzed in PBS with 1% DMSO to promote re-oxidation. The non-disulfide linked form was separated from the disulfide linked natalizumab by SEC on a Superdex 200 column in sodium citrate buffer (pH 3.5, 25 mM) with NaCl (0.3 M). Under these acidic chromatographic conditions the non-disulfide linked antibody disassociates into half-antibody, 75 kDa heavy–light chain pairs, separable from the intact 150 kDa mAb. The separated half-antibody was then dialyzed into PBS to allow the heavy chains to non-covalently re-associate to re-form non-disulfide linked bivalent molecules as demonstrated by SEC analysis before testing in the ELISA assays.

2.4. Production of natalizumab IgG4 S228P hinge variant for rat pharmacokinetic study

Natalizumab IgG4 S228P mAb was expressed in Chinese hamster ovary cells as described previously [11] and purified by Protein A Sepharose and SEC on Superdex 200 using pyrogen-free solutions and depyrogenated chromatography resins. Clarified and filtered culture medium was loaded onto a Protein A Sepharose column, which was washed with PBS, and then eluted with sodium phosphate buffer (pH 2.8, 0.1 M) and neutralized by addition of 0.1 volume of HEPES buffer (pH 8.0, 1 M). After dialysis into PBS, the sample was concentrated and further purified by SEC on a Superdex 200 column. Endotoxin levels were tested using the Limulus amebocyte lysate endotoxin kit from Associates of Cape Cod (East Falmouth, MA) as per the manufacturer's instructions.

2.5. Total natalizumab assay

The total natalizumab assay measures molecules that have at least one heavy–light chain pair of natalizumab, including non-exchanged intact natalizumab, and exchanged bispecific natalizumab/IgG4 molecules. Nunc Maxisorp ELISA plates were coated with 5 μg/mL of a murine monoclonal anti-natalizumab idiotype monoclonal antibody (12C4) for 16–18 h at 2–8 °C, washed one time with PBS, 0.05% Tween 20, pH 7.2 (PBST) wash buffer, and subsequently blocked with PBST, 1% bovine serum albumin (BSA) for 1–4 h at ambient temperature. After removing the block buffer the standards, controls, and samples, diluted 1:50 in assay diluent (PBST, 1% BSA), were added to the plates in duplicate. Samples that

resulted in values above the assay limit of quantitation were pre-diluted in human serum prior to 1:50 dilution in assay diluent, and re-tested in the assay. The standard curve was prepared by spiking a concentration series of natalizumab into pooled normal human serum. Controls were made by spiking natalizumab and bispecific natalizumab/mAbX into the pooled normal human serum at different ratios. After a 2 h incubation at ambient temperature, the plates were washed with PBST wash buffer, and then a mixture of biotinylated mouse anti-human IgG4 (Southern Biotech, Birmingham, AL) and streptavidin conjugated to horseradish peroxidase (HRP) (Jackson Immunoresearch, West Grove, PA) was added to plates. Following another 30 min incubation plates were washed again, and the bound HRP conjugated complex was detected with one step tetramethylbenzidine (TMB) substrate (BioFX Laboratories, Owings Mills, MD). The substrate reaction was stopped with 1 N sulfuric acid and the level of color development determined by absorbance at 450 nm using a Spectromax Plus plate reader (Molecular Devices, Sunnyvale, CA). The concentration of antibody with at least one heavy–light chain pair of natalizumab was obtained by back-fitting the absorbances of the samples relative to the standard curve using the Molecular Devices SoftMax Pro 5-Parameter curve fit.

2.6. Intact natalizumab assay

The intact natalizumab ELISA measures only non-exchanged, monospecific bivalent intact natalizumab molecules utilizing a bridging assay format. Nunc Maxisorb ELISA plates were coated with human anti-natalizumab idiotype Fab (MorphoSys 03657) at 1 μg/mL for 16–18 h at 2–8 °C, washed with PBST and blocked with PBST, 1% BSA to minimize non-specific binding. After removing the block buffer, the same standards and controls as prepared for the total natalizumab assay were added to the plates in duplicate and incubated for 2 h at ambient temperature. The plates were then washed, and bound natalizumab was detected with the monoclonal anti-natalizumab antibody (12C4) at 1 μg/mL. Following the 30 min incubation at ambient temperature and subsequent washing, a goat anti-murine IgG Fcγ conjugated to HRP (Jackson Immunoresearch, West Grove, PA) was added and incubated for another 30 min at ambient temperature. The plates were processed and the concentration of intact natalizumab was determined as described in Section 2.5.

2.7. Validation of intact and total natalizumab assays

Validation for both total and intact natalizumab assays was performed as per industry guidelines [12,13] and included assessments of the following parameters: performance of standards and regression model, accuracy and precision, upper and lower limits of quantitation and assay range, dilutional linearity; selectivity, specificity, robustness and ruggedness, and reagent stability. In addition, the limit of detection of exchanged bispecific natalizumab/IgG4 antibody was established following the statistical analysis of the accuracy and precision data for quality control (QC) samples in both intact and total natalizumab assays. The detailed methods for validation of the assays are provided in Supplementary Information.

2.8. Rat pharmacokinetic study

The described animal experiments were approved by the Biogen Idec Institutional Animal Care and Use Committee. Six Sprague–Dawley rats, three per group (Charles River Breeding Laboratories, Wilmington, MA), were used in the pharmacokinetic study. Rats were given an intravenous bolus injection of the natalizumab wild type IgG4 or the natalizumab S228P hinge mutant at 1 mg/kg in PBS, with 10 mg/kg of mAbX as an exchange partner. Blood samples (0.25 ml) were collected into serum separator tubes at several timepoints: prior to injection, and then at 5 min, 6 h, 24 h, 48 h, 96 h, and 168 h following injection. Sample collection timepoints were guided by the pharmacokinetic profile of natalizumab observed in clinical studies. Serum was separated by centrifugation and stored at −70 °C. On the day of the assay, serum samples were thawed and analyzed in both total and intact natalizumab assays as described in Sections 2.5 and 2.6, respectively.

3. Results and discussion

3.1. Assay strategy

The strategy for measuring the concentration of exchanged bispecific natalizumab/IgG4 antibody in serum samples involved the development of two ELISAs (Fig. 1). The first ELISA (total natalizumab assay) was designed to measure molecules that have at least one heavy–light chain pair of natalizumab, including non-exchanged intact natalizumab, and exchanged bispecific natalizumab/IgG4 molecules. The second ELISA (intact natalizumab ELISA) was designed to measure only non-exchanged, monospecific bivalent intact natalizumab molecules utilizing a bridging assay format. The extent of natalizumab half-molecule exchange in serum samples is then calculated from the difference in the values obtained in the two assays. Both assays utilized anti-idiotype antibodies instead of the antigen (human α4 integrin) to capture natalizumab, as they were earlier demonstrated to provide specific detection of natalizumab without the complexity of producing human α4 integrin. Successful quantitation using the two-assay strategy required an equivalent, or close to equivalent dose–response relationship in the total natalizumab assay for all molecules having at least one heavy–light chain pair of natalizumab. In order to ensure the performance of the two-assay system, a control exchanged human IgG4 antibody was created. The control antibody comprising a heavy–light chain pair derived from natalizumab, and a heavy–light chain pair derived from another irrelevant humanized monoclonal IgG4 antibody, mAbX, was designed to mimic half-antibody exchanged natalizumab/IgG4 in serum. The exchanged antibody was used for preparation of QC samples for both assays by spiking it and intact natalizumab into normal human serum at different ratios and over a range of concentrations that would cover the expected concentrations of natalizumab in patients undergoing treatment. Additionally, in order to successfully compare results from the two assays and accurately calculate concentrations of exchanged natalizumab/IgG4, both assays were designed to have similar ranges, utilize the same preparation of standards and controls, and require the same minimal serum dilution.

3.2. Generation of bispecific reagents

Since IgG4 antibodies do not exchange in vitro in the absence of reducing agents [2], the exchanged bispecific natalizumab/mAbX antibody with the re-established disulfide bond was considered to be a suitable stable control reagent for the assays. The bispecific natalizumab/mAbX was prepared by mixing mAbX with excess natalizumab and reducing under acidic conditions to accelerate exchange, followed by re-oxidation. The exchanged bispecific antibody was purified by capture on an affinity column coupled with the mAbX ligand. The eluted fraction contained 99% natalizumab/mAbX bispecific exchanged antibody, and intact mAbX represented less then 1% of the total amount of the eluted antibody. The preparation was characterized by SDS-PAGE, analytical SEC, and mass spectrometry. SDS-PAGE evaluation confirmed that the bispecific antibody had a 1:1 ratio of light chains for natal-

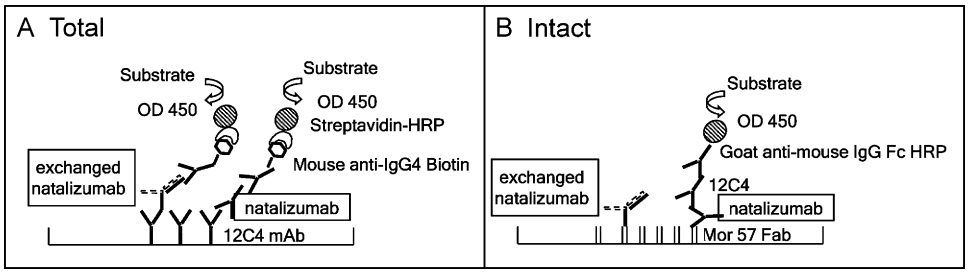

Fig. 1. Assays to detect total (A) and intact (B) natalizumab in serum.

izumab and mAbX (Fig. 2). Mass spectrometry revealed <2% each of intact mAbX and intact natalizumab present in the preparation (Fig. 2C) and analytical SEC demonstrated 3.2% dimer aggregate of antibody (data not shown). Since this amount of aggregate was found to produce an inappropriate signal in the intact natalizumab assay, the bispecific antibody preparation was further purified by SEC. The resulting final product had only 0.6% aggregate (Fig. 2B) and performed as desired in both assays (see below).

3.3. Development of total natalizumab assay

The total natalizumab assay was developed with intent to generate equivalent dose–responses for intact natalizumab, for exchanged bispecific natalizumab, and for mixtures of both at different concentrations when spiked into human sera. During initial assay development different capture and detection antibodies, their concentrations, and timing of the incubation steps were optimized and dose–responses of natalizumab and natalizumab/mAbX were compared in the assay. The concentration of the capture anti-natalizumab antibody was found to be crucial for the intended assay performance: lower concentrations of the capture antibody (0.5–2 μg/mL) produced natalizumab curves that are shifted to the left (better sensitivity) compared to that of the bispecific natalizumab/mAbX, while higher concentration of the capture antibody (5 μg/mL) resulted in reduced sensitivity for natalizumab, and in two overlapping dose–response curves as required for subsequent analyses (Fig. 3). The reason for this phenomenon is unknown, but may potentially be attributed to the concentration of the capture antibody on the plate increasing to the point that binding is no longer influenced by the difference in avidity between two-armed and one-armed molecules. As a result of the experiments, 5 μg/mL was selected to be an optimum concentration of the capture antibody albeit with an approximately 10-fold loss of sensitivity relative to the curve obtained with lower coating concentrations (Fig. 3D). Pre-mixing biotin labeled anti-IgG4 antibody and streptavidin HRP for detection further minimized the difference between natalizumab and natalizumab/mAbX curves and achieved better assay sensitivity than using anti-IgG4 antibody directly conjugated to HRP (data not shown).

Upon finalization of the assay format, a minimum required serum dilution was selected based on a comparison of dose–response curves generated in buffer containing human serum at concentrations from 0.1% to 10% (1:1000 to 1:10 dilutions). While evaluated serum dilutions resulted in similar background signals and curve shapes, the minimal required serum dilution was chosen to be 1:50 in consideration of the expected natalizumab concentration in patient serum samples. Also, based on the assay performance during development, the range of the assay was estimated to be between 0.5 μg/mL and 15 μg/mL (Fig. 4A) of natalizumab. The relatively limited concentration range of the assay was a result of targeting similar assay dose responses for both intact and half-antibody exchanged forms of natalizumab. However, since the concentration of natalizumab is approximately 5–20 μg/mL for patient samples drawn four weeks post-infusion of the drug, this sensitivity and assay range were deemed appropriate.

3.4. Development of intact natalizumab assay

The assay for intact natalizumab is a bridging ELISA format with anti-natalizumab idiotype-specific antibodies used for capture and detection. It was found that capture with an intact murine anti-natalizumab antibody resulted in high non-specific binding and reduced assay sensitivity (data not shown). Therefore, a panel of

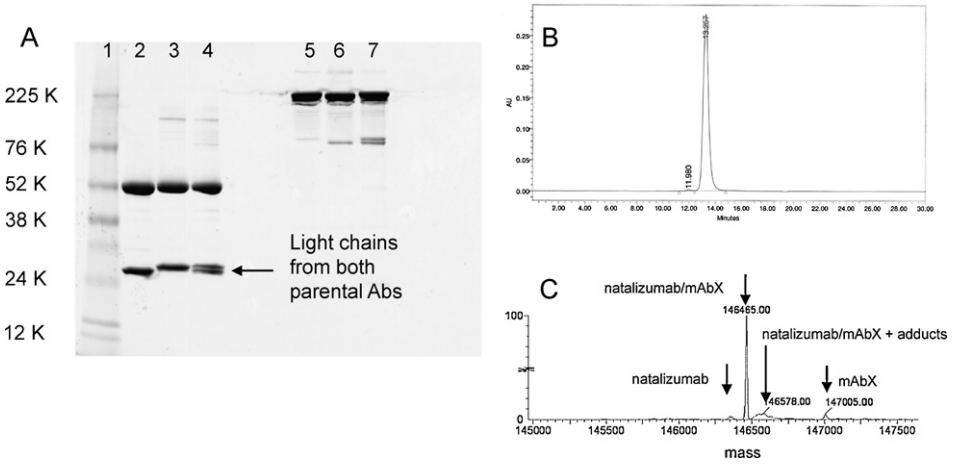

Fig. 2. Characterization of bispecific natalizumab/mAbX. (A) SDS-PAGE: lanes 2–4 reduced. Lanes 5–7 non-reduced. Lane 1: standards, lanes 2 and 5: natalizumab; lanes 3 and 6: mAbX; lanes 4 and 7: the bispecific natalizumab/mAbX, (B) analytical SEC and (C) LC–MS analysis.

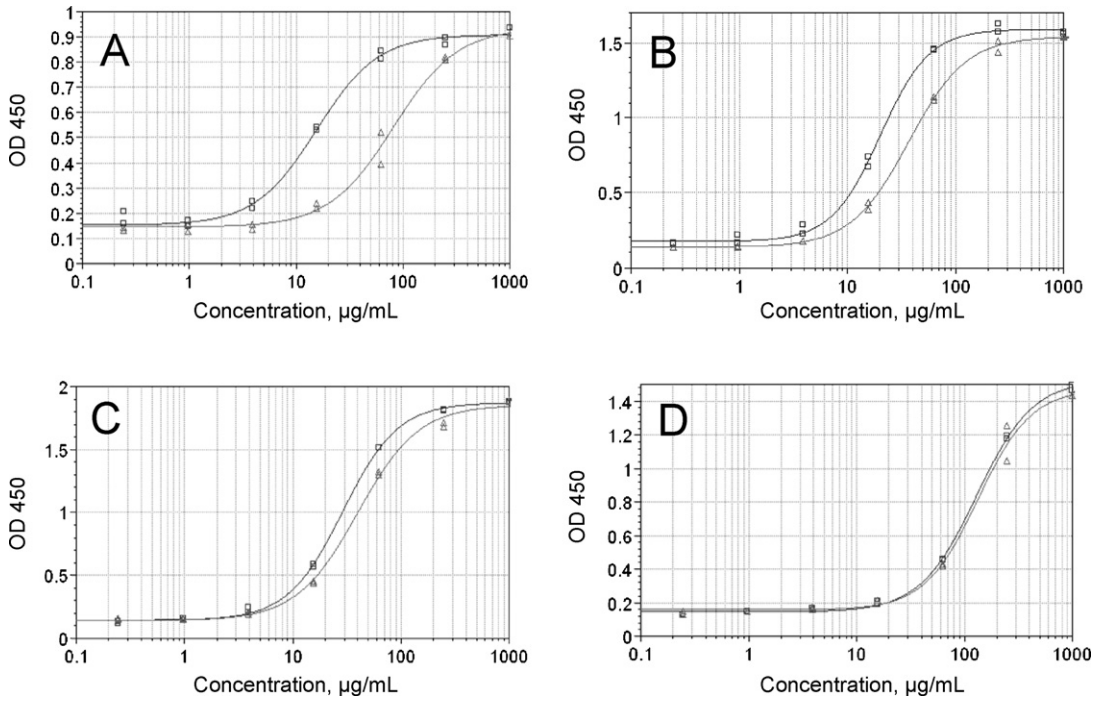

Fig. 3. Optimization of anti-natalizumab idiotype mAb coating concentration for the total natalizumab assay. (A) 0.3 μg/mL, (B) 1 μg/mL, (C) 2 μg/mL and (D) 5 μg/mL. Curves with square data points represent natalizumab standards and curves with triangle data points represent the natalizumab/mAbX bispecific antibody standards.

anti-natalizumab Fab fragments was screened to select the best pair for achieving desired sensitivity and specificity, including minimal binding to mAbX and endogenous serum IgG4. Calibration curves of natalizumab, natalizumab/mAbX, mAbX, and human IgG4 were evaluated in assays that utilized all these available Fabs as capture antibodies with 12C4 anti-natalizumab mAb as a primary detection antibody, and the goat anti-murine IgG Fcγ conjugated to HRP as a secondary antibody. None of the evaluated capture antibody Fabs cross-reacted with mAbX or polyclonal IgG4; however the sensitivity of natalizumab detection varied greatly with C values (midpoint of the curve) ranging from 8 to 122 μg/mL (data not shown). MorphoSys 03657 and 12C4 Fab possessed the best sensitivity. Because MorphoSys 03657 Fab did not require additional preparation by papain digestion, it was chosen for further assay development. Using these reagents, concentrations of all the assay components were optimized by standard checkerboard titration. A minimum required serum dilution of 1:50 was selected after comparing dose–response curves generated in buffer containing varying concentrations of serum, and matching the serum dilutions for both assays. The working range of the intact natalizumab assay was 0.5–15 μg/mL (Fig. 4B), which was appropriate for measuring natalizumab concentrations in serum samples taken from patients at four weeks following administration of the drug. The exact ranges of the assays were established during the assay validation described in Section 3.7.

3.5. Quality controls for both total and intact natalizumab assays

In both total and intact natalizumab assays, natalizumab was used to generate a standard curve. Controls were made for both assays by spiking pooled normal human serum with intact natalizumab and natalizumab/mAbX into serum at ten ratios ranging from 100% to 25% of natalizumab with five levels (1.5, 3, 6, 9 and 12 μg/mL) of the total natalizumab concentrations (Tables 1 and 2). The extensive set of controls was necessary to fully evaluate performances of both assays, and the data demonstrated that the two assays are able to accurately detect total and intact natalizumab and to accurately infer the concentration of half-antibody exchanged natalizumab in serum (see Fig. 5 for an example of response of spiked samples at 3 μg/mL of total natalizumab).

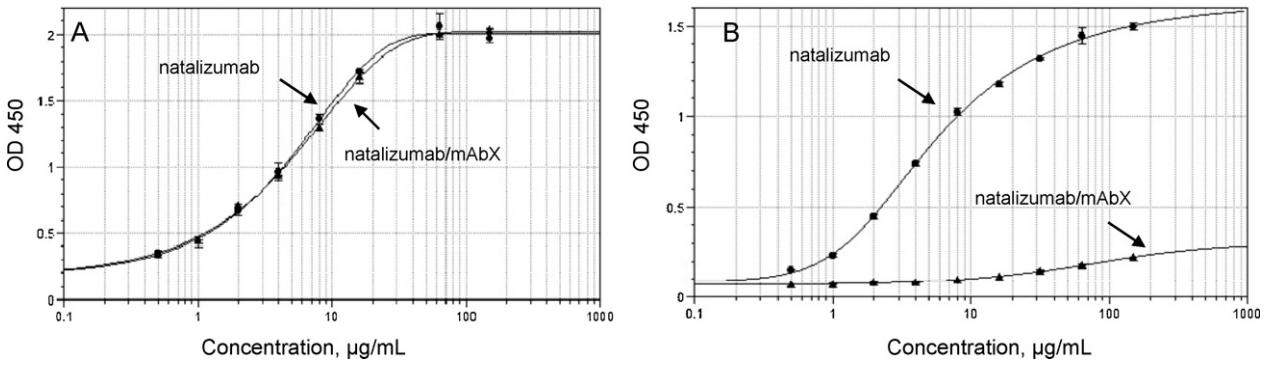

Fig. 4. Example of final standard curves from total (A) and intact (B) natalizumab assays. Curves with solid circles represent natalizumab, curves with solid triangles represent natalizumab/mAbX.

Table 1
Accuracy and precision of total natalizumab assay, example of 6 μg/mL QC samples.

	% Intact natalizumab									
	100	90	85	80	75	70	65	60	50	25
# Of runs	31	10	10	10	31	10	10	10	10	31
Intra-assay precision, %	6.7	6.4	8.7	6.5	10.1	11.5	8.1	6.5	7.2	7.4
Inter-assay precision, %	2.3	2.3	3.2	2.4	2.6	1.7	2.0	3.3	2.1	1.8
Accuracy (% nominal)	104.7	102.8	103.6	103.2	101.8	101.4	98.9	96.9	93.1	88.3

Table 2
Accuracy and precision of intact natalizumab assay, example of 6 μg/mL QC samples.

	% Intact natalizumab									
	100	90	85	80	75	70	65	60	50	25
# Of runs	30	10	10	10	30	10	10	10	10	30
Intra-assay precision, %	4.1	3.2	2.1	2.1	2.6	1.4	6.5	1.7	1.3	2.7
Inter-assay precision, %	9.8	7.3	7.3	10.1	7.2	6.7	9.4	4.4	5.2	8.8
Accuracy (% nominal)	113.1	106.3	107.3	104.4	104.0	101.7	98.7	101.2	95.5	99.1

3.6. Detection of non-disulfide linked IgG4 in the ELISA assays and in human serum

The reduction of the hinge disulfides of IgG4 antibodies is a necessary step in the exchange reaction, but the level of non-disulfide linked IgG4 in circulation has not heretofore been reported. Thus we investigated how well experimentally non-disulfide linked natalizumab would be detected in the two assays. The non-disulfide linked natalizumab gave approximately 65% and 33% of the expected responses in the total and intact natalizumab assays, respectively, suggesting that this form dissociates to some extent to half-molecules under assay conditions. This necessitated an additional experiment to determine whether non-disulfide linked IgG4 is present in serum at sufficiently high concentrations to cause under reporting of the true concentration of natalizumab in the assays. To do this, serum samples from healthy donors were run on non-reducing SDS-PAGE and blotted with anti-IgG4 antibody. Under these conditions, non-disulfide linked IgG4 runs as half-antibody (75 kDa) and is separated from disulfide linked antibody which runs at 150 kDa. As shown in Fig. 6, greater than 95% of the IgG4 antibody in serum has the intact disulfide structure indicating that non-disulfide linked IgG4 is present in human serum only at low levels (<5%), and apparently exists only transiently to allow IgG4 heavy chain exchange. Therefore the lower detection of this form of IgG4 in the assays should have minimal impact on the quantitation of natalizumab half-antibody exchange using the developed two-assay strategy.

3.7. Validation of the total and intact natalizumab assays

The results of the validation experiments demonstrated that the performance of both total and intact natalizumab assays is accurate, precise and specific (as per recommended acceptance criteria for ligand binding assays for macromolecules [12]) in human serum within the determined ranges of the assays. A five-parameter curve-fit model was shown to be acceptable for both assays, with the overall mean of back-calculated concentrations for standards within the assay range between 95% and 113% of the nominal concentrations, and the mean precision (% coefficient of variation (CV)) ≤11%.

The ranges of the assays were established using accuracy and precision characteristics of the numerous QC samples containing natalizumab and natalizumab/mAbX at ten different ratios for five concentrations of total natalizumab (see Section 3.5). Both total and intact natalizumab assays, when used independently, were demonstrated to have working ranges between 0.5 μg/mL and 12 μg/mL. Additionally, in order to ensure the ability of the two-assay strategy to accurately measure the concentration of the exchanged

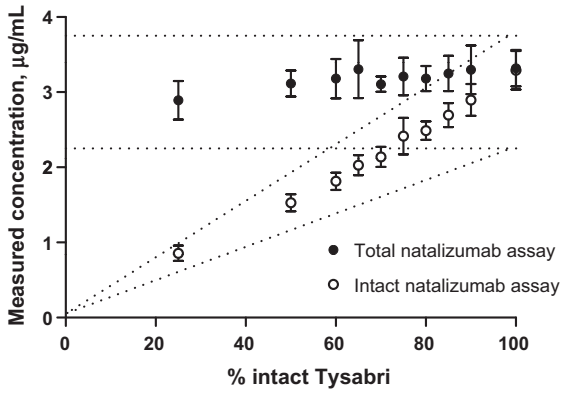

Fig. 5. Example of performance of quality control (QC) samples prepared by spiking natalizumab and natalizumab/mAbX into human serum at different ratios (total concentration 3 μg/mL). Data plotted as mean and standard deviation. Solid circles represent total natalizumab assay, empty circles represent intact natalizumab assay. Dotted lines represent 25% acceptance intervals for accuracy of the assays.

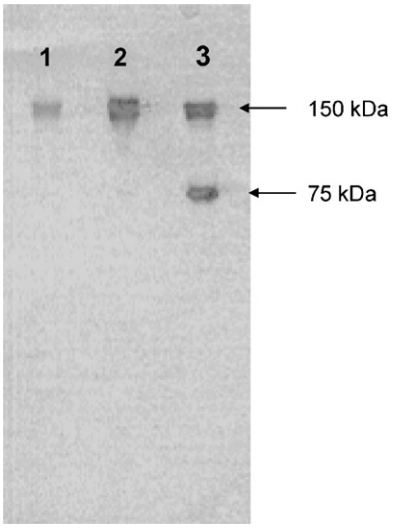

Fig. 6. Western blot of normal human serum detected with anti-human IgG4 antibody. Lane 1: normal human serum; lane 2: normal human serum spiked with natalizumab and lane 3: normal human serum with natalizumab half-antibody.

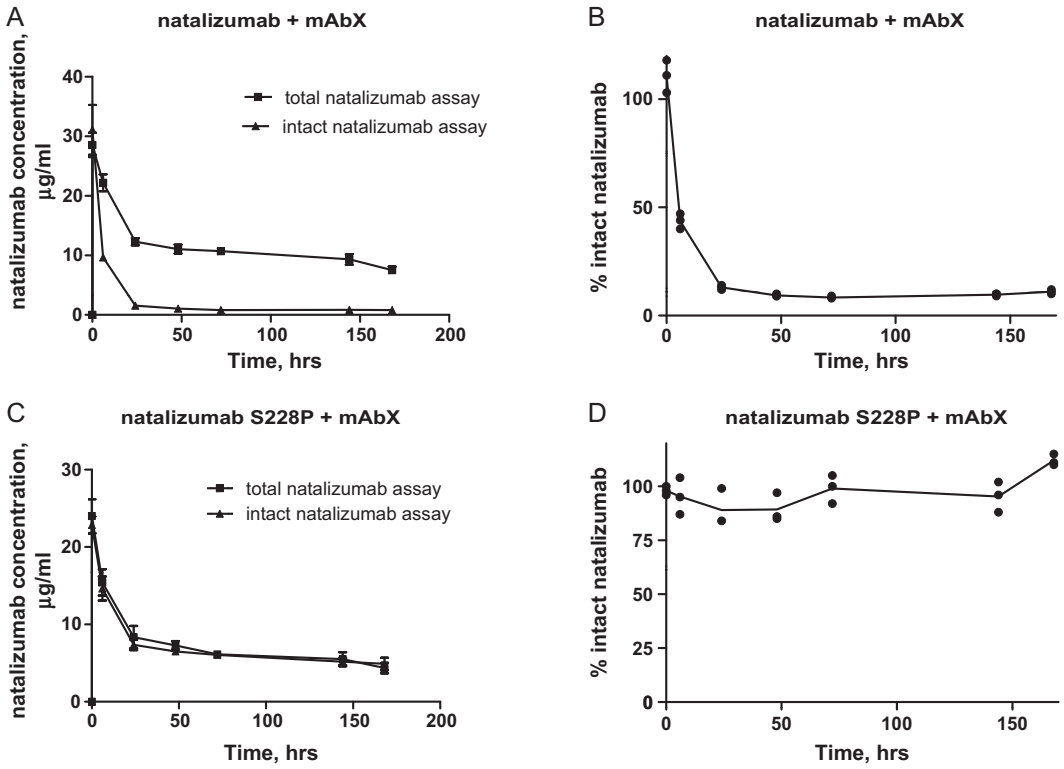

Fig. 7. Pharmacokinetic profile of natalizumab (A and B) and natalizumab P228S variant (C and D) when co-administered with mAbX to Sprague–Dawley rats. (A) Serum concentration of natalizumab in total and intact natalizumab assays (median and standard deviation, $n = 3$). (B) % Intact natalizumab in rat serum (median and individual values, $n = 3$). (C) Serum concentration of natalizumab S228P in total and intact natalizumab assays (median and standard deviation, $n = 3$). (D) % Intact natalizumab S228P in rat serum natalizumab (median and individual values, $n = 3$).

natalizumab/IgG4, the assay ranges were evaluated using statistical methods (see Supplementary Information), and restricted the working ranges for the total natalizumab assay to 1.5–10 μg/mL and for the intact assay to 0.5–12 μg/mL. In addition, the limit of detection of exchanged natalizumab/IgG4 was described as a function of the results obtained in the total natalizumab assay and the ratio of the pre-dilution factors used in the intact and total natalizumab assays (see Supplementary Table 1). The pre-dilution factors are additional dilutions (prior to 1:50 dilutions in the assay buffer) performed for samples with the natalizumab concentration above the limit of quantitation of the assays.

Accuracy and precision were evaluated by the performance of QC samples at five levels: 1.5, 3, 6, 9 and 12 μg/mL. The mean accuracy from all the runs performed for the total natalizumab assays was between 82% and 111%, while for the intact natalizumab assay was between 98% and 113%. The intra-assay precision (repeatability) and inter-assay precision (intermediate precision) for the total natalizumab assay were ≤6% and ≤17%, and for the intact natalizumab assay were ≤9% and ≤20% (see Tables 1 and 2 for examples of the precision and accuracy of the assays at 6 μg/mL of total natalizumab). Dilutional linearity for the assays was also demonstrated using three spiked samples prepared in pooled human sera with different ratios of intact natalizumab and natalizumab/mAbX: 100:0, 75:25 and 25:75 (total natalizumab concentration of 200 μg/mL). For more than 94% of the samples diluted into the range of the assays, recoveries of natalizumab and natalizumab/mAbX were within 100 ± 25% of the respective nominal values with %CV ≤ 25%. All samples diluted to yield the levels of natalizumab or natalizumab/mAbX above the assay's upper limit of quantitation resulted in values above the range of the assays and demonstrated the absence of a "hook effect" in both assays (which would have been manifested as lower signals at high concentrations). Additionally, the assays were shown to possess excellent specificity and selectivity, including no observed matrix interference. Natalizumab and natalizumab/mAbX spiked into individual lots of human sera demonstrated recoveries within 100 ± 25% of the respective nominal values, and precision ≤25% for both the total and intact natalizumab assays. Neither assay demonstrated interference by another human IgG4 antibody (mAbX) which provided additional evidence of specificity. Finally, both total and intact natalizumab assays were demonstrated to be robust and rugged as they were shown to be tolerant toward incubation time differences, performed consistently well when run by different analysts, and when different equipment was used. Short term stability was demonstrated for assay controls subjected to three freeze/thaw cycles when stored at −70 °C, for 6 weeks when stored at 2–8 °C, and for 27 h when stored at ambient temperature. Coated plates were shown to be stable for eight days when stored at 2–8 °C. In addition, the natalizumab/mAbX stock solution used to make controls was shown to remain free of aggregates when monitored by analytical SEC over the two year assay development period.

3.8. Proof of concept: in vivo natalizumab exchange and its dependence on structure

To confirm the utility of the two-assay strategy for evaluation of natalizumab half-antibody exchange, 1 mg/kg natalizumab or a natalizumab variant with the S228P hinge mutation were co-administered with 10 mg/kg of an irrelevant human IgG4 antibody (mAbX) to Sprague–Dawley rats. Analysis of serum samples from rats given natalizumab clearly revealed half-antibody exchange in vivo. Fig. 7A shows a time course of the measured concentrations for both total and intact natalizumab while Fig. 7B shows the percent of remaining intact natalizumab. At 6 h, 44% of the natalizumab remained intact while only 13% remained intact by 24 h following administration. In contrast, serum samples from rats given natal-

izumab S228P "fixed hinge" mutant did not exhibit the presence of half-antibody exchanged species (Fig. 7C and D). These data confirmed the ability of the developed assays to measure half-antibody exchange of natalizumab in serum.

4. Conclusions

A novel two assay strategy was developed and validated to accurately measure the concentration of the half-antibody exchange of natalizumab, an IgG4 antibody, with endogenous IgG4. The intact natalizumab ELISA measures only non-exchanged (intact, monospecific bivalent) natalizumab molecules, while the total natalizumab ELISA equally measures molecules comprised of at least one heavy–light chain pair of natalizumab, including both intact bivalent monospecific natalizumab and exchanged bispecific natalizumab/IgG4 molecules. The amount of exchanged natalizumab/IgG4 in serum is calculated by comparing values obtained in both assays. The assays utilize a set of elaborate controls consisting of an exchanged bispecific natalizumab/mAbX human IgG4 antibody and intact natalizumab spiked into serum at different ratios representing a range of anticipated in vivo concentration ratios for these molecules. The two ELISAs utilize the same minimal serum dilution and standard and control preparations, and have similar assay ranges to assure accurate quantitation of the exchanged natalizumab/IgG4 in serum. The validation of the assays, including statistical analyses, demonstrated their accurate, precise and specific performance and established their accuracy and limits of detection of half-molecule exchanged natalizumab/mAbX. The ability to accurately measure the concentrations of the major forms of natalizumab (intact bivalent monospecific and half antibody exchanged bispecific) in human serum using this two-assay system has enabled the full assessment of the in vivo disposition of natalizumab. Although the recent confirmation of half-antibody exchange between natalizumab and native IgG4 is interesting, there is no data suggesting that this phenomenon has an impact on the clinical effects of natalizumab. We have, however, used this assay to assess samples from multiple sclerosis patients and the data showing the time course and extent of natalizumab half-antibody exchange in serum collected from these patients is the subject of a planned future publication.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2011.01.006.

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