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A kinetic enzyme immunoassay for the quantitation of antibodies to a humanized monoclonal antibody in human serum

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Abstract

A kinetic enzyme immunoassay was developed and validated to quantitate human antibodies to the humanized monoclonal antibody CAMPATH[1]-1H (C1H) in human serum. The assay was configured using C1H-coated 96-well plates which were blocked with bovine serum albumin, and incubated with dilutions of human serum containing anti-C1H antibody. Antibody was detected using biotinylated C1H followed by streptavidin-conjugated alkaline phosphatase and *p*-nitrophenyl phosphate. Absorbance data were collected for 10 min, and mOD min^{-1} data were exported to MultiCalc data analysis software. A 4-parameter logistic-log algorithm was shown to model the data through the range of the standard curve within 15% of nominal values. The overall assay performance coefficient of variation by ANOVA was 9.2%. The lower limit of detection was defined at 160 Units ml^{-1}. The anti-idiotype antibody standard stock solution is stable at 4°C and at −80°C for at least 11 months in buffer. The anti-idiotype antibody controls are stable for at least seven freeze–thaw cycles and at least 6 months in human serum stored at −20°C. A strategy was devised by which to establish the specific antibody potency for any given batch of anti-C1H antibody standard relative to the Reference Standard. This EIA has been used to quantify and characterize anti-C1H antibody in human serum in support of clinical safety and efficacy studies.

Keywords: Kinetic enzyme immunoassay; Humanized monoclonal antibody; C1H antibody; Immunogenicity; Human serum

1. Introduction

Antibodies against tumor target cell phenotypes have been exploited as therapeutic agents over the last 15 or more years, but an overriding problem which has limited their success has been the inherent immunogenicity of murine and chimeric anti-

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[1] C1H is a trademark of Wellcome group companies. Registered in U.S. Patent and Trademark Office.

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bodies. This area has recently been reviewed by Khazaeli [1]. To address these concerns, antibodies having the desired specificity have been 'humanized', i.e. genetically re-engineered to carry the same antigen recognition sequences on a human-like immunoglobulin framework, in the hope of eliminating immune responses to the therapeutic protein and improving pharmacokinetic properties [2]. The evaluation of the success of this approach requires analytical methods to quantify human antibodies to the humanized therapeutic antibody in human serum or plasma.

CAMPATH-1H (C1H) is a humanized IgG1 monoclonal antibody which recognizes the CD52 antigen on human lymphocytes and monocytes, and is cytolytic for those cells [3]. Non-Hodgkin's lymphoma patients treated with C1H showed clearance of their tumor cells [4]. Treatment of rheumatoid arthritis patients with C1H produced some clinical responses which were transient [5] or lasted for several months [6]. Early trials of CAMPATH-1G, a rat antibody having the same specificity as C1H, showed that it was highly immunogenic even in immunosuppressed transplant patients [7]. The humanized version was expected to be immunologically inert; however, antibodies to C1H still develop against the idiotype (sequences associated with the antigen-binding domains) in patients who receive this antibody [6,8]. The presence of anti-C1H antibodies may alter the pharmacokinetics of C1H, especially the C_{max} and possibly the terminal elimination phase, thereby reducing the efficacy of the antibody. In addition, an antibody response may theoretically pose the risk of anaphylactic or immune complex sequelae.

This paper details the validation of a kinetic enzyme immunoassay for the quantitation of human antibodies to the humanized monoclonal antibody C1H. The method is similar to that first described by Cobbold and used to detect antibodies against CAMPATH-1G [9]. The method relies on a rat anti-C1H idiotypic antibody standard, and may be expanded to characterize the specificity of the human antibody for the C1H idiotype or for the C1H isotype.

2. Materials and methods

2.1. CAMPATH-1 antibodies

C1H was obtained at 10 mg ml^{-1} in phosphate-buffered saline (PBS) and EDTA (Wellcome, Beckenham, UK), and was stored in the original glass ampoules at 4°C. Ampoules were opened as needed after visual inspection in daylight confirmed the contents as a clear bright liquid with no particulates. The contents of opened ampoules were transferred to clean amber screw-topped vials having a rubberized closure and stored at 4°C for up to 6 months. CAMPATH-1G (rat antibody against the CAMPATH-1 antigen) was supplied at 10 mg ml^{-1} in PBS (Therapeutic Antibody Centre, Cambridge, UK). This antibody was obtained for use as a competitor for establishment of antibody as anti-idiotype.

Biotin-conjugated C1H was prepared as follows. C1H was diluted to 0.5 mg ml^{-1} in 0.05 M sodium bicarbonate buffer (pH 8.5) with 190 μl of 1 mg ml^{-1} sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-Biotin; Pierce, Rockford, IL) in a glass tube which resulted in a molar ratio of 55 for the reaction. After incubation for 2 h on ice, the mixture was transferred to a Centricon-30 filtration unit (Amicon, Beverly, MA) to be washed and concentrated by ultrafiltration. The absorbance at 280 nm was used to determine the final protein concentration. The biotin substitution ratio was determined by displacement of the HABA dye from avidin by the biotinylated protein [10]. Briefly, the absorbance of HABA bound to avidin was measured at 500 nm. Then, aliquots of biotin standard or biotinylated C1H were added stepwise to the HABA-avidin, and the absorbance decrease was measured. A calibration curve was plotted of the biotin concentration versus the change in absorbance. The unknown biotinylated C1H was read from the calibration curve. The biotin–protein conjugation ratio was found to be between six and 15 as used in this assay method.

2.2. Anti-C1H antibody standard

The anti-idiotypic antibody standard was prepared from the rat hybridoma cell line YID13.9.4 (Wellcome, Beckenham, UK). The cell line was derived in H. Waldmann's laboratory (Cambridge University, UK) from the fusion of Y3 myeloma cells (which secrete their own non-specific immunoglobulin light chain) with splenocytes from a rat immunized with CAMPATH-1G. The cell line secretes a rat monoclonal antibody which recognizes the idiotype of both C1H and CAMPATH-1G, but the specific heavy chains can associate randomly with either the specific or the nonspecific light chain.

Cells were grown in low-protein (0.5% bovine serum albumin) serum-free WRC935 media (Amicon) to confluency and the culture supernatant was harvested twice weekly and frozen at −20°C. Antibody standard was purified by affinity chromatography on a Protein G matrix. Pooled supernatants were filtered through a 0.22 μm filter and 150 ml were pumped through a Protein G capsule (Amicon). The capsule was rinsed with 100 ml tris-buffered saline, pH 7.4 (TBS assay buffer), and then the bound antibody was eluted with 30 ml glycine-HCl buffer (pH 3). The eluate was neutralized with 1 M NaOH to pH 7.2, and then concentrated using a Centriprep-30 ultrafiltration system (Amicon) followed by a buffer exchange into TBS assay buffer containing 0.02% NaN_3 and 10 mM EDTA. The absorbance at 280 nm was used to determine the final protein concentration.

Samples of individual batches of anti-idiotypic antibody standard were diluted in non-reducing sample buffer or reduced in sample buffer containing 2-mercaptoethanol and subjected to electrophoresis on a pre-cast 12% SDS–PAGE gel (Bio-Rad, Richmond, CA). Gels were stained overnight in 5% Coomassie Brilliant Blue G250 in 10% acetic acid and 40% methanol, then destained in acetic acid–methanol until the background was clear. Stained protein bands in the gel were quantified using a densitometer (FisherBiotech Model FB910, Raleigh, NC) interfaced to a Hewlett Packard Integrator (Model 3394, FisherBiotech). The immunoglobulin heavy chain migrated with an apparent molecular weight of 55 kd, whereas a doublet of light chain bands appeared at 28 and 31 kd. The fusion partner used in the creation of the hybridoma secretes its own non-specific light chain, which migrates at 28 kd and can be distinguished from the specific light chain on protein gels. The 31 kd band has been shown to be the specific light chain on the basis of amino terminal sequencing (Dr. B. Merrill, personal communication).

Three batches of purified anti-C1H antibody standard were pooled and established as the Reference Standard based on acceptable performance[2] in the assay and immunoglobulin light chain doublet ratio for specific:nonspecific light chain ratio of >1. Protein concentration was determined by absorbance at 280 nm and by BCA protein assay (Pierce). The potency of the Reference Standard was assigned unity based on protein mass units, i.e. 1 ng = 1 Unit. The Reference Standard was prepared in phosphate-buffered saline (PBS; Mediatech, Washington, DC) containing 1% human serum albumin (Sigma, St. Louis, MO), 10 mM EDTA, and 0.02% NaN_3 and stored frozen at −80°C.

The specific antibody potency was determined for each new batch of anti-C1H antibody standard against the Reference Standard. New batches of anti-idiotype working standard were calibrated against a standard curve of the Reference Standard, and an average potency value was obtained for the new batch from at least three determinations. The potency is calculated as below:

$$\text{Potency, Units ng}^{-1} = \frac{\text{Units ml}^{-1}\ \text{IC}_{50}\text{, Reference Standard}}{\text{ng ml}^{-1}\ \text{IC}_{50}\text{, working standard}}$$

Anti-C1H antibody data were reported as antibody activity in Units ml^{-1} based on the potency of the working standard.

[2] Acceptable assay performance was defined as the production of a Reference Standard calibration curve between 20 and 1280 ng ml^{-1} which could be transformed using a logit-log 4-parameter algorithm into a linearized data set.

2.3. Anti-C1H antibody assay procedure

Antibody to C1H was measured in a kinetic enzyme immunoassay run in 96-well plates. Briefly, plates were coated with 1 μg ml^{-1} C1H in carbonate buffer at pH 9.3 overnight. Plates were washed with TBS containing 0.01% Tween-20 (TBS/Tween), and 2% bovine serum albumin was added to all wells to block non-specific binding sites. After incubation for 2 h, plates were washed with TBS/Tween and 1/8 dilutions of patient serum, positive control sera, or working standard were prepared in Specimen Diluent Green (Alerchek, Portland, ME) containing 20% fetal bovine serum, and 100 μl were added to triplicate wells. Final serum concentrations were always 12.5% (as 1/8 final dilution); normal human serum was used when necessary to adjust final concentrations. The antibody standard was the affinity-purified rat anti-C1H idiotypic antibody, YID13.9.4. After overnight incubation at 4°C, the plates were washed with TBS/Tween and 100 μl of 200 ng ml^{-1} biotinylated C1H in TBS was added. After incubation for 4 h, plates were washed again and 50 μl of a 1/1000 dilution of streptavidin-alkaline phosphatase (which had been previously diluted 1/2 with glycerol and stored at 4°C; Jackson ImmunoResearch, West Grove, PA) in TBS was added for 30 min. Approximately every 15 min, the next plate was washed with TBS/Tween, and 50 μl of an excess of *p*-nitrophenylphosphate in 10% diethanolamine was added. Each plate was loaded into the plate reader (V_{max}, Molecular Devices, Menlo Park, CA) and the absorbance was measured over a 10-min reaction time for each well. The data (mOD min^{-1}) were analyzed using a 4-parameter logistic-log curve as calculated by algorithms in MultiCalc version 1.42 (Wallac, Gaithersburg, MD).

2.4. Validation experiments

Calibration standard curves were prepared at nominal concentrations of 1280, 640, 320, 160, 80, 40, and 20 Units ml^{-1} in triplicate. Five independent standard curves were set up on one day to evaluate intra-assay variation, and ten independent standard curves were set up over 5 days to evaluate inter-assay variation. All assays were run using two sets of controls (3600, 1200, and 400 Units ml^{-1}) in triplicate. No data were omitted from these analysis. The standard curve raw mOD min^{-1} were fit to a weighted 4-parameter logistic-log curve.

The anti-C1H antibody working standard was run periodically and compared to the Reference Standard anti-C1H antibody. Two sets of quality control (QC) samples (anti-C1H antibody spiked into normal human serum at three different concentrations) were run each time the Reference Standard was used.

The stability of the anti-C1H antibody in human serum when repeatedly frozen and thawed was assessed by submitting QC samples to seven cycles of freeze–thaw, then assaying aliquots from each cycle simultaneously. All 21 samples were run in triplicate, and in 63 wells on the same assay plate in order to compare actual mOD min^{-1}. Rows A and H of plates for this assay were never used in order to avoid edge effects with the enzyme kinetics, so only 72 wells were available per plate; thus, a standard curve was not run for this experiment.

QC samples were prepared at various times over the course of clinical sample analysis. QC samples were prepared in pooled normal human serum, and aliquots were stored frozen at −20°C. The stability of anti-C1H antibodies in human serum was assessed by the apparent retention of nominal activity in these samples.

2.5. Interferences

Matrix effects were evaluated using pre-treatment serum obtained from ten randomly selected rheumatoid arthritis patients. Individual sera were spiked with two concentrations of anti-C1H standard antibody, and were compared against a standard curve and spiked samples prepared using commercially available pooled normal human serum (Sigma).

The interference of the therapeutic monoclonal antibody C1H with detection of the anti-C1H antibody standard was investigated by preparing the calibration standards in the presence of vari-

ous amounts of C1H. Each calibration curve was examined for changes in shape and the lowest calibration standard response (mOD min^{-1}) inhibited by a given concentration of C1H was determined.

The interference of soluble antigen was investigated indirectly by an attempt to demonstrate it in patient serum samples. Samples of purified unquantified CD52 antigen (Glaxo Wellcome Inc.) were spiked into normal human serum and separated by electrophoresis as described above for anti-idiotype antibody characterization. Serum from rheumatoid arthritis and non-Hodgkin's lymphoma patients was also examined. Gels were western blotted with biotin-C1H, and stained with streptavidin-alkaline phosphatase and visualized using nitroblue tetrazolium. Blots were examined for bands staining around 21–28 kd (the molecular weight of the CD52 antigen).

The potential interference of rheumatoid factor in the sera from patients with rheumatoid arthritis was investigated. Commercially available IgM rheumatoid factor-positive serum (Binding Site, Birmingham, UK) was spiked into normal human serum at 150 Units ml^{-1} and assayed for false-positive anti-C1H antibody.

2.6. Characterization of human anti-C1H antibody specificity

Specificity analysis to identify the immunodominant domains of C1H was used to characterize antibody responses to C1H isotype (immunoglobulin constant region) or idiotype (antigen-binding region). Antibody specificity is demonstrated by the inhibition of binding to solid-phase C1H in the presence of an excess of appropriate competitor immunoglobulins, i.e. CAMPATH-1G which has the C1H complementarity-determining regions (CDRs) in a murine framework, to compete with anti-idiotypic antibody, or human IgG (having only the human IgG1 constant region) to compete with anti-isotype antibody. Plates were coated and blocked as described above for the routine quantitation assay. Duplicate wells then received 50 μl of standard, buffer, or unknown, and 50 μl of either assay buffer, or 50 μg ml^{-1} of C1H, CAMPATH-1G, or human IgG. After overnight incubation at 4°C, the plates were washed and the assay was completed as described above.

The mOD min^{-1} data were exported into an Excel (Microsoft) spreadsheet for calculations. Statistical analysis of the data was done using Lord's range test which is able to evaluate the means and ranges of 2 normal populations having a very small n [11] to generate the test statistic L as follows:

$$L = \frac{|\text{mean}_{\text{control}} - \text{mean}_{\text{competitor}}|}{(\text{range}_{\text{control}} - \text{range}_{\text{competitor}})}$$

For $n = 2$ for both control and competitor, the difference is significant when $L > 1.71$ [11]. All antibody responses in the buffer control should be significantly inhibited by excess C1H; antibody to the C1H idiotype should also be inhibited by excess CAMPATH-1G; antibody against the human IgG isotype should be inhibited by excess human IgG.

3. Results

3.1. Characterization of antibody standard

The Reference Standard was established from a pool of three separate purification batches of rat anti-C1H antibody and characterized as described above. The final protein concentration was 560 μg ml^{-1}, and the ratio of specific:nonspecific immunoglobulin light chain was 1.1 as estimated from SDS–PAGE. The performance of the Reference Standard was established in 12 independent calibration curves done over a 2-week period. The average IC_{50} was 379 ng $ml^{-1} = 379$ Units ml^{-1}, with a coefficient of variation of 8.8%. Additional standard curves were run periodically thereafter, when needed to calibrate new working standards or to evaluate new batches of QC samples.

Six batches of working standard were calibrated for potency in Units ml^{-1} against the Reference Standard, and had potency values which ranged from 0.560 to 1.805 Units ng^{-1}. This variation was presumed to reflect differences

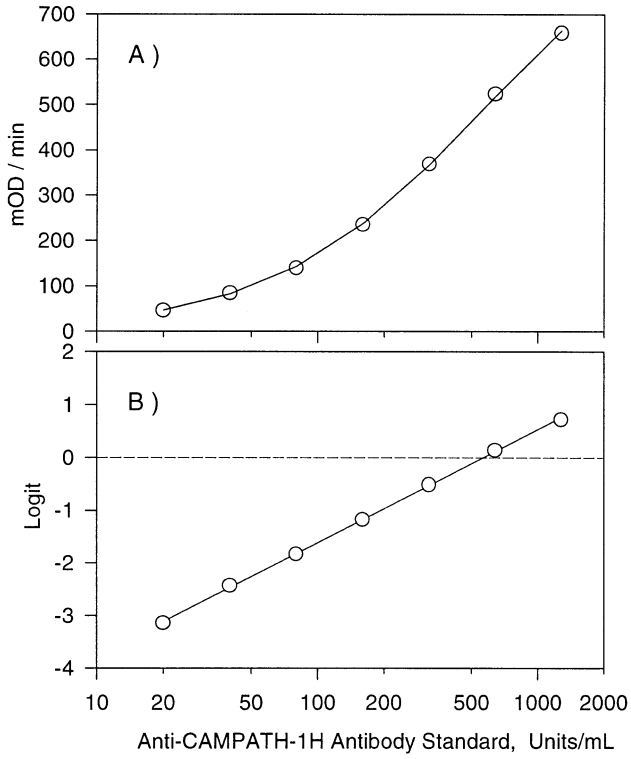

Fig. 1. Typical 4-parameter logistic weighted curve fit to the response data for the calibration standards. Analyses were done using MultiCalc (Wallac) software as described in the Materials and Methods and were graphed using SigmaPlot (Jandel). Response data are shown in A) mOD min^{-1} and in B) the logit transformation of the logistic function for mOD min^{-1} calculated as follows:

$$\text{Logit} = \ln \frac{(\text{Response} - \text{Estimated blank})}{(\text{Estimated reference} - \text{Estimated blank})}$$

in the relative production of the specific and nonspecific light chains by the cell line. Standard curves using the working standard were prepared in Units ml^{-1}, as were QC samples.

Fig. 1 shows a typical calibration curve fit using a 4-parameter logistic-log function. Actual response data (mOD min^{-1}) are shown (Fig. 1A), but results were routinely analyzed using the linearized logic-transformed data (as shown in Fig. 1B). Calibration standards having concentrations greater than 1280 Units ml^{-1} could not be read reliably throughout the 10 min kinetic assay, which was reflected in a lack of linearity of the enzyme kinetics within each well and in exceeding the capabilities of the plate reader software to capture the data.

3.2. Accuracy and precision

Intra-assay accuracy and precision were evaluated for 5 standard curves run the same day (designated I through V) using the back-calculated concentrations of two sets of controls (designated 1 and 2) (Table 1). The accuracy as a percentage difference is calculated as follows:

$$\text{Accuracy} = \frac{(\text{Back} - \text{calculated conc.} - \text{Nominal conc.})}{(\text{Nominal conc.})} \times (100)$$

The precision as the percentage coefficient of variation is calculated as follows:

$$\text{Precision} = \frac{(\text{Standard deviation})}{(\text{Average calculated conc.})} \times 100$$

The data in Table 1 show that intra-assay accuracy ranges from 11.2 to −10.8%, and that the loss of precision is <10%.

Inter-assay accuracy and precision were evaluated using ten independent standard curves (designated I through X) run on five different days using the back-calculated concentrations of two sets of controls (designated 1 and 2) (Table 2). The data in Table 2 show that inter-assay accuracy ranged from 8.9 to −10.4% for the QC samples. Fig. 2 shows the plot of the percentage difference versus the nominal concentration for each standard concentration for a weighted 4-parameter logistic-log algorithm and reflects the accuracy across the range of the calibration standards. The back-calculated concentrations determined for each standard under the 4-parameter calibration model were used to derive the percentage difference for each standard. The accuracy of the curve fit was not improved by using a 5-parameter logistic-log algorithm (data not shown), and so the simpler curve fit model was selected for use. The precision as a percentage coefficient of variation of the QC samples ranged from 6.5 to 12.3% (Table 2). The precision across the range of the calibration standards is shown in Fig. 3.

ANOVA statistics calculated for the assay performance overall resulted in a coefficient of variation of 9.2%.

Table 1
Intra-assay accuracy and precision of anti-C1H antibody EIA

Plate/replicate	Back-calculated concentrations (Units ml^{-1})		
	400 Units ml^{-1}	1200 Units ml^{-1}	3600 Units ml^{-1}
I/1	435	1207	3215
I/2	426	1182	3252
II/1	415	1100	3053
II/2	459	1108	3225
III/1	408	1219	3298
III/2	493	1248	3340
IV/1	467	1172	3607
IV/2	468	1115	3573
V/1	404	880	2508
V/2	468	996	3039
Geometric mean	444	1118	3197
Arithmetic mean	445	1123	3212
SD	30.6	112.5	309.7
%CV	6.9%	10.0%	9.6%
Accuracy	11.2%	−6.4%	−10.8%

Table 2
Inter-assay accuracy and precision of anti-C1H antibody EIA

Assay/replicate	Back-calculated concentrations (Units ml^{-1})		
	400 Units ml^{-1}	1200 Units ml^{-1}	3600 Units ml^{-1}
I/1	436	1037	2990
I/2	464	1079	3379
II/1	419	1006	3101
II/2	441	1050	3164
III/1	435	1207	3216
III/2	426	1182	3253
IV/1	416	1101	3053
IV/2	459	1108	3225
V/1	408	1219	3298
V/2	493	1249	3341
VI/1	468	1172	3608
VI/2	468	1115	3574
VII/1	439	987	2961
VII/2	390	1102	3190
VIII/1	405	880	2508
VIII/2	469	996	3039
IX/1	386	990	3556
IX/2	424	1075	4606
X/1	444	1137	3222
X/2	440	982	3530
Geometric mean	436	1076	3269
Arithmetic mean	437	1079	3291
SD	28.2	88.5	401.2
%CV	6.5%	8.2%	12.3%
Accuracy	8.9%	−10.4%	−9.2%

3.3. Stability

The stability of the anti-C1H antibody standard is shown in Fig. 4. Each working antibody standard was defined as a separate batch of YID13.9.4 anti-C1H idiotype antibody which was

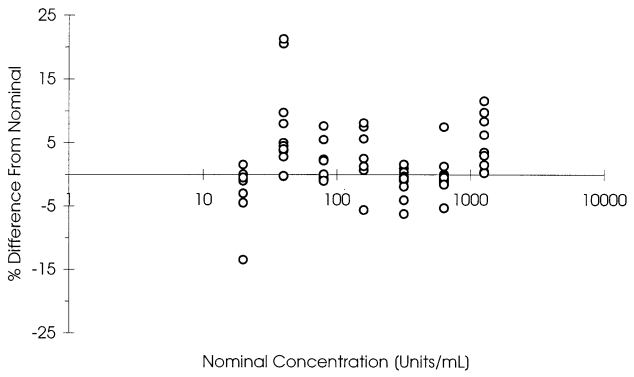

Fig. 2. Inter-assay percentage difference between back-calculated and nominal concentrations of the calibration standards using a 4-parameter logistic weighted model. The data are the means for each calibration standard run in triplicate in ten independent assays.

assigned a potency based on the Reference Standard. Each working standard, which was stored at 4°C in PBS/HSA, was used to set up the standard curves for routine analysis. The IC_{50} for standard curves generated over a period of 11 months was shown to vary around that of the Reference Standard. All experiments shown were run with acceptable QC samples (QC data not shown).

The stability of the QC samples throughout seven freeze–thaw cycles is shown in Fig. 5. No significant change in the mOD min^{-1} was detected, which suggests that human study samples may be likewise frozen and thawed for re-analysis.

The stability of the anti-C1H antibody standard in human serum was evaluated in the context of QC samples prepared and used within 1 week to 6 months from date of preparation. QC samples were within 20% of their nominal value when stored frozen for as long as 6 months (data not shown).

3.4. Interferences

Serum samples were obtained prior to treatment from ten randomly selected rheumatoid arthritis patients. Sera were spiked with two concentrations of anti-C1H antibody standard and compared against a standard curve and spiked samples of pooled normal human serum. For sera spiked at 450 Units ml^{-1}, the apparent anti-C1H concentration of the spiked rheumatoid arthritis patient sera is 13% lower than that of a similar spiked pooled normal human serum. This difference is magnified at 45 Units ml^{-1} (apparent average concentration is 24% lower) near the lower end of the calibration curve.

If the CAMPATH-1 antigen CD52 was present in patient sera, it could in theory interfere with detection of anti-C1H antibody by providing a bridge between solid phase C1H and biotinylated C1H, causing artificially high apparent anti-C1H concentrations. Although the antigen is not known to be shed or internalized, abundant lysis of target cells could release antigen into the circulation. In pooled normal human sera spiked with the purified antigen, a wide band near 25 kd was detected which corresponded to the molecular weight of the antigen. No such band was detected in similar western blots of patient sera. Since soluble CD52 could not be demonstrated in a subset of patient sera (and purified CD52 was in severely limited supply and its detection by blotting was unquantified), the direct interference of soluble CD52 was not further tested.

Interference by rheumatoid factor in the anti-C1H antibody assay would have appeared as high apparent anti-C1H antibody activity in the pretreatment serum specimen. Commercially available IgM rheumatoid factor-positive serum used within a standard curve at the highest possible concentration (150 Units ml^{-1}) did not interfere with the assay performance.

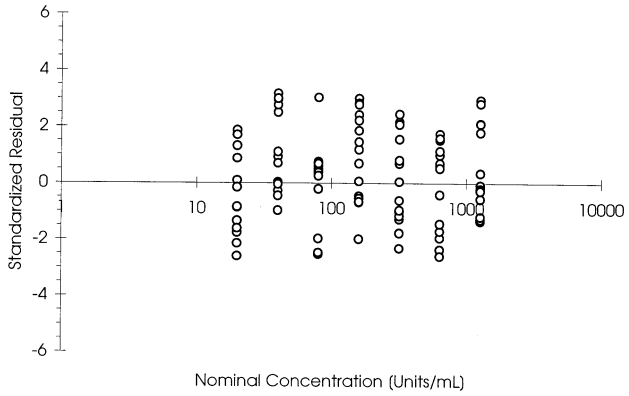

Fig. 3. Standardized residuals plot for the calibration standards using a 4-parameter logistic weighted model. The data are the standardized residuals for each calibration standard run in triplicate in ten independent assays. The standardized residual was calculated as

$$\text{Standardized residual} = \frac{(\text{Back} - \text{calculatedconc.} - \text{nominal conc.})}{(\text{Standard error})}$$

where the standard error was calculated as

$$\text{Standard error} = \frac{(\text{Mean back} - \text{calculated conc.}) \times (\%\text{cv})}{(\sqrt{n})}$$

3.5. Anti-C1H antibody specificity

Specificity analysis of antibody to C1H was conducted during routine analysis. The presence of antibody specific for C1H was confirmed by the addition of an excess of unlabeled C1H to the serum sample, which significantly inhibited binding. Antibody recognition of the C1H isotype (immunoglobulin constant region) or idiotype (antigen-binding region) was defined using human IgG or CAMPATH-1G, respectively. Binding of the rat anti-C1H idiotypic antibody standard was clearly inhibited in the presence of either C1H and CAMPATH-1G, as expected.

4. Discussion

A precise and reproducible kinetic enzyme immunoassay specific for antibodies to C1H was developed and validated to quantify and characterize human antibodies to C1H in serum. The challenge of validating an immunoassay method for a protein of interest is heightened in this case by the lack of species difference between the therapeutic (C1H) or elicited (anti-C1H) protein in the biological matrix (human serum), the absence of a matched standard antibody, and the unknown antibody class or affinity of the elicited immune response. In addition, it has been recognized that degradation products and inhibitory substances in biological matrices confound bioanalysis of therapeutic proteins [12,13]. Notwithstanding these considerations, the present data were modeled by a 4-parameter logistic-log algorithm throughout the range of the standard curve from 1280 Units ml^{-1} down to 20 Units ml^{-1} based within 15% of nominal values. A method was devised by which to establish the specific antibody potency for any given batch of anti-C1H antibody standard relative to the Reference Standard. The anti-idiotype antibody standard is stable at 4°C and at -80°C for at least 11 months in buffer. The anti-idiotype antibody controls were stable through at least seven freeze–thaw cycles in normal human serum. The lower limit of quantitation for the assay was defined at 160 Units ml^{-1}.

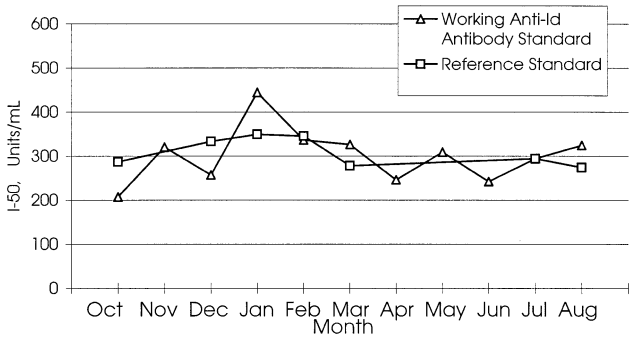

Fig. 4. Stability over time for anti-C1H antibody standard solutions. Data are the EC_{50} values (Units ml^{-1}) for a standard curve run during each month.

The anti-C1H antibodies are quantified in Units ml^{-1}. The primary reason for doing so is that the class and affinity of antibody formed are unknown, and the antibody response is presumed to be polyclonal. Thus, comparisons on a mass basis to the available rat monoclonal anti-C1H idiotypic antibody standard are inappropriate. A secondary reason for the use of a Unit scale is that batches of rat anti-C1H standard vary unpredictably in their light chain composition (see Section 2.2) and the use of a Reference Standard permits the assignment of a potency concentration based on Units ml^{-1}.

There was a consistent bias in the QC sample accuracy for both intra- and inter-assay evaluations which tended to overestimate the low control and underestimate the medium and high controls. Because the noted biases were less than 15%, the assay was considered acceptable for use. The ANOVA intra-assay and inter-assay coefficients of variation were 7.1 and 5.8%, respectively, whereas the overall assay performance coefficient of variation was 9.2%.

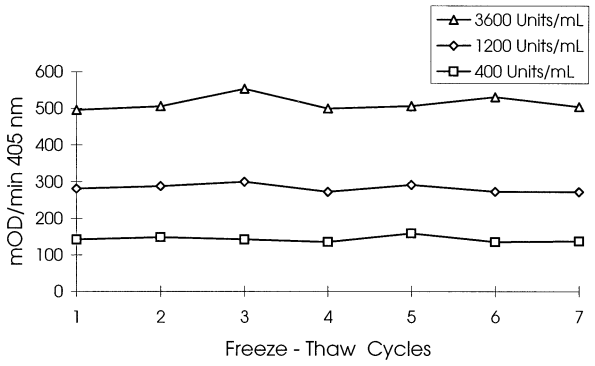

Fig. 5. Stability of anti-C1H antibody quality control samples to repeated cycles of freezing and thawing. Prepared controls were frozen and thawed and assayed simultaneously on the same assay plate. Data are the means of triplicate determinations of mOD min^{-1}.

The usual approach to validation of an assay is to assure that the assay is specific for the analyte, and that interfering substances are understood. Rheumatoid factor is an endogenous IgM or IgG (less common) antibody produced against an individual's own IgG immunoglobulin. Levels of rheumatoid factor were measured at the clinical trial sites, and results ranged from titers of 0–5120 in the rheumatoid arthritis patients enrolled in the Phase I clinical trials, and were extremely variable. Interference by rheumatoid factor in the anti-C1H antibody assay could cause artifactually high apparent anti-C1H antibody activity in the pre-treatment serum specimen. Rheumatoid factor at the levels seen in rheumatoid arthritis patients did not produce false-positive results in this assay, since all patient pre-treatment specimens were negative (<20 Units ml^{-1}) for anti-C1H antibody [8]. The existing rheumatoid factor might have been of low affinity or might simply have not recognized the C1H allotype. Furthermore, normal serum samples spiked with rheumatoid factor did not yield artifactual anti-C1H results. Matrix effects with normal human serum were negligible, as evidenced by $100 \pm 20\%$ recovery of nominal concentrations of anti-C1H spiked

into the individual sera. The analysis of spiked pre-treatment sera obtained from rheumatoid arthritis patients was problematic in that the apparent anti-C1H concentrations were somewhat lower than that of spiked pooled normal human serum. This discrepancy was possibly due to a serum antibody cross-reactive with rat immunoglobulin (i.e. the anti-C1H standard species), but which may not reflect the patient's antibody reactivity to C1H itself.

Anti-C1H antibody cannot be reliably quantified if more than about 1 ng ml^{-1} of C1H is present in the serum sample. The serum half-life of C1H has recently been estimated to be approximately 40 h (manuscript in preparation), and if one assumes a 1 mg dose, then the residual serum C1H should be below the threshold of interference by 2 weeks after the last dose. Anti-C1H would not be expected to be produced much earlier than 2 weeks after dosing, except upon a secondary immune response. If it was present, however, it would be expected to shorten the clearance time of the circulating C1H due to involvement of the reticuloendothelial system. The CD52 antigen does not modulate [3] and so would not be expected to be normally shed into the blood; furthermore, the antigen was not demonstrated in the rheumatoid arthritis patients' sera. Therefore, it is unlikely that soluble antigen is responsible for false-positive responses in the anti-C1H assay.

In the present assay, specificity is obtained by virtue of the capture reagent (C1H coated onto the microtiter plate) and the specific interactions of the analyte for the capture reagent. Indeed, it is incumbent in the definition of the anti-C1H analyte that it is a C1H-binding protein. The immunologic specificity of anti-C1H antibody can be further addressed by identification of regions of the C1H molecule which present immunodominant epitopes. The present assay can be modified by the addition of an excess of competitors which will only block the binding of anti-C1H antibody to solid phase C1H when the same epitopes are carried on the competitor. Thus, a non-specific antibody which carries human γ chain (e.g. purified human IgG) can be used to identify anti-isotype (anti-human IgG) antibody, and an immunoglobulin which carries the CDRs of C1H but not having the same constant region (e.g. rat CAMPATH-1G) can be used to identify anti-idiotypic antibody. Using this approach, most anti-C1H antibody responses were directed against the idiotypic regions containing the CDRs rather than the constant region. Even in an excess of competitor, the binding inhibition may not be complete and cannot reveal absolute percentages of epitope reactivity due to affinity differences, but can yield information regarding epitope presentation which may be useful in the evaluation of clinical events or in the redesign of humanized therapeutic proteins.

This EIA has been used to quantify units of anti-C1H antibody in human serum in support of clinical safety and efficacy studies [5,8,14]. Approximately half of the rheumatoid arthritis patients treated with C1H in single-dose or multiple-dose regimens produced anti-C1H antibody. Those patients became antibody-positive at about 3 weeks post-dose in most cases. The anti-C1H antibody was characterized using the present assay, and was found to be predominantly anti-idiotypic antibody [8], as might be expected. Few patients had antibody which recognized the C1H isotype. The demonstration of immune responses to biopharmaceuticals such as humanized therapeutic antibodies is an important facet of the clinical management and interpretation of pharmacokinetic data obtained in these studies.

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