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Qualification and application of a surface plasmon resonance-based assay for monitoring potential HAHA responses induced after passive administration of a humanized anti Lewis-Y antibody

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Abstract

A sensitive, surface plasmon resonance (SPR)-based assay monitoring potential human–anti-human antibody (HAHA) reactions against the monoclonal antibody (mAb) IGN311 is presented. The latter is a fully humanized Lewis-Y carbohydrate specific mAb that is currently tested in a passive immune therapy approach in a clinical phase I trial.

For the SPR experiments a BIACORE 3000 analyzer was used. The ligand IGN311 was covalently coupled to the carboxy-methylated dextran matrix of a CM5 research grade chip (BIACORE). In the course of a fully nested experimental design, a four parameter logistic equation was identified as appropriate calibration model ranging from 0.3 μg/mL (lower limit of quantitation, LLOQ) to 200 μg/mL (upper limit of quantitation, ULOQ) using an anti-idiotypic mAb ('HAHA mimic') as calibrator. The bias ranged from −2.4% to 5.5% and the intermediate precision expressed as 95% CI revealed values from 5.6% to 8.3%. Specificity was evaluated using six human serum matrices from healthy donors spiked with calibrator at the limit of quantitation (LOQ) with >80% of values being recovered with less than 25% relative error.

The qualified assay was applied to monitor potentially induced HAHA reactivity in 11 patients from a clinical phase I trial with passively administered IGN311. Of the 11 patients, one high HAHA responder and several low responders were identified. Protein-G depletion experiments with human serum samples revealed that the observed response is predominantly caused by IgG binding to the ligand. The characteristics of these HAHA responses were all of the so-called 'Type I' which is defined by a peak response around day 15 that decreases from this point steadily suggesting that some kind of tolerance is established. Therefore, this type of HAHA response is regarded as non critical for the patient's safety.
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Keywords: Lewis-Y; HAHA; BIACORE; Qualification

1. Introduction

The human–anti-human antibody response (HAHA) elicited to various degrees after repeated administration of therapeutic monoclonal antibodies (mAbs) is perceived as a major challenge in their clinical development because it can be, in to worst case, life-threatening for the individual patient. Therefore, accurate methods for the timely detection of HAHA are mandatory for clinical trials to ensure the patients safety. Also the FDA makes specific recommendations on monitoring the development of HAHA responses [1] further stressing the importance of this issue.

For passive cancer immunotherapy, igeneon has developed IGN311, a fully humanized IgG1/κ monoclonal antibody directed against the Lewis-Y carbohydrate antigen. The challenge encountered with the humanization strategy is that the obtained IgG molecule is still potentially immunogenic and will therefore induce a human–anti-human antibody response [2] that is most likely directed against the specificity determining regions, the residues most critical in antigen–antibody recognition [3].

Therefore, a clinical phase I study with IGN311 was designed to define the maximum tolerated dose and to determine the

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immunogenicity. Clinical trials applying humanized antibodies traditionally have used ELISA-based methods for the assessment of HAHA reactivity [4–6]. ELISA, however, has several inherent problems: in the case of direct ELISA, the use of an anti-human detection reagent is not applicable for drugs such as humanized antibodies. For double antigen ELISA, significant challenges include the careful optimization of the reagent concentration, the availability of the labelled antibody drug with comparable affinity and the potential interaction with excess of therapeutic antibody resulting in a delayed monitoring of HAHA responses. Moreover, the method is considerably complex and laborious.

Besides ELISA, the use of surface plasmon resonance (SPR)-based assays for the determination of the induced HAHA response has been reported [7,8]. SPR measures binding to an immobilized ligand in real time without the need of a secondary antibody [9] and have been effectively used for the characterization of antigen–antibody interactions [10–12]. The use of SPR-based assays to support clinical phase I studies was described for Cetuximab (anti-EGF receptor antibody) where the circulating levels of this mAb were determined [13] and for the humanized anti-EpCAM mAb A33 measuring induced HAHA response following administration [14].

We here report the qualification of a sensitive and robust BIACORE assay comprising criteria such as specificity, response function (linearity), range, LOQ, bias, and precision. The assay is applied to serum samples of patients from a phase I study measuring potentially induced HAHA responses against the passively administered mAb IGN311.

2. Experimental

2.1. Materials and equipment

HBS-EP (0.01 M HEPES, 0.15 M NaCl, pH 7.4 containing 3.4 mM EDTA and 0.05% surfactant P-20), 10 mM NaAc-Buffer, pH 6.5, and the 'Amine Coupling kit' were obtained from BIACORE AB, Uppsala, Sweden. Blood from six healthy donors were obtained from Red Cross, Vienna, Austria. As sample dilution buffer PBS_{def} containing 0.2 M NaCl, 0.5% Nonidet P40 and 10% normal human serum (NHS). The NHS obtained from igeneon donor 'I' was used. IGN311, a fully humanized IgG1/κ mAb specific for the Lewis-Y antigen, was derived from murine ABL364 antibody (BR55-2 hybridoma) by CDR grafting (Protein Design Labs). Herceptin (Genentech, CA, USA), a human IgG1/κ antibody specific for the HER2 antigen, was used as isotype control antibody. MMA383, a murine Lewis-Y mimicking IgG1/κ mAb, was obtained from Novartis Austria. Human serum samples were obtained from a phase I dose escalation study with IGN311 performed from 2002 to 2005 in Augsburg, Germany. The study was approved by the institutional ethic committee review board and was performed according to the Declaration of Helsinki and the Good Clinical Practice guidelines. The study was designed to assess safety parameters (like adverse events and HAHA response), to determine a safe dosage range and to gather pharmacokinetic data. Two infusions of IGN311 were given on days 1 and 15 at three dose levels (50 mg/infusion, 100 mg/infusion and 200 mg/infusion). For SPR analysis, a BIACORE 3000 apparatus equipped with a CM5 sensor chip research grade was used (BIACORE AB, Sweden).

2.2. SPR assay set-up

The ligand, IGN311 ('ref 1' 10.5 mg/mL) was diluted with 10 mM NaAc-Buffer (pH 6.5) to a concentration of 20 μg/mL. The immobilization onto a CM5 sensorchip Research Grade was performed at a flow rate of 10 μL/min for 2 min using *N*-hydroxy-succinimide and *N*-ethyl-*N*′-dimethylaminopropyl-carbodiimide to activate the dextran layer for coupling via primary amines. After saturation of unreacted binding sites with ethanolamine, the obtained immobilization level was 9622 relative response units (RUs). Control lanes included Herceptin (19,999 RUs immobilized) and an ethanolamine treated lane. Samples and standards were analyzed in randomized order (except for the pre-qualification experiment) by injecting 40 μL at a flow rate of 10 μL/min. Prior to sample measurements, the chip surface was conditioned with three cycles of 10% NHS "I" diluted in sample dilution buffer (PBS_{def} containing 0.2 M NaCl and 0.5% Nonidet P40). After washing with HBS-EP for 2.5 min, analyte binding was measured as relative response units over baseline. Regeneration of the chip surface was accomplished by injecting 10 μL of 10 mM HCl. The stabilization time between two cycles was 4 min.

2.3. Assay qualification

In a pre-qualification experiment, six successive calibration experiments (two calibrations per day) were carried out with MMA383 at equally log-spaced concentrations of 0.061 μg/mL, 0.244 μg/mL, 0.977 μg/mL, 3.906 μg/mL, 15.025 μg/mL, 62.5 μg/mL, 250 μg/mL and 1000 μg/mL in duplicates. Calibrators were prepared in dilution buffer (PBS_{def}, 0.2 M NaCl, 0.5% Nonidet P40) containing 10% NHS (obtained from igeneon donor 'I') and each experiment was run from the lowest to the highest concentration standard. In addition to response function and linearity, this set-up provided a preliminary accuracy estimate for the assay.

For qualification, a (operator and time different) nested design was used for the evaluation of bias, precision, range, and LOQ. Two operators, each on three different days, analysed duplicate qualification samples of MMA383 in 10% NHS (obtained from igeneon donor 'I') at concentrations of 0.3 μg/mL, 0.5 μg/mL, 15 μg/mL and 200 μg/mL on a calibration with equally log-spaced concentrations of 0.244 μg/mL, 0.977 μg/mL, 3.906 μg/mL, 15.025 μg/mL, 62.5 μg/mL and 250 μg/mL. Between the experiments of the two operators, the chip was stored in HBS-EP for 3 days at 4 °C in order to simulate typical routine operations. For specificity testing, MMA383 was diluted into six different sera obtained from healthy Red Cross donors (age and sex matched) at the LLOQ and analysed in duplicates on a calibration with equally log-spaced concentrations

of 0.244 μg/mL, 0.977 μg/mL, 3.906 μg/mL, 15.025 μg/mL, 62.5 μg/mL and 250 μg/mL MMA383.

2.4. Clinical sample testing

After conditioning the chip surface with three cycles of 10% NHS "I" diluted in sample dilution buffer (PBS_{def} containing 0.2 M NaCl and 0.5% Nonidet P40), 1:10 diluted (in dilution buffer) patient samples were injected. As standard, MMA383 was diluted with dilution buffer containing 10% NHS to provide evenly log-spaced concentrations of 0.244 μg/mL, 0.977 μg/mL, 3.906 μg/mL, 15.025 μg/mL, 62.5 μg/mL and 250 μg/mL. As QC-samples, 0.5 μg/mL, 15 μg/mL and 200 μg/mL MMA383 were analyzed in duplicates. All samples and standards were randomly analyzed.

2.5. Statistical evaluation

For the determination of bias, repeatability, and intermediate precision, a one-way ANOVA was used.

Bias was calculated as following:

$$\%\mathrm{RE} = 100 \times \left(\frac{z-\mu}{\mu}\right)$$

where z is the overall mean and μ the nominal concentration.

Repeatability (intra-day precision) was calculated as:

$$\%\mathrm{CV} = 100 \times \left(\frac{s_\mathrm{w}}{\mu}\right)$$

where s_w is derived from the repeatability variance component (see below).

Intermediate precision was calculated as:

$$\%\mathrm{CV} = 100 \times \left(\frac{s_\mathrm{IP}}{\mu}\right)$$

where s_IP is derived from the repeatability and (operator and time) variance components (see below).

The total error (accuracy) of the method was calculated as following:

$$\%\mathrm{RE} = \left(\frac{100}{\mu}\right) \times [(z-\mu) \pm 2 \times s_\mathrm{IP}]$$

This provides a concentration-dependent confidence interval within a future single value that may be anticipated with a likelihood of ~95%. Variance components were calculated from ANOVA mean square errors as follows:

$$\text{Repeatability variance}: \quad \mathrm{MS_w} = s_\mathrm{w}^2 = \frac{1}{t}\sum_{i=1}^{t} s_i^2$$

where t is the number of days and s_i^2 is the variance of the ith day.

$$\text{(Operator and time) variance}: \quad \frac{\mathrm{MS_b} - \mathrm{MS_w}}{n} = s_\mathrm{b}^2$$

where n is the number of replicates per day and:

$$\mathrm{MS_b} = \frac{n}{t-1}\sum_{i=1}^{t}(z_i - z)^2$$

where z_i is the mean of the ith day.

(Operator and time)—different intermediate precision :

$$s_\mathrm{IP}^2 = s_\mathrm{w}^2 + s_\mathrm{b}^2$$

Data evaluation was carried out using the following software packages: Sigma Plot 8.0, Sigma Stat 3.0, Statgraphics 5.0 and GraphPad Prism 4.2.

2.6. Operational safety

While working with serum samples, protective clothing (lab coat, goggles and gloves) was worn. Initial serum dilutions were prepared under laminar flow to avoid contact with aerosols. Waste was deposited according to the 'Abfallwirtschaftsgesetz'.

3. Results

3.1. Response function and linearity

For the evaluation of the response function, six successive calibration experiments were conducted with eight concentrations in duplicates ranging from 0.061 μg/mL to 1000 μg/mL. Calibrators prepared in dilution buffer containing 10% NHS 'I' were diluted evenly spaced on a log-scale. Fig. 1 shows the relationship between concentration and response. A four parameter logistic equation was applied and suggested as appropriate model which was also confirmed by the distribution of the

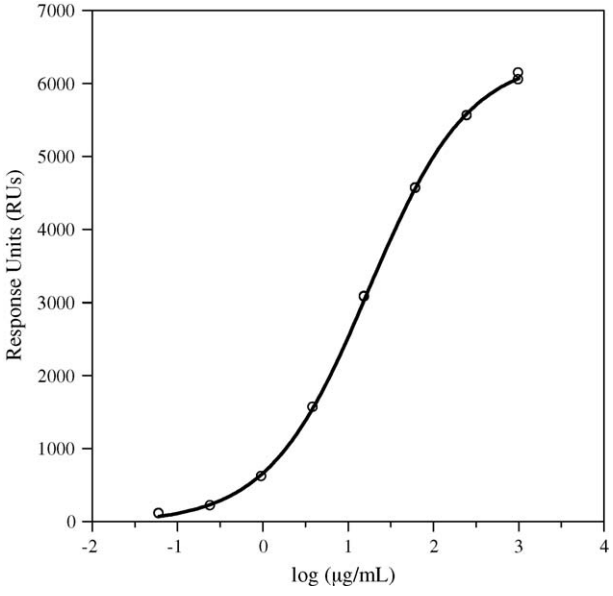

Fig. 1. Anti-idiotype mAb MMA383 in 10% NHS: response function. A four-parameter logistic model was applied as response function to describe the relationship between concentration and RUs.

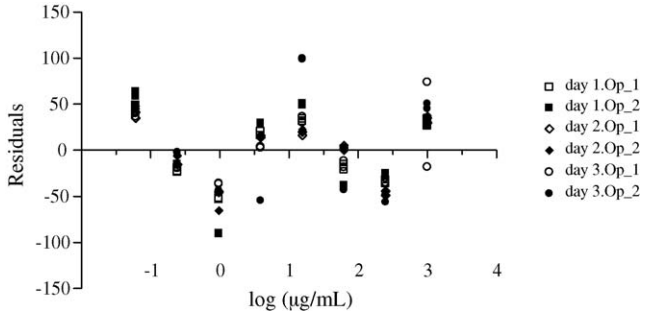

Fig. 2. Anti-idiotype mAb MMA383 in 10% NHS: residual plot. The corresponding residual plot of the non-linear regression of legends to figures (Fig. 1) is shown. Based on visual inspection, no significant trend in the residual distribution was observed.

residuals which revealed no significant trend based on visual inspection (Fig. 2). In addition, runs test performed on all six regressions revealed no deviation from linearity at $P = 0.01$.

3.2. Preliminary accuracy determination

The above experimental data were used to calculate preliminary accuracy (total error) by reading off calibrators of all six sequentially conducted calibration experiments on the first regression. Fig. 3 shows the total error for all evaluable concentrations of the assay performed under the above conditions. The bias ranged from −11% to −32% and the precision calculated as 95% CI ($2 \times s_{IP}$) from 17% to 33% (except for the 0.061 μg/mL calibrator). The persistent high negative bias was found to be due to a continuous reduction of mean recovery with increasing experimental time as exemplarily shown for the 250 μg/mL calibrator in Fig. 4. This finding was further supported by the analysis of RUs of a chip subjected to more than 600 runs and 142 days with a control sample containing 15 μg/mL MMA383 (data not shown). The reason for this phenomenon may be a small but distinct loss of immobilized IGN311 ligand as a consequence of multiple chip regeneration. For comparison, Mason et al. [15] reported the validation of a BIACORE 3000-based assay for the determination of antibodies against erythropoietic

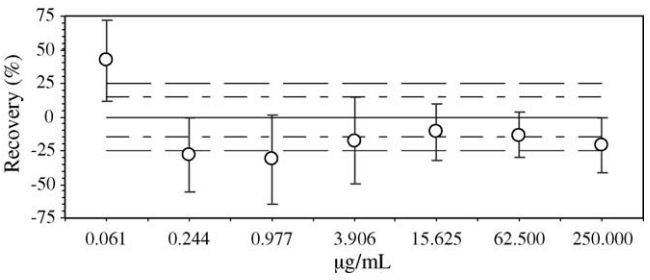

Fig. 3. Anti-idiotype mAb MMA383 in 10% NHS: total error (accuracy = precision ± bias). Preliminary accuracy plot displaying the total error (accuracy = precision and bias) for the entire experimental range (1000 μg/mL was not evaluable). All calibrators were read off on the first regression; bias is given as dots, error bars are 95% CI ($2 \times s_{IP}$). Dotted hatched lines indicate the 15% CI and hatched lines indicate the 25% CI.

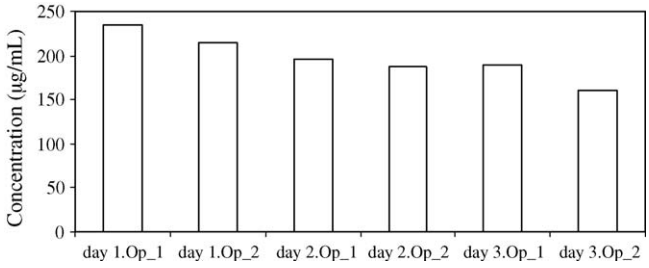

Fig. 4. Anti-idiotype mAb MMA383 in 10% NHS: sensor chip stability. Antibody recovery reduction with increasing experimental time for the 250 μl/mL calibrator is shown ('day 1.Op_1' stands for 'day 1, Operator 1').

agents in human serum samples that was stable through only 180 sample/regeneration cycles.

When calculating preliminary accuracy by back-calculation of calibrators on their individual regression, the systematic error could be significantly reduced (−12.9% to 3.6%; except for the 0.061 μg/mL calibrator) and the negative trend was eliminated (Fig. 5). Not surprisingly, the random error component could also be reduced considerably to 3.2–9.2% (95% CI ($2 \times s_{IP}$) – except for the 0.061 μg/mL calibrator).

3.3. Method qualification

Based on the findings from the preliminary experiments, we applied a fully nested design with two operators measuring duplicate qualification samples each on three different days. The calibration range was narrowed from 0.244 μg/mL to 250 μg/mL and replicates were omitted which had no significant effect on the residual standard deviation of the regressions. Thus, every sequence (day) comprised six calibrators and eight qualification samples. Importantly, and as a consequence from the findings above, samples and calibrators were introduced in randomized order.

As can be seen in Fig. 6, all concentrations applied for the qualification of the assay revealed total errors significantly below 15% (20% at the LLOQ), limits that were recommended in the 1990 Crystal City conference report for bioanalytical methods [16]. For immunoassays, even broader limits of <20% (25%

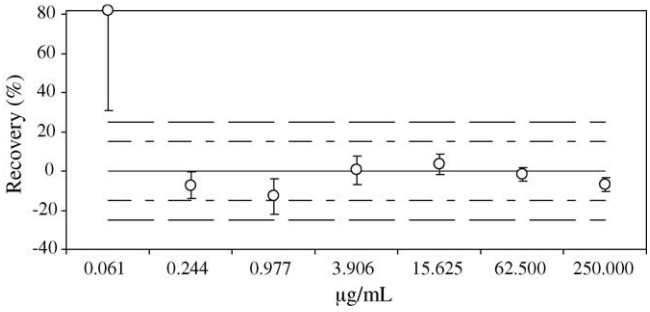

Fig. 5. Anti-idiotype mAb MMA383 in 10% NHS: total error/back-calculation. Preliminary accuracy plot displaying the total error for the entire experimental range (1000 μg/mL was not evaluable). All calibrators were back-calculated on their individual regression; bias is given as dots, error bars are 95% CI ($2 \times s_{IP}$). Dotted hatched lines indicate the 15% and hatched lines indicate the 25% CI.

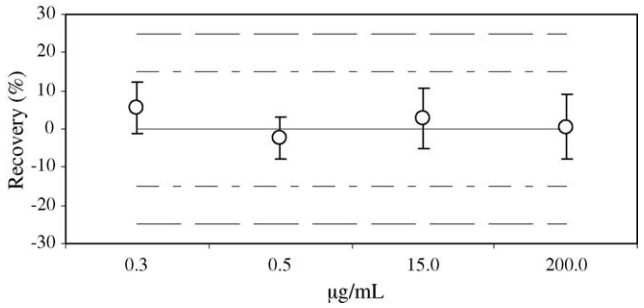

Fig. 6. Anti-idiotype mAb MMA383 in 10% NHS: accuracy plot for the assay in the qualification experiment. Bias is given as dots, the error bars represent 95% CI ($2 \times s_{IP}$). Dotted hatched lines indicate the 15% and hatched lines indicate the 25% CI.

at the LLOQ) were suggested by Findlay et al. [17]. Moreover, the bias showed no trend over the tested range confirming both, the application of the appropriate response function (linearity) and the randomized sample/calibrator introduction strategy as discussed above. Thus, the range of the assay from 0.3 µg/mL to 200 µg/mL almost spans three orders of magnitude allowing to circumvent dilutional linearity and parallelism testing ('bringing sample into range'). The LLOQ is 0.3 µg/mL and bias and precision ($2 \times s_{IP}$) are presented in Table 1.

For routine testing, it was suggested that a sequence comprising one calibration should be limited to 20–25 cycles including samples, calibrators, and QC standards. For the latter, duplicates in the low, mid, and high concentration range should be used and follow a 4–6–25 QC-rule [17]. For evaluation of specificity, MMA383 was diluted into six different sera obtained from Red Cross donors at the LLOQ (0.3 µg/mL) and analysed in duplicates (Table 2). Ten out of twelve samples (>80%) were within 25% RE (LLOQ) – a specificity criterion suggested by DeSilva et al. [18].

3.4. Measurement of clinical serum samples

Within this study, patients were assigned to three cohorts: cohort 1 (patients 1–3) received 50 mg/dose IGN311, cohort 2 (patients 4–6) received 100 mg/dose IGN311 and cohort 3 (patients 7–11) received 200 mg/dose IGN311. Patients were infused with IGN311 at days 0 and 15. To monitor HAHA response, blood samples were collected before (day 0) and after (days 7, 14, 21 and 42) treatment. Serum was prepared and assayed for HAHA reactivity against immobilized IGN311 as described above. HAHA titers are expressed in MMA383 equivalents. The relative errors of all QC samples were below

Table 1
Bias and precision ($2 \times s_{IP}$) in percent for the assay over the tested range

	0.3 µg/mL	0.5 µg/mL	15 µg/mL	200 µg/mL
Bias	5.5	−2.4	2.7	0.6
Precision	6.9	5.6	8.0	8.3

Table 2
Relative errors (RE) for the 0.3 µg/mL MMA383 (LLOQ) spiked into six different normal human sera (NHS 1–NHS 6)

Matrix	RE (%)
NHS 1	87 84
NHS 2	−8.0 −9.8
NHS 3	−9.5 −12
NHS 4	−13 −13
NHS 5	−24.98 −24
NHS 6	−0.05 −0.45

10% compared to the nominal values which is well in line with the accuracy results from the qualification runs (Fig. 6) and by far fulfils the required 4–6–25 QC-rule as suggested above. Only one individual (patient 6) had detectable HAHA levels prior to treatment with IGN311, which slightly increased after repeated treatment—these preformed reactivity might be caused by rheumatoid factors. The obtained data (Fig. 7) indicates that patient 1 developed a HAHA response of approximately 15 µg/mL MMA383 equivalents. This patient belonged to the low dose (50 mg) cohort indicating that the strength of the HAHA response does not correlate with the applied antibody dose because patient 7 – belonging to the high dose cohort – developed a HAHA response with only ∼3.5 µg/mL

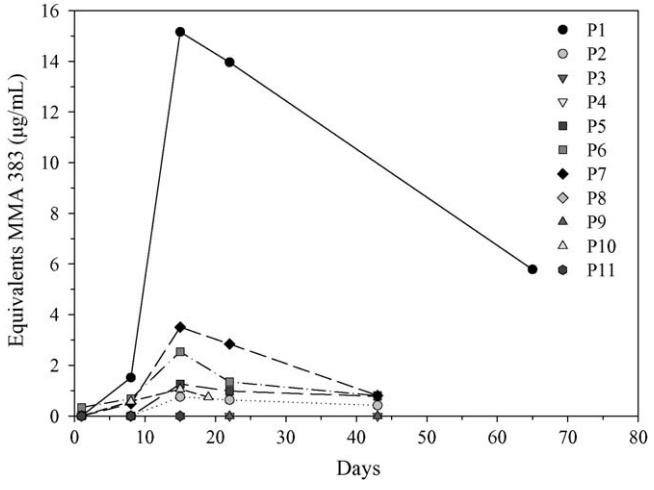

Fig. 7. Determination of HAHA reactivity in patient sera (BIACORE). The following doses of IGN311 were applied at days 1 and 15: patients 1–3 (P1, P2, P3) received 50 mg/dose, patients 4–6 (P4, P5, P6) received 10 mg/dose and patients 7–11 (P7, P8, P9, P10, P11) received 200 mg/dose. Serum from all patients was prepared from blood drawn on days 1, 8, 15, 22 and 43. Only for patient 1, serum prepared from blood taken on day 66 was available. Patient 10 had to be removed from the trial after day 14 because of progressing disease. As negative control, binding to a Herceptin coated lane was monitored—none of the serum samples showed elevated binding to Herceptin (data not shown).

MMA383 equivalents. Patients 2, 5, 6 and 10 showed only a marginal but detectable increase of HAHA reactivity ranging from 0.75 μg/mL to 2.54 μg/mL. In five patients (3, 4, 8, 9 and 11) no HAHA response was detectable. As isotype control, a lane with covalently immobilized Herceptin was used. Interestingly, none of the sera, which showed elevated binding to IGN311, did bind to Herceptin (data not shown) although the amount of immobilized antibody was in the same order of magnitude (IGN311: 9622 RUs and Herceptin: 19,999 RUs). This result suggests that the observed HAHA reactivity is directed against the antigen binding site of IGN311.

In order to verify that the measured signal increase is caused by immunoglobulins binding to immobilized IGN311, the serum sample with the highest reactivity was subjected to Protein-G chromatography. Using serum samples from patient 1, analysis revealed that the obtained SPR signal is generated by IgG binding since binding reactivity was found only in the column eluate and not in the flow-through fraction (data not shown).

4. Discussion

An SPR-based method to measure HAHA response was developed and qualified to support a clinical phase I trial using the passively administered humanized mAb IGN311. The method allows for direct measurement of specific anti-human Ig antibodies in serum of patients using a label-free real time approach. Monitoring the induced HAHA response is of paramount importance for the safety of the treated patients. The qualified assay for HAHA determination is easy to perform and the automated instrument significantly reduces 'hands on' time. The qualification was designed to assess response function, linearity, specificity, bias, precision, assay range and the LOQ. Qualification of the assay revealed that the method is considerably accurate and linear over three orders of magnitudes using a 4PL equation as response function. For comparison, the ELISA described by Hale et al. [6] to measure the anti-alemtuzumab immune response revealed a limit of detection of 488 ng/mL anti-idiotype reference standard; an overall precision of ±14% and an overall accuracy of 94% were reported. Regarding BIACORE, Wong et al. [19] reported the validation of a method that simultaneously measures the serum concentrations of a humanized antibody in mouse serum and induced antibodies. Their assay covered a concentration range of only one log (0.05–0.5 μg/mL) with a LOD of 1 μg/mL in mouse serum, an accuracy from 92.2% to 105.8% and a precision (%CV) from 0.96 to 7.39 (intra-assay) and 5.88 to 17.56 (inter-assay).

Qualification of the current assay further demonstrated that a time dependent signal decrease must be considered—whether this is due to loss of biological activity of the immobilized IGN311 due to repeated regeneration or due to the release of immobilized IGN311 from the surface was not further investigated. To take this issue into account, samples and standards were applied in randomized order. Additionally, an analysis sequence is suggested to be limited to approximately four consecutive days with a total of ca. 25 samples. Long-term studies using one chip revealed an appropriate ligand activity of at least 500 cycles. However, chip quality is suggested to be monitored using QC samples allowing consequent performance qualification (system suitability testing) for the individual chip.

Regarding the HAHA response measured in serum samples, two types have been described by Ritter et al. [14] who investigated the induced HAHA response after repeated administration of the human, EpCAM specific, Ab A33 in patients suffering from colon cancer. Onset of all measured HAHA responses was observed after day 7 and had peak values at day 14; upon subsequent administration, the HAHA reactivity decreased indicative for the 'HAHA response Type I'. In contrast, 'HAHA response Type II' is characterized by delayed onset and a steadily increasing reactivity upon repeated human Ab administration.

In the current study, MMA383 (IGN311 idiotype specific antibody) was used as calibrator for the quantitation of the observed HAHA response and expressed as 'MMA383 equivalents'. Out of 11 patients, six developed a HAHA response which can be assigned to Type I. Furthermore, we observed no induced reactivity against a human IgG1/κ isotype matched control antibody indicating that the HAHA response is directed against the antigen binding site of IGN311. In patient 1 (belonging to the low dose group), a HAHA response of about 15 μg/mL MMA383 equivalents was observed. The HAHA response in the five other patients which received higher doses of IGN311 was generally 10-fold lower than in patient 1 indicating that the magnitude of HAHA response is not dose-dependent.

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