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Evaluation of a biosensor immunoassay for simultaneous characterization of isotype and binding region of human anti-tocilizumab antibodies with control by surrogate standards

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ABSTRACT

This article describes the simultaneous Biacore analysis of human anti-human antibodies (HAHAs) with respect to the binding region and the isotype by a combination of 11 single measurements per sample. The multiplexing single assay setup made efficient use of the four parallel flow cells on one biosensor chip by immobilization of full-length antibody and its constant (Fc) and antigen binding (Fab) fragments for differential binding analysis of anti-drug antibodies (ADAs). Thereby, a complete time-specific immunogenicity profile (intensity, isotype, specificity, and kinetics) of a patient could be obtained by assessing the response patterns of serially collected samples analyzed in a single measurement run. The use of functionally active standard conjugates allowed control of the assay performance throughout the whole procedure. The positive control standard conjugates mimicking polyclonal human ADAs of different isotypes were obtained by conjugating polyclonal rabbit antibodies against the therapeutic antibody to human immunoglobulin (Ig) M, IgG, or IgE. In this article, the qualification of the assay is demonstrated and the application of the methodology to six representative rheumatoid arthritis patients treated with the therapeutic humanized IgG1 antibody tocilizumab (anti-IL-6R) is shown to illustrate the versatility of the assay. The presented method allows one to differentiate specific ADAs from drug-unspecific responses (e.g., rheumatoid factors). In addition, the method can be used to discriminate between isotype responses of the IgG, IgM, and IgE types and, thereby, allows one to describe the time course of specific ADA formation and its disappearance on the single patient level.

Human monoclonal antibodies are established as a key therapeutic modality for a wide range of diseases [1]. However, despite the human nature of therapeutic proteins, immunogenic reactions against biological products, including therapeutic antibodies, are common and need to be monitored in clinical studies. Such immunogenic reactions can potentially lead to serious side effects and/or loss of efficacy [2]. Several techniques have been developed to detect and measure anti-drug antibodies (ADAs),[1] including enzyme-linked immunosorbent assays (ELISAs), electrochemiluminescence immunoassays (ECLIAs), radioimmunoprecipitation assays, surface plasmon resonance (SPR) assays, and bioassays [3]. The complexity of immunogenic reactions requires careful selection, validation, and interpretation of methods used for their evaluation [4]. The most widely used technique to measure ADAs are ELISA formats, whereas reports on the use of SPR methods to detect antibodies against therapeutic proteins are less frequent [5–10].

SPR-based methods (e.g., performed on Biacore instruments) analyze binding of an ADA to the immobilized drug in real time, and a secondary antibody is currently used only to prove the antibody nature of the response. So far, Biacore methods typically have been set up for the semiquantitative analysis of ADAs but do not characterize the binding region and the isotype in the same experimental run [6]. Knowledge of the binding region and the isotype may be helpful to differentiate specific ADAs from drug-unspecific responses such as unspecific immunoglobulin (Ig)M antibodies directed against the Fc fragment of immunoglobulins (i.e., rheumatoid factors) and to help in the interpretation of clinical events by identifying the isotype of the ADA response (e.g., IgE response in allergic reactions). Qualitative SPR-based methods for antibody isotype or subclass analysis of ADAs have been reported for

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[1] *Abbreviations used:* ADA, anti-drug antibody; ELISA, enzyme-linked immunosorbent assay; ECLIA, electrochemiluminescence immunoassay; SPR, surface plasmon resonance; Ig, immunoglobulin; Fc, fragment, crystallizable (constant part of immunoglobulin); HAHA, human anti-human antibody; Fab, antigen-binding fragment; IL-6R, interleukin-6 receptor; CM–dextran, carboxymethyl–dextran; H_3PO_4, phosphoric acid; NHS, *N*-hydroxysuccinimide; SATP, *N*-succinimidyl-3-acetylthiopropionate; MHS, maleimidohexanoyl-*N*-hydroxysuccinimide; HPLC, high-performance liquid chromatography; MAB, monoclonal antibody; PAB, polyclonal antibody; LC, light chain; FC, flow cell; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; RU, resonance units; SD, standard deviations; CV, coefficient of variation; LLRD, lower limit of reliable detection; UV, ultraviolet; RF, rheumatoid factor.

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therapeutic proteins [11] and antibodies [7] but were performed in follow-up assays or by use of off-line sample pretreatment and not in a single assay setup. Typically, epitope analyses using the Biacore methodology have been described as individual competitive experiments that were performed by the addition of peptides or protein fragments competing with the ADA in the sample for binding to the full-length drug immobilized to the chip surface [7,9] or used isotype depletion steps [5]. Such sample pretreatment methods could cause false-negative or false-positive results and substantially consumed sample volume.

This report describes an SPR technology-based assay setup that combined the characterization of the binding region and the isotype identification of human anti-human antibodies (HAHAs) directed against a humanized therapeutic antibody in one single analytical run consisting of 11 measurements per sample. The method was qualified and each run was quality-controlled by using surrogate control antibodies based on rabbit anti-tocilizumab conjugated to human IgE, IgM, or IgG as specific positive controls. To the best of our knowledge, the use of such conjugates as positive controls is novel and has not yet been published. Simultaneous analysis of antibodies to the whole IgG, Fab, and Fc fragments was demonstrated as an effective way to obtain data about antibody specificity and facilitate the isotype analysis. To illustrate the versatility of the assay, this article describes the ADA analysis of a set of clinical serum samples from six representative patients treated with the therapeutic antibody tocilizumab (a humanized monoclonal antibody directed against interleukin-6 receptor [IL-6R]) in phase III clinical trials. By interpreting the response patterns at several time points, the analyses revealed a complete immunogenicity profile for each patient and illustrated a range of different types of response to a therapeutic antibody.

Materials and methods

Equipment and materials

A Biacore 2000 instrument and corresponding sensor chips of the CM5 type were used for all experiments (Biacore, Uppsala, Sweden). Sample evaluations were done using BIAevaluation and Control (version 4.1) software obtained from Biacore. An Amine Coupling Kit, HBS-P buffer (10 mM Hepes [pH 7.4], 150 mM NaCl, and 0.005% surfactant P20), and 10 mM Na-acetate buffer (pH 4.5) were obtained from Biacore. Carboxymethyl–dextran (CM–dextran) and phosphoric acid (H_3PO_4), used as regeneration solution, were acquired from Fluka/Sigma–Aldrich (Taufkirchen, Germany). A human pooled serum basematrix (TRINA Bioreactives, Naenikon, Switzerland) was used to prepare a sample dilution buffer consisting of HBS-P buffer (adjusted to 300 mM NaCl), 1.5 mg/ml CM–dextran, and 5% normal human serum that was used for dilution of all standards except the anti-Fc–IgG positive control, which was diluted with HBS-P/CM–dextran buffer without human serum. The buffer used for the dilution of serum samples was also serum free. Control samples of healthy human volunteers and of patients suffering from rheumatoid arthritis were used to evaluate the cut point of the assay and were obtained from Huntingdon Life Sciences (Huntingdon, UK). The biotin binding protein neutravidin was purchased from Pierce (Rockford, IL, USA). Soluble IL-6R was obtained from R&D Systems (Wiesbaden, Germany).

Ligands

The recombinant humanized monoclonal IgG1 antibody tocilizumab was produced by Chugai (Tokyo, Japan) and is directed against the human IL-6R [12]. The monovalent antigen binding (Fab) fragment and the constant (Fc) fragment of tocilizumab were prepared by papain digestion and subsequent purification according to standard techniques. Coupling of the full-length tocilizumab and its Fab and Fc fragments to biotin was performed by *N*-hydroxysuccinimide (NHS) coupling chemistry. A low labeling rate was chosen to avoid epitope masking. The content of the Fc part in the Fab preparation was less than 1% as determined in an Fc-specific ELISA using an anti-human Fc antibody. Binding of biotinylated Fab to IL-6R was comparable to that of nonbiotinylated Fab and tocilizumab–IgG.

Positive controls

For the purpose of quality control and use as calibration standards, isotype-specific ADA conjugates were prepared as positive controls. In brief, five rabbits were immunized with tocilizumab–Fab fragments according to standard methods. The lipid components in rabbit raw serum were removed by delipidation with 1.5% (w/v) Aerosil. The immunoglobulins in the delipidated serum were precipitated with 1.7 M ammonium sulfate. After acid treatment (30 min, pH 5.5) and dialysis against 15 mM potassium phosphate buffer supplemented with 50 mM NaCl (pH 7.0), the mixture was separated by diethylaminoethyl ion exchange chromatography at pH 7.0. The immunoglobulin fraction with the polyclonal anti-tocilizumab antibodies was in the flow-through and concentrated to approximately 25 mg/ml. Negative immunosorption using a column with polyclonal human antibodies pooled from different donors and subsequent positive immunosorption using a column packed with tocilizumab were applied to the concentrated rabbit IgG fraction. The affinity-purified antibody was coupled to *N*-succinimidyl-3-acetylthiopropionate (SATP) and covalently linked to a purified human polyclonal IgG, IgM, or IgE preparation that was itself coupled to maleimidohexanoyl-*N*-hydroxysuccinimide (MHS) ester. The coupling stoichiometries of the affinity-purified antibody with the respective human immunoglobulins were 1:3 with IgM, 1:1.7 with IgE, and 1:2 with IgG. The slight excess of human immunoglobulins served to ensure coupling of the rabbit anti-tocilizumab antibody. The conjugates were separated and purified via size exclusion chromatography (Sephacryl S-300, Pharmacia, Uppsala, Sweden). Only those fractions that were demonstrated to bind simultaneously to tocilizumab and the respective anti-human immunoglobulin isotype antibody, and had an apparent molecular mass of more than 300 kDa, were pooled and used. The conjugates were analyzed by ELISA for their concentration of human immunoglobulin and of the rabbit anti-tocilizumab. Results showed similar concentrations of both components of the conjugates, suggesting a final molar ratio of 1:1. The conjugates were separated with high-performance liquid chromatography (HPLC) on size exclusion columns TSK 5000 for IgM, TSK 4000 for IgE, and TSK 3000 for IgG to determine the molecular mass based on calibration curves of standard proteins. The molecular masses of conjugates containing IgM were in the range of 1–4 MDa, those containing IgE were in the range of 0.3–1.5 MDa, and those containing IgG were in the range of 0.5–1.5 MDa. The data suggest that the conjugate pools contained a mixture of heterodimers up to hexamers with an average molar ratio of 1:1 of rabbit anti-tocilizumab to human immunoglobulin. The method was reproduced several times. This resulted in the following conjugates: polyclonal rabbit anti-tocilizumab–human IgG, –human IgM, and –human IgE. As a positive control for a human anti-Fc binding antibody, an additional conjugate was prepared with the same technique; a mouse monoclonal antibody recognizing the Fc part of all human IgG subclasses (IgG1–4) [13] was coupled to purified polyclonal human IgG Fab fragments, resulting in an anti-human Fc human IgG(Fab) antibody. Specificities and isotypes of all positive control conjugates are shown in Table 1. A direct comparison of the conjugates of rabbit anti-tocilizumab and human immunoglobulin with

Table 1
Specificity and isotypes of positive control samples.

Conjugate	Specificity	Isotype
Anti-tocilizumab–IgG	Tocilizumab CDR	Human IgG
Anti-tocilizumab–IgE	Tocilizumab CDR	Human IgM
Anti-tocilizumab–IgM	Tocilizumab CDR	Human IgE
Anti-Fc–IgG (Fab)	Human IgG Fc fragment	Human IgG (Fab)

Note. CDR, complementarity-determining region.

affinity-purified ADAs from the same patients was not feasible due to very limited volumes of patient samples.

Secondary antibodies

Polyclonal rabbit antibodies PAB<human IgG> (Cat. No. 109-005-098) and PAB<human IgM> (Cat. No. 109-005-129) directed against the Fc fragment of human IgG (anti-IgG(Fc)) isotype and against the IgM (anti-IgM) isotype were obtained from Dianova (Hamburg, Germany). Monoclonal mouse antibodies MAB<human IgE> and MAB<human light chain> directed against the human IgE (anti-IgE) isotype and the human light chain of IgG (anti-IgG(LC)), commercially available from Roche Diagnostics (Penzberg, Germany), were used.

Clinical samples

Serum samples were obtained from six patients suffering from rheumatoid arthritis who participated in a clinical study for the treatment with tocilizumab. All study patients had given their informed consent for participation in the study, including blood withdrawal and sample analysis according to the study protocol. Samples were taken before (baseline) and repeatedly during and after treatment. Each of the six patients was tested ADA positive in a dedicated screening and confirmatory ELISA for at least one time point (data not shown).

Chip preparation

Fig. 1 illustrates the setup of the biosensor method and the sequence of injections. All four flow cells (FCs) of the CM5 chip were coated with neutravidin by injection of 100 μg/ml neutravidin over all four FCs in parallel at a flow rate of 20 μl/min for 5 min after previous activation of the FCs by injection of 125 μl NHS–EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide) (Amine Coupling Kit). To inactivate residual esters, 140 μl of 1 M ethanolamine (Amine Coupling Kit) was injected. Coupling of neutravidin resulted in at least 6000 resonance units (RU). Regeneration of the chip was done by four injections of 20 μl of regeneration solution (100 mM H_3PO_4 solution). Binding of the different ligands to the three FCs was done by separate injections of 75 μg/ml biotinylated full-length Fc or Fab fragmented tocilizumab in HBS-P buffer at a flow rate of 20 μl/min for 5 min. At least 4000 RU, but typically between 6000 and 8000 RU, of the ligands were immobilized. FC1 was left unbound as a blank control. All FCs were conditioned by repeated injection of 20 μl of regeneration solution to remove unbound and unstable ligand. The use of biotinylated ligands bound to neutravidin resulted in better assay sensitivity compared with direct ligand binding to the chip (unpublished data). Neutravidin is a derivate of streptavidin that had been used to reduce blank signals.

Application runs

Positive controls or samples were injected to all four FCs with a volume of 20 μl injected for 1 min (primary response). For isotype determination (secondary response), the secondary detection antibodies were subsequently injected without prior regeneration in the sequence of (i) anti-IgE, (ii) anti-IgM, (iii) anti-IgG(LC), and (iv) anti-IgG(Fc) as depicted in Fig. 1 with a volume of 20 μl each injected for 1 min. Afterward, one injection of 20 μl of regeneration solution dissolved the bound complex, and the chip surface containing the immobilized drug was conditioned for the next sample.

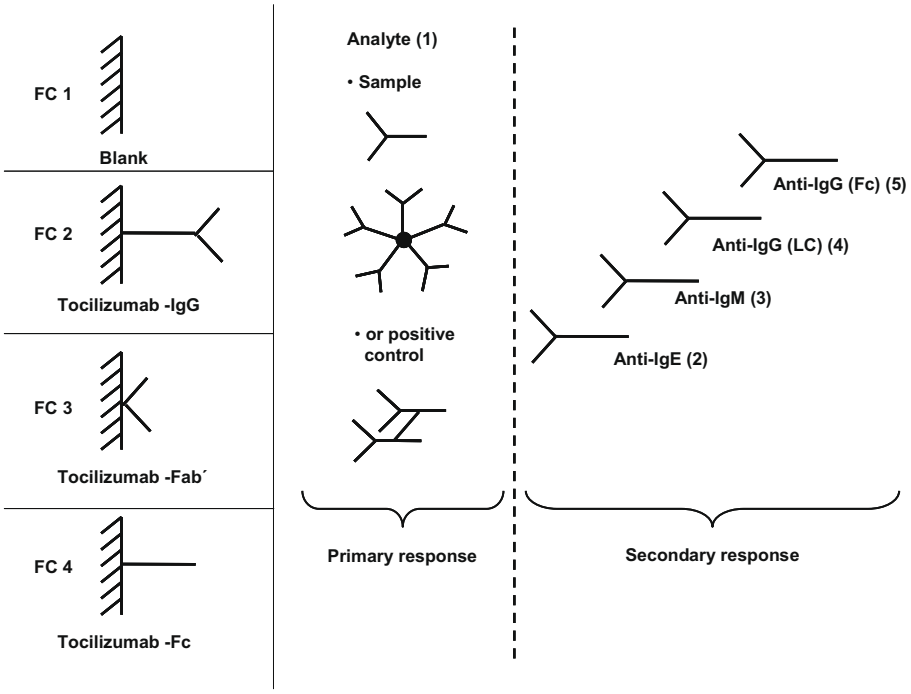

Fig. 1. Schematic view of the Biacore assay setup and sequence of injections. For better illustration, the symbol for (Fab′)2 (<) is used for Fab′.

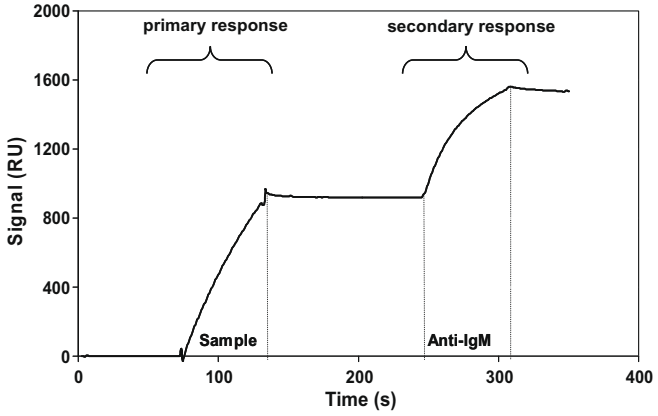

Fig. 2. Typical sensogram for an IgM positive control conjugate injected over FC3 containing the tocilizumab–Fab fragment as ligand (primary response), followed by the injection of the anti-IgM detection antibody (secondary response). Data shown are not referenced (data with no blank subtraction).

A typical complete set of cycles of a sample measurement series with a new chip starts with 10 dilution buffer runs followed by the analysis of 12 positive controls (three concentrations for each of the four conjugates listed in Table 1) and 30 individual human blank serum samples to determine the assay-specific cutoff value. Although two dilution buffer runs already resulted in at least 90% elimination of incompletely immobilized protein, 10 conditioning cycles were applied to optimize conditioning as recommended by the manufacturer. Subsequently, up to 45 different test samples were analyzed, after which all 12 positive controls were repeated to ensure remaining chip quality. Patient and control samples were analyzed as single replicates in the order of their time sequence.

Positive controls were prepared at concentrations around the expected cutoff values (final concentrations in the assay after 1:20 dilution: 0.5, 1.0, and 1.5 μg/ml for anti-tocilizumab–IgE and anti-tocilizumab–IgG; 1.0, 2.0, and 3.0 μg/ml for anti-tocilizumab–IgM; and 0.5, 1.0, and 2.0 μg/ml for anti-Fc–IgG positive controls). Serum samples were diluted 1:20 with dilution buffer and placed in the temperature-controlled sample rack at 10 °C, whereas the chip was kept at 25 °C. Each analytical run consisted of a first step to detect the ADA and to describe its binding region (primary response) followed by a second step to confirm that the ADA was a human immunoglobulin as well as to determine its isotype (secondary response). Antibody primary and secondary responses were recorded as relative RU above the respective baseline value.

Results and discussion

Method qualification

Validation of Biacore assays for ADA analysis according to ICH guidelines [4] so far has been published only for those measuring a primary response (binding) but not for measuring secondary responses as described in the current article for use as a qualitative, differential ADA analysis. The method described here did not serve only as an ADA screening or confirmatory assay; rather, it served to also analyze, in an exploratory manner, the nature of ADAs identified in previous assays. The rather complex logistics of a measurement cycle, including dilution buffer runs, positive control runs at the beginning and end of the cycle, and blank serum runs for intra-assay determination of the assay-specific cutoff value, considerably limited the number of possible sample measurements within a cycle. For these practical reasons, only single measurements of samples were performed. Repeat measurements of 5% of the initially analyzed samples confirmed the findings of the first measurement. Furthermore, the same sample was analyzed in three FCs, with primary and secondary reactions allowing a plausibility check of the results. The use of the four positive controls primarily served to assess the validity of each analytical run. In this qualitative assay, a signal of at least 50% of the initial signal needed to be recovered for each control at the end of the analytical run and a signal below 100 RU should be obtained for the blank FC. Fig. 2 illustrates a typical result of an anti-tocilizumab–IgM positive control conjugate injected over FC3, with the primary response being the binding of the conjugate to the Fab fragment of tocilizumab and the secondary response being the binding of the anti-IgM antibody to the IgM part of the conjugate. A representative typical primary and secondary response pattern for the IgM conjugate that can be obtained is listed in Table 2. Positive responses in parentheses represent false-positive signals due to a cross reaction of the anti-IgG(LC) to tocilizumab–IgG (FC2) and tocilizumab–Fab (FC3) and of the anti-IgG(Fc) antibody to tocilizumab–IgG (FC2) and tocilizumab–Fc (FC4). An example for a primary and secondary response sequence on FC3 (tocilizumab–Fab) of a patient is given in Fig. 3.

During method qualification, the stability of the three immobilized tocilizumab ligands during 120 regeneration cycles of one chip was evaluated by injecting all positive control conjugates after every 10th cycle. Each regeneration cycle was preceded by injection of a serum sample or a standard conjugate. Residual ligand activity after 120 cycles typically was approximately 100% and only in rare cases was lowered to approximately 50%. Therefore, no significant influence on the binding of all four standard conjugates was observed. For routine use of the method, the maximum number of regeneration cycles before change of the chip was limited to 109 (10 preruns, 24 positive controls, 30 individual cutoff sera, and 45 samples). Residual ligand activity needed to be higher than 50% for acceptance, which was sufficient to detect ADA re-

Table 2
Primary and secondary response pattern for anti-tocilizumab–IgM conjugate.

FC	Immobilized tocilizumab	Primary response	Secondary response			
			Anti-IgE	Anti-IgM	Anti-IgG(LC)	Anti-IgG(Fc)
1	—	—	—	—	—	—
2	IgG	+	—	+	(+)	(+)
3	Fab	+	—	+	(+)	—
4	Fc	—	—	—	—	(+)

Note. Parentheses represent a false-positive signal due to a shortcut reaction. In the absence of sample ADA, the secondary reaction antibodies directly bind to the immobilized tocilizumab–IgG/Fab/Fc.

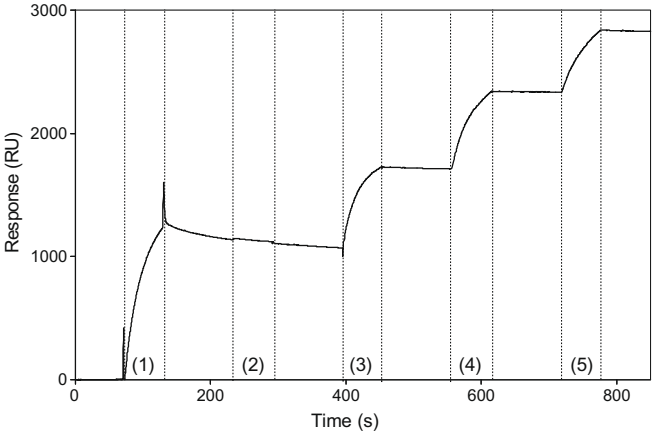

Fig. 3. Primary (1) and secondary (2–4) response signals of a serum sample of patient A on the FC containing the immobilized tocilizumab–Fab fragment (FC3).

Table 3
Sensitivity and LLRD of all responses.

	Tocilizumab–IgG		Tocilizumab–Fab		Tocilizumab–Fc	
	Sensitivity	LLRD	Sensitivity	LLRD	Sensitivity	LLRD
(a) Primary response: sensitivity and LLRD						
Anti-tocilizumab–IgE	6.8	10	14.8	20	n.a.	n.a.
Anti-tocilizumab–IgM	21.0	40	36.0	40	n.a.	n.a.
Anti-tocilizumab–IgG	9.2	10	14.0	25	n.a.	n.a.
Anti-Fc–IgG(Fab)	17.8	n.a.	n.a.	n.a.	21.6	n.a.
	IgG		Fab		Fc	
	Sensitivity	LLRD	Sensitivity	LLRD	Sensitivity	LLRD
(b) Secondary response: sensitivity and LLRD						
Anti-IgE	8.6	10	9.2	10	n.a.	n.a.
Anti-IgM	20.0	40	17.0	20	n.a.	n.a.
Anti-IgG(LC)	n.a.	n.a.	11.2	15	n.a.	n.a.
Anti-IgG(Fc)	n.a.	n.a.	n.a.	n.a.	12.2	n.a.

Note. Values are in μg/ml reference standard equivalents. n.a., not applicable.

sponses. A total of 78 measurement series (78 different chips) demonstrated that residual activity of ligands always was higher than 50%.

Short-term analyte stability for 48 h in the cooled sample rack, which was the maximum duration of a complete analytical run, was assessed by showing that signal recovery of the standard conjugates analyzed at two different concentrations stored at 4 and 10 °C and analyzed at 3, 24, and 72 h was between 81.2% and 106.9% compared with the controls stored at −80 °C. Long-term stability of positive controls stored at 4 and 25 °C for 4 months was demonstrated by showing that signal recovery was between 80.2% and 117.7%.

Cutoff value determinations for all five responses of each FC were performed using serum samples from 30 healthy human volunteers and 30 rheumatoid arthritis patients for each response. The cutoff value was defined as the arithmetical mean + 3 standard deviations (SD). Interassay precision of the cutoff value ("chip-to-chip" variability) was evaluated by using three different CM5 chips at three different days to determine the cutoff values. The chip-to-chip variability expressed as the coefficient of variation (CV) over all three measurements was between 3% and 27% and, therefore, was not considered as acceptable variability. To minimize the influence of the variability of different chip lots observed in pilot experiments, it was decided to evaluate the cutoff value for each analytical run of a new chip.

Although the method is intended to be used as a qualitative method, its sensitivity and lower limit of reliable detection (LLRD) were evaluated by spiking different concentrations of positive controls into pooled serum and individual serum samples. A series over a range of 400 ng/ml to 50 μg/ml (neat serum concentrations) was used to establish a calibration curve for each positive control conjugate. The theoretical assay sensitivity was defined as the back-calculated value of the cutoff value and is expressed in micrograms per milliliter (μg/ml) reference standard equivalents. The assay sensitivity ranged from 6.8 μg/ml for anti-tocilizumab–IgE bound to full-length tocilizumab to 36 μg/ml for anti-tocilizumab–IgM bound to tocilizumab–Fab. For the secondary responses, theoretical sensitivity was in the range of 8.6–20.0 μg/ml. The LLRD was defined as the lowest positive control concentration that consistently gave a response above the cutoff value. For this purpose, positive controls were spiked into the individual serum of 10 healthy volunteers and 10 patients at three different concentrations near the cutoff point. For primary and secondary responses, the LLRD was in the range of 10–40 μg/ml, depending on the respective conjugate. Values for sensitivity and LLRD are listed in Table 3. Because the concentration of the standard conjugates was based on ultraviolet (UV) spectroscopy instead of an active concentration, the maximum sensitivity to be expected was 50% assuming a 1:1 stoichiometry (rabbit anti-tocilizumab:human IgG/IgE). Thus, these sensitivity values could not directly be compared with data for other types of positive controls. The use of neutravidin instead of streptavidin for coating reduced a potentially high background level induced by unspecific binding.

Selectivity of the method for detection of ADA was evaluated by testing interference with the drug target (IL-6R) in spiked human serum samples over a range of 10–600 ng/ml IL-6R. Typical predose and placebo group soluble IL-6R levels in the studies were in the range of 10–100 ng/ml but increased up to 250–600 ng/ml in tocilizumab-treated patients. The soluble human IL-6R spiked into blank samples did induce a primary false-positive result at IL-6R concentrations higher than 0.31 μg/ml (for tocilizumab–IgG) and 0.63 μg/ml (for tocilizumab–Fab). However, it did not generate a positive secondary response, allowing one to identify the primary response as false positive. As expected, cospiking soluble IL-6R and positive controls led to an overestimation of the

Table 4
Binding regions of ADAs evidenced by primary responses.

Patient	FC	Immobilized tocilizumab	Primary response (RU) at										
			Cutoff	0 weeks	2 weeks	4 weeks	6 weeks	8 weeks	12 weeks	24 weeks	28 weeks	36 weeks	40 weeks
A	2	IgG	75	78*	74	74	960*	730*	1179*	n.a.	n.a.	n.a.	n.a.
A	3	Fab	81	33	22	26	1610*	1112*	1754*	n.a.	n.a.	n.a.	n.a.
A	4	Fc	77	75	75	72	73	75	102*	n.a.	n.a.	n.a.	n.a.
B	2	IgG	105	21	n.a.	349*	n.a.	187*	103	23	n.a.	n.a.	n.a.
B	3	Fab	103	17	n.a.	523*	n.a.	205*	84	21	n.a.	n.a.	n.a.
B	4	Fc	86	26	n.a.	39	n.a.	42	35	23	n.a.	n.a.	n.a.
C	2	IgG	33	43*	n.a.	45*	n.a.	45*	65*	1276*	n.a.	n.a.	n.a.
C	3	Fab	69	11	n.a.	13	n.a.	12	13	1639*	n.a.	n.a.	n.a.
C	4	Fc	48	62*	n.a.	68*	n.a.	66*	66*	82*	n.a.	n.a.	n.a.
D	2	IgG	73	89*	91*	92*	93*	93*	n.a.	n.a.	n.a.	n.a.	n.a.
D	3	Fab	55	20	20	14	17	19	n.a.	n.a.	n.a.	n.a.	n.a.
D	4	Fc	86	118*	125*	139*	137*	137*	n.a.	n.a.	n.a.	n.a.	n.a.
E	2	IgG	69	47	n.a.	44	n.a.	352*	385*	n.a.	n.a.	n.a.	n.a.
E	3	Fab	82	37	n.a.	39	n.a.	515*	370*	n.a.	n.a.	n.a.	n.a.
E	4	Fc	79	51	n.a.	50	n.a.	48	57	n.a.	n.a.	n.a.	n.a.
F	2	IgG	50	48	n.a.	n.a.	n.a.	n.a.	40	33	207*	84*	66*
F	3	Fab	55	15	n.a.	n.a.	n.a.	n.a.	14	15	260*	91*	66*
F	4	Fc	69	45	n.a.	n.a.	n.a.	n.a.	45	48	222*	99*	82*

Note. Single asterisk (*) indicates positive response; n.a., not applicable.

positive primary response. The secondary response allowed one to discriminate between false-positive and negative results, but it cannot identify overestimation. Therefore, the method should be regarded as a qualitative method and does not allow semiquantification.

Analysis of patient samples

The purpose of the current Biacore method was to confirm the presence of human ADAs of tocilizumab-treated patients that were identified previously by ELISA screening assays and to simulta-

Table 5
Immunoglobulin isotypes of ADAs evidenced by secondary responses.

(a) Patient D: Unspecific IgM response

FC	Immobilizedtocilizumab	IgM secondary response (RU) at					
		Cutoff	0 weeks	2 weeks	4 weeks	6 weeks	8 weeks
2	IgG	46	70*	79*	105*	107*	74*
4	Fc	57	90*	101*	147*	144*	112*
		IgG secondary response (RU) at					
		Cutoff	0 weeks	2 weeks	4 weeks	6 weeks	8 weeks
4	Fc	35	13	14	13	13	33

(b) Patient C: unspecific IgM response converting to specific IgM and IgG response

FC	Immobilized tocilizumab	IgM secondary response (RU) at					
		Cutoff	0 weeks	4 weeks	8 weeks	12 weeks	24 weeks
2	IgG	24	27*	29*	28*	26*	593*
3	Fab	50	8	9	8	7	446*
4	Fc	37	52*	59*	55*	52*	71*
		IgG secondary response (RU) at					
		Cutoff	0 weeks	4 weeks	8 weeks	12 weeks	24 weeks
3	Fab	49	34	34	33	34	736*
4	Fc	35	13	14	13	13	33

(c) Patient E: Specific IgM and IgG response

FC	Immobilizedtocilizumab	IgM secondary response (RU) at				
		Cutoff	0 weeks	4 weeks	8 weeks	12 weeks
2	IgG	34	14	15	139*	136*
3	Fab	39	10	12	112*	46*
4	Fc	46	23	24	20	25
		IgG secondary response (RU) at				
		Cutoff	0 weeks	4 weeks	8 weeks	12 weeks
3	Fab	49	55**	52**	367*	364*
4	Fc	13	10	10	10	16*

(d) Patient B: Transient specific IgG response

FC	Immobilized tocilizumab	IgM secondary response (RU) at					
		Cutoff	0 weeks	4 weeks	8 weeks	12 weeks	24 weeks
2	IgG	40	6	32	27	15	9
3	Fab	38	11	20	25	15	12
4	Fc	55	4	11	14	10	4
		IgG secondary response (RU) at					
		Cutoff	0 weeks	4 weeks	8 weeks	12 weeks	24 weeks
3	Fab	45	27	387*	212*	113*	29
4	Fc	28	24	12	12	12	23

(e) Patient F: Specific IgE and IgM response

FC	Immobilized tocilizumab	IgE secondary response (RU) at						
		Cutoff	0 weeks	12 weeks	24 weeks	28 weeks	36 weeks	40 weeks
2	IgG	8.3	7.8	7.8	12.9*	17.3*	10.2*	9.2*
3	Fab	6.2	4.8	5.7	n.d.	18.6*	9.9*	8.5*
4	Fc	16	10	11	10	22*	14	14
		IgM secondary response (RU) at						
		Cutoff	0 weeks	12 weeks	24 weeks	28 weeks	36 weeks	40 weeks
2	IgG	38	13	13	14	231*	84*	63*
3	Fab	47	10	11	11	308*	112*	80*
4	Fc	44	22	21	25	241*	91*	73*
		IgG secondary response (RU) at						
		Cutoff	0 weeks	12 weeks	24 weeks	28 weeks	36 weeks	40 weeks
3	Fab	66	44	44	45	52	53	53
	Fc	18	12	12	11	12	12	13

Note. Single asterisk (*) indicates positive response; double asterisk (**) indicates positive secondary response but negative primary response.

neously further characterize ADAs for the binding region and immunoglobulin isotype. Table 4 summarizes the primary response results for different samples of six patients. Five of the six patients (all except patient D) show a positive ADA response against tocilizumab–IgG and tocilizumab–Fab but differ in the time course of ADA appearance and disappearance. Patient B displays an early and transient immune response, whereas patients A, E, and F do not show a positive result prior to weeks 6, 8, and 28, respectively. Samples that were positive for tocilizumab–Fab also tested positive for tocilizumab–IgG. Positive results for tocilizumab–IgG but not for tocilizumab–Fab or tocilizumab–Fc would be indicative of a false-positive signal and hint to a technical failure. Positive results for tocilizumab–IgG and tocilizumab–Fc but not for tocilizumab–Fab are indicative of a less drug-specific signal: Patients C and D follow this pattern even in the baseline sample. The response of patient C evolved to a specific anti-tocilizumab response in week 24. Due to the similar intensity of the anti-Fc response over the complete time course, including pretreatment, the possibility of antibodies against an IgG allotype being induced by the therapeutic antibody in individuals of a different allotype is unlikely. Therefore, the method allowed one to discriminate between reactions of high and low specificity such as those caused by rheumatoid factor (RF) that are known to be anti-Fc-binding antibodies of the IgM isotype.

The secondary response analyses were used to characterize the isotype of the ADAs. Detection antibodies against IgE and IgM revealed the respective isotypes. The IgG isotype identification required a special assay setup and was possible only for tocilizumab–Fab and tocilizumab–Fc due to false-positive cross-reactivity with the secondary anti-IgG antibodies to tocilizumab–IgG; an ADA of the IgG isotype directed against tocilizumab–Fab was detectable with the anti-IgG(Fc) antibody, whereas an ADA against tocilizumab–Fc was detectable with the anti-IgG(LC) antibody. Patient D, whose primary response was positive against tocilizumab–Fc from predose baseline to the last sample, displayed an IgM isotype response but not an IgG isotype response (Table 5a), a result again indicative of an RF response given that RFs are known to be IgM antibodies [14]. Because the pretreatment sample already showed high IgM levels, it appeared to be unlikely that the IgM detected was specific for the study drug. Patient D also showed a highly elevated baseline serum concentration of RF of 6140 U/ml, confirming this assumption. Patient C showed a similar unspecific early IgM response but developed a late Fab (antigen)-specific immune response of the IgG isotype at week 24 (Table 5b). Patient E represents an example of a tocilizumab-specific early IgM response against the Fab fragment simultaneously converting to an IgG isotype response (Table 5c). The ADA response observed in patient B at weeks 4 and 8 (Table 4b) was found to be an IgG response against Fab showing that IgG responses also can be of a transient nature (Table 5d). The method was also able to detect ADAs of the IgE isotype, as can be seen in Table 5e for patient F, who in parallel developed an IgM response against tocilizumab–Fab. Interestingly, this patient did not develop ADAs of the IgG isotype during the observed time course of the study but directly switched from an IgM response to an IgE response. However, it must be noted that, due to relatively low IgE levels in serum samples even in the case of an IgE-mediated adverse event, the sensitivity of this Biacore method might not be sufficient to detect ADA of the IgE isotype. The detection of IgE ADAs is further complicated by coexisting ADAs of the IgM and IgG classes. Typically, the levels of IgG and IgM are 100- to 1000-fold higher than those of IgE [15]. Therefore, the primary response is dominated by IgG and IgM. The detection of small amounts of bound IgE with a secondary anti-IgE antibody is also hampered by the simultaneous dissociation of ADAs with lower affinity. This dissociation results in a baseline drift that could mask the small signal increase of a secondary anti-IgE response.

This article has described a qualified method for the combined characterization of the binding region and isotype determination of human ADAs against a human therapeutic antibody in one single analytical run consisting of 11 individual measurements per sample. Current SPR-based methods used for the characterization of ADAs [5–10] are set up for the semiquantitative screening and confirmation of ADAs without further characterization of the binding region and the isotype. The assay described in this article made use of all four FCs of the chip, allowing one to separately use full-length antibody and its Fab and Fc fragments as binding targets on the chip for the evaluation of differential binding of ADAs in one single experiment. By using different detection antibodies for tocilizumab–Fab and tocilizumab–Fc, unspecific binding of IgG detection antibodies to the immobilized therapeutic IgG antibody can be avoided. In addition, knowledge of the binding region, the isotype, and the time course of the response allows one to differentiate drug-unspecific responses such as IgM-type RF-mediated responses against the Fc fragment of immunoglobulins from drug-specific ADA responses. The Biacore method described here was quality-controlled by developing four positive controls mimicking anti-tocilizumab ADAs of human IgM, IgE, and IgG isotypes. A response directed against the Fc part (e.g., RF) was mimicked by generating a conjugate of anti-human Fc antibodies coupled to human IgG.

Conclusions

A surrogate antibody-controlled SPR method has been developed to qualitatively characterize ADAs directed against the human therapeutic IgG1 antibody tocilizumab. The method combines, in a single assay setup, the characterization of the binding region and the isotype of ADAs in human serum samples by a label-free protein interaction analysis, thereby avoiding follow-up assay runs and competition experiments. The assay was qualified by using unique positive controls mimicking human ADAs obtained by conjugating rabbit anti-tocilizumab with human IgE, IgM, and IgG. This method was applied to the analysis of representative clinical samples of patients treated with tocilizumab to demonstrate its versatility. This is the first article describing differential characterization of human ADAs in a single Biacore assay setup controlled by functional positive standards. The application of this method enabled patient-specific determination of the ADA response and the correlation with clinical events. Although this assay has specifically been developed for analyzing anti-tocilizumab antibodies, the principle of the method may be used for other IgG-type human therapeutic antibodies. The study also demonstrated the utility of functionally active surrogate standard conjugates as positive controls for ADAs.

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