

THE RECOVERY-ELISA—A NOVEL ASSAY TECHNIQUE TO MONITOR THERAPY WITH HUMANIZED ANTIBODIES: THE EXAMPLE OF OMALIZUMAB

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□ *The therapeutic use of antibodies has grown exponentially, providing new treatment options for various diseases. Monitoring treatment with therapeutic antibodies is a particular challenge because often the target antigen is no longer measurable or the results are unreliable. To overcome this problem, a recovery-ELISA was developed to quantify therapeutic antibody and antigen by a modification of the traditional sandwich immunoassay using omalizumab as an example. Standard serum samples were spiked with IgE and omalizumab in a certain concentration range to create standard curves. After incubation and washout procedures, the reaction was stopped and the plate read with bichromatic absorbance. This approach defines reference functions for serum IgE without and with antibody, respectively. Based on these functions, free IgE and omalizumab concentrations can be calculated in serum samples of patients treated with omalizumab. The recovery-ELISA allows us to easily quantify antigen (free IgE) and therapeutic antibody (omalizumab) simultaneously in the same sample and thus to monitor patients treated with the specific antibody.*

Keywords antigen, asthma, ELISA, IgE, omalizumab, therapeutic antibody

INTRODUCTION

Over the past 10 years, the therapeutic use of antibodies has grown exponentially. This new generation of pharmaceuticals provides new treatment options for malignant, autoimmune, and inflammatory diseases. Many therapeutic antibodies have been approved in these indications, and many more are currently in advanced stages of clinical development. The large majority of these antibodies are monoclonal antibodies, and most of them are partly (human/mouse) or fully humanized to improve efficacy and safety.^[1]

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Omalizumab is a humanized monoclonal anti-immunoglobulin E (IgE) antibody indicated for the treatment of severe persistent allergic (IgE-mediated) asthma despite optimal treatment with inhaled corticosteroids and long-acting beta-2-agonists.^[2] Upon injection, omalizumab interrupts the allergic cascade by binding to the Fc region of free serum IgE, thereby blocking the binding of IgE to its receptors on effector cells. Omalizumab and IgE molecules form small, biologically inactive complexes.^[3,4] Consequently, free IgE serum concentrations are rapidly reduced, the expression of the high affinity IgE receptor (FcεRI) on inflammatory cells are downregulated, and mast cell and basophil activation and subsequent release of their inflammatory mediators are inhibited.^[5,6] The ability of omalizumab to lower free IgE in serum depends on dose and the patients' IgE load, defined by body weight and serum IgE concentration. Clinical trials suggest that benefit with omalizumab is observed when serum-free IgE concentrations are reduced to 50 ng/mL (20.8 IU/mL) or less.^[7,8] However, not all asthma patients receiving omalizumab respond to this treatment. This is even more consequential since, once omalizumab treatment is initiated, it is currently impossible to quantify free serum IgE as a means to confirm successful IgE elimination and guide future omalizumab dosing. Commercially available assays to quantify IgE recognize both free IgE and IgE molecules as part of omalizumab-IgE-complexes, leading to false IgE measurements. Thus, the decision as to whether omalizumab treatment is effective is currently solely based on the physicians' judgment.^[9,10]

To overcome this problem, a new recovery–enzyme linked immuno sorbent assay (R-ELISA) was developed to quantify both the therapeutic antibody (omalizumab) and the antigen (free IgE) in serum samples of patients treated with the antibody (omalizumab). The recovery-ELISA is a modification of the traditional sandwich immunoassay to account for the presence of the therapeutic antibody in the sample by a two-dimensional calibration.

EXPERIMENTAL

The experiment was undertaken with the following parameters:

Streptavidine-coated microplate: 96 cavities, SC-coating (BioTeZ method), coated with biotinylated capture antibody B388/030610, our own preparation

Assay buffer: 0.05 M PBS, pH = 7.2 + 0.01% caseine

hIgE standard: Calibrated against the WHO Reference Reagent, NIBSC code: 75/502

Control sample: IgE free serum

Therapeutic antibody (TA): Omalizumab

Signal antibody: Horseradish peroxidase (HRP)-omalizumab conjugate, our own preparation: P295, 110 ng/mL

Washing buffer: 0.01 M PBS, pH = 7.2/0.1% RSA +0.1% Tween 20

Chromogen dilution: tetramethylbenzidine (TMB)

Stopping dilution: 5 M sulfuric acid

The assay includes calibration and sample measurements on one microtiter plate as demonstrated in Table 1. The cavities of the microplate were precoated with streptavidine and biotinylated capture antibody. One-third of the plate was used for calibration measurements, the other two-thirds were used for measuring serum samples of patients. The calibration curves are based on IgE concentrations of 0, 20, 60, and 120 IU/mL and addition of omalizumab in concentrations of 0, 0.2, 1.0, and 4.0 µg/mL. Measurements were performed in duplicate according to ISO 9001 and the rules of Good Laboratory Practice.

Standard samples were spiked with antigen (IgE) in a certain concentration range with and without addition of therapeutic antibody (omalizumab), and standard curves were created. Blinded serum samples of patients treated with omalizumab were diluted 1:20 with dilution buffer and spiked with increasing amounts of IgE standard (20, 60, and 90 IU/mL). Standards, samples, therapeutic antibody, and the signal antibody (25 µL/well each) were pipetted into the cavities of the microplate (standards: 50 µL HRP-omalizumab +25 µL omalizumab +25 µL IgE into wells A–H, 1–4,

TABLE 1 Diagrammatical Representation of the Microtiter Plate Allocation for Standards, Samples, and Therapeutic Antibodies

Standards					Samples							
	1	2	3	4	5	6	7	8	9	10	11	12
					IgE-Spiking				[IU/ml]:			
IgE [IU/ml]	0	20	60	120	0	0	20	20	60	60	90	90
TA [µg/ml]:					Str. 5-12: Samples							
A	0				—				—			
B	0								Sample 1			
C	0.2								Sample 2			
D	0.2								Sample 3			
E	1								Sample 4			
F	1								Control			
G	4								Sample 5			
H	4								Sample 6			
									Sample 7			
									Dilution			

TA: therapeutic antibody.

samples: 50 μ L HRP-omalizumab +25 μ L sample +25 μ L IgE into wells A–H, 5–12 according to Table 1).

The plate was incubated for 18 h at 2–8°C in darkness. During the incubation, the biotinylated capture antibody and the signal antibody-peroxidase-conjugate reacted with two different binding IgE epitopes. Thus a complex of biotinylated capture antibody, IgE, and signal antibody-peroxidase-conjugate (sandwich) was formed, which was immobilized at the capture antibody coated surface of the cavities of the microplate. The incubation was terminated by a washing procedure. Cavities were washed three times with washing solution (100 μ L buffer: 0.01 M PBS, pH = 7.2/0.1% BSA + 0.1% Tween 20), and 100 μ L of TMB chromogen solution were added. Subsequently, the plate was incubated for 30 min at room temperature in the dark. During this phase, TMB and the complex of biotinylated anti-IgE-antibody, IgE, and signal antibody-peroxidase-conjugate bound to the streptavidine surface of the microplate. Upon addition of the chromogen dilution to the cavities, a blue stain developed. This reaction was stopped by adding H₂SO₄ (5 M), and the color changed to yellow. The plate was read with bichromatic absorbance at 450 nm with 620 nm as reference (TECAN Spectra Flour; serial number: 93385). Measured values were calculated using XLSTAT-Pro2010 (Addinsoft, Andernach, Germany). The extinction rises with increasing concentrations of IgE and falls with increasing concentration of unlabeled omalizumab.

RESULTS

1. Reference Function for Serum IgE Without TA (Table 2)

In a first step, standard samples spiked with increasing amounts of IgE (0, 20, 60, and 90 IU/mL) were measured in duplicate, and the average of the extinctions (O.D. = optical density) was calculated. The nonspecific binding (NSB; means of the extinctions at standard 0 = 0.046) was subtracted from all average extinctions. Using a nonlinear regression model (BioTeZ software program) with the function $y = a \cdot x / (b + x)$ (Michaelis–Menten equation),

TABLE 2 Reference Function for IgE (TA = 0 μ g/mL)

X=IgE [IU/mL]	Extinctions (O.D.)	Y O.D.-NSB	Calculated IgE concentration [IU/mL]	Recovery (calculated/spiked IgE)
0	0.046	0.000		
20	1.103	1.056	19.56	0.978
60	1.861	1.815	62.66	1.044
120	2.182	2.136	116.13	0.968
Average of recovery (AR)				0.997
Standard deviation of recovery (SDR)				0.042

O.D.: optical density, NSB: non-specific binding.

the reference function $Y = 2.693 * X / (30.287 + X)$ could be designed [$R^2 = 0.9996$ (R^2 = coefficient of determination)]. Using the inverse function $X = 30.287 * Y / (2.693 - Y)$, the X (IgE) values could be calculated from all Y (O.D. – NSB) values (calculated IgE concentration). Subsequently the recovery of IgE was determined by dividing calculated IgE concentration by spiked IgE concentration (recovery = calculated IgE concentration / spiked IgE concentration).

2. Reference Function for IgE with TA (Table 3)

Similar to step one, standard samples were spiked with increasing IgE concentrations (0, 20, 60, and 90 IU/mL) and also with increasing TA

TABLE 3 Reference Function for IgE and Different TA Concentrations

Results for TA = 0.2 µg/mL				
X=IgE [IU/mL]	Extinctions (O.D.)	Y O.D.-NSB	Calculated IgE [IU/mL]	Recovery
0	0.042	−0.004		
20	0.869	0.823	13.32	0.666
60	1.616	1.570	42.34	0.706
120	1.975	1.929	76.48	0.637
			AR:	0.670
			SDR:	0.034
Standard curve for TA = 1 µg/mL				
X=IgE [IU/mL]	Extinctions (O.D.)	Y O.D.-NSB	Calculated IgE [IU/mL]	Recovery
0	0.044	−0.002		
20	0.487	0.441	5.93	0.296
60	1.074	1.028	18.70	0.312
120	1.416	1.370	31.35	0.261
			AR:	0.290
			SDR:	0.026
Standard curve for TA = 4 µg/mL				
X=IgE [IU/mL]	Extinctions (O.D.)	Y O.D.-NSB	Calculated IgE [IU/mL]	Recovery
0	0.041	−0.005		
20	0.188	0.142	1.68	0.084
60	0.517	0.471	6.43	0.107
120	0.825	0.779	12.32	0.103
			AR:	0.098
			SDR:	0.012

TA: therapeutic antibody, O.D.: optical density, AR: average recovery, SDR: Standard deviation of recovery.

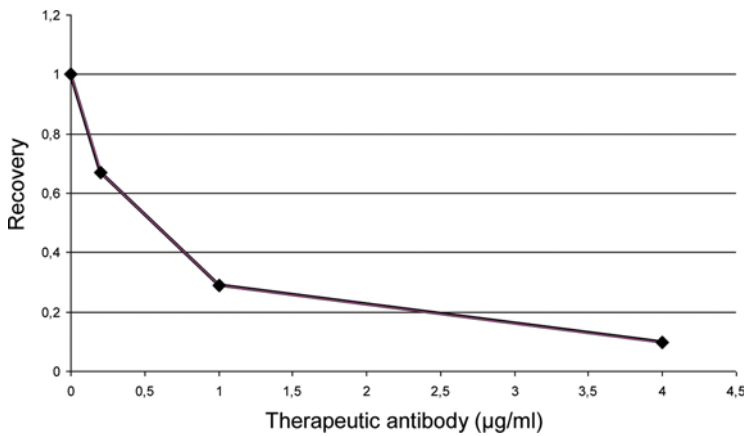


FIGURE 1 Recovery curve (color figure available online).

concentrations (0, 0.2, 1.0, and 4.0 µg/mL). Average recovery (AR) was calculated as described in step 1 for every TA concentration (Table 3). As expected, AR decreased with increasing TA concentrations, i.e., it is possible to create a recovery curve for the different TA concentrations (Figure 1).

Using a nonlinear regression model (BioTeZ software program) with the function $AR = 1 / (1 + b * TA) + a$, the recovery function $AR = 1 / (1 + 2.47 * TA) + 0.002$ was calculated ($R^2 = 0.99995$). Determination of AR using the reference function of step 1 allows the calculation of the concentration of TA by using the recovery function.

3. Calculation of Free Serum IgE and TA in Patient Samples (Table 4)

As proof of concept, samples from patients treated with omalizumab and healthy controls were chosen. To determine free serum IgE and TA in these samples, the reference function and the recovery function are needed. Unknown samples were spiked with IgE concentrations of 0, 20, 60, and 90 IU/mL. Extinctions were measured as described in step 1. Using the inverse of the reference function $X = 30.287 * Y / (2.693 - Y)$, the IgE concentrations of samples could be calculated. The recovery for each added standard IgE concentration was calculated as described above [recovery = calculated / spiked IgE; IgE value without addition of IgE (IgE = 0) had to be subtracted from the other IgE values before recovery calculation], and the average recovery was determined for every sample. Using the inverse of the recovery function $AR = 1 / (1 + 2.47 * TA) + 0.002$, the omalizumab content in the diluted unknown samples could be estimated.

TABLE 4 IgE Concentration in Unknown Samples (P0163, 238, P0399, Control: Healthy Volunteer) and Recovery

Sample	IgE-Addition [IU/mL]				AR	TA ($\mu\text{g/mL}$) Rounded
	0 IgE [IU/mL]	20 IgE [IU/mL]	60 IgE [IU/mL]	90 IgE [IU/mL]		
P0163	0.3	18.6	58.3	79.9	0.899	0.00
238 (4°C)	26.4	31.8	37.2	42.2	0.167	2.04
238 (−20°C)	26.8	32.1	39.3	42.9	0.177	1.90
Control	0.0	20.0	63.4	91.0	1.022	0.00
P0399	20.6	26.5	31.9	34.2	0.155	2.2

TA: therapeutic antibody.

The values without IgE addition are the free-antigen concentrations in a dilution of 1:20. For the control sample a recovery of 1 ± 0.15 is expected. It is necessary to account for the sample dilution by multiplication with the dilution factor. For the calculation of free antigen (IgE) concentrations, the therapeutic antibody content in the undiluted serum has to be taken into account. The effect on free-antigen content for undiluted serum is described by the recovery function (undiluted) $R_u = 1 / (1 + 2.47 * T_{Au}) + 0.002$. For estimation of free antigen, the following formula is needed: $A_u = A_d / R_d * R_u * f$ (A_u : free antigen in undiluted serum; A_d : free antigen in diluted serum, R_u : Recovery in undiluted serum; R_d : Recovery in diluted serum; f : dilution factor) (Table 5).

In undiluted serum, the R-ELISA is able to assay for free IgE in a concentration range of 1–2000 IU/mL in samples without omalizumab and of 1–500 IU/mL in samples containing omalizumab, as well as for omalizumab concentrations in a range of 0.4–80 $\mu\text{g/mL}$. Inter- and intravariability of the assay is displayed in Tables 6a and 6b.

TABLE 5 Calculation of Free Serum IgE of Unknown Samples (P0163, 238, P0399, Control: Healthy Volunteer) (Dilution Factor 1:20)

Sample	TAd in Diluted Serum [$\mu\text{g/mL}$]	TAu in Undiluted Serum [$\mu\text{g/mL}$] ($\times$ Dilution Factor)	Ad (IgE), IU/mL	Rd	Ru	Au (IgE), IU/mL
P0163	0.00	0.0	0	0.899	1.000	6
P0005	0.00	0.0	3	0.980	1.000	63
238 (4°C)	2.04	40.9	26	0.167	0.010	31
238 (−20°C)	1.90	38.1	27	0.177	0.011	32
Control	0.00	0.0	0	1.022	1.000	0
P0399	2.2	44.0	20.6	0.155	0.01	27

TAd: therapeutic antibody (omalizumab) in diluted serum, TAu: therapeutic antibody (omalizumab) in undiluted serum, Ad: free antigen in diluted serum, Rd: recovery in diluted serum, Ru: Recovery in undiluted serum, Au: free antigen in undiluted serum.

TABLE 6a Intra-assay Variability (One Test in Sevenfold Determination)

Sample	Mean Value IgE (IU/mL)	CV (%)	Mean Value Omalizumab (µg/mL)	CV (%)
Serum, Measured in Dilution (1:20)				
1 (n = 7)	4.8	7.6	<LOQ	–
2 (n = 7)	2.0	9.2	1.3	12.1
3 (n = 7)	19.0	6.2	1.9	11.3
4 (n = 7)	10.4	3.2	0.2	14.3
Serum, undiluted (after recalculation)				
1 (n = 7)	96.7	7.6	<LOQ	–
2 (n = 7)	2.6	7.2	26.5	12.1
3 (n = 7)	22.8	6.1	38.5	11.3
4 (n = 7)	32.5	9.0	3.7	14.4

CV: coefficient of variation, LOQ: limit of quantification.

TABLE 6b Interassay Variability (Three tests/Four Determinations Each, Variance between Three Tests)

Sample	Mean value IgE (IU/mL)	CV (%)	Mean value Omalizumab (µg/mL)	CV (%)
Serum, measured in dilution (1:20)				
1 (n = 12)	4.3	5.9	<LOQ	–
2 (n = 12)	30.5	7.3	0.9	13.3
3 (n = 12)	1.9	4.2	1.0	6.9
4 (n = 12)	19.3	7.3	2.3	12.4
5 (n = 12)	22.9	8.7	2.7	13.1
Serum, undiluted (after recalculation)				
1 (n = 12)	86.5	5.9	<LOQ	–
2 (n = 12)	44.7	9.6	17.4	13.2
3 (n = 12)	2.6	5.2	20.7	7.4
4 (n = 12)	22.3	9.4	45.2	12.9
5 (n = 12)	25.7	12.1	53.0	13.4

CV: coefficient of variation, LOQ: limit of quantification.

The assay requires a stable equilibrium that is reached after 16 hours. The verification of stability of equilibrium in a time depending assay is demonstrated in Table 7. These results confirm the stability of the complex of capture antibody, IgE, and omalizumab.

TABLE 7 Stability of Equilibrium

Time [h]	16 h	20 h	22 h	24 h	Mean	C.V.
IgE [IU/mL]	O.D.	O.D.	O.D.	O.D.	O.D.	%
15	1.233	1.159	1.171	1.139	1.175	3.44
50	1.714	1.875	2.003	1.791	1.846	6.70
100	2.268	2.004	2.301	2.045	2.154	7.03

O.D.: optical density, C.V.: coefficient of variation.

DISCUSSION

Monitoring treatment with therapeutic antibodies is a particular challenge, because often either the target protein is no longer measurable with conventional assays or results are unreliable due to interference with the therapeutic antibody in the sample. To overcome these problems, a new recovery-ELISA (*recovery*ELISA IgE/omalizumab) was developed to quantify therapeutic antibody and antigen as well as the recovery rate as a readout for the neutralization of the target protein. To validate the assay, serum samples from asthma patients treated with omalizumab were used, and omalizumab (therapeutic antibody) and free IgE (antigen) were quantified.

The therapeutic efficacy of omalizumab is mainly attributed to a reduction of free IgE secondary to the formation of IgE-anti-IgE immune complexes, thereby preventing downstream immunological reactions that eventually lead to allergic and asthmatic symptoms.^[7,11] Unfortunately, not all asthma patients receiving omalizumab experience the desired clinical benefits.^[12] In omalizumab nonresponders, it is critical to know whether the omalizumab dose has reduced free serum IgE levels into the therapeutic target range. To answer this clinical question, a serological measurement of free IgE is needed, since free IgE serum concentrations identify patients who have been underdosed and in whom noncomplexed free IgE continues to arm FcεR1 on effector cells. Presently, no licensed clinical serological assays to quantify free IgE in serum are available, highlighting the unmet clinical need and relevance of the R-ELISA developed to assay for free IgE and omalizumab in sera of asthma patients receiving omalizumab.

Dedicated assays to monitor patients treated with the therapeutic monoclonal antibodies rituximab and alemtuzumab have already been described.^[13,14] In the past several years, attempts were made to quantify free serum IgE in the presence of omalizumab. In clinical trials, concentrations of omalizumab and free IgE were measured using a solid-phase ELISA with an IgE receptor alpha-chain IgG chimera that only captures free IgE not in complex with omalizumab.^[15,16] Currently, even though the human FcγR1a-IgG chimera may be a useful reagent for an immunoassay to measure free IgE, it is not available for use by clinical laboratories. A potential alternative is a recombinant form of the extracellular domain of the alpha chain of the FcγR1 produced in a baculovirus expression system.^[17] However, when purified FcγR1a is insolubilized on plastic surfaces, it appears to lose its reactivity for IgE, most likely due to steric hindrance. Another approach proposed by Hamilton et al. is based on a reciprocal assay configuration to capture IgE with a solid phase anti-IgE and detect bound free IgE with biotin labeled-FcγR1a.^[12] This assay does not measure the therapeutic antibody, thus can only be used indirectly to monitor omalizumab therapy, and is not commercially available.

The R-ELISA has strengths and limitations: First, it was investigated if and how the therapeutic antibody influences the antigen calibration curves of a sandwich assay. The knowledge about this systematic effect and its mathematic description provides the basis for the development of the R-ELISA. Second, an internal multidimensional calibration procedure was developed to enable the simultaneous measurement of antigen and therapeutic antibody in unknown samples. Third, the feasibility and validity of these concepts were confirmed in patient samples spiked with antigen.

The most important optimization of the R-ELISA was the selection of appropriate assay conditions that allow the application of a Michaelis–Menten equation, and which guarantee that within the frame of experimental error the recovery is independent of the antigen (IgE) concentration.

The chemistry of a free IgE assay involves binding of IgE via an IgE heavy chain epitope that interacts with the high affinity IgE receptor. The affinity of omalizumab for IgE is not very high. The dissociation constant is around 2–3 min. Further, there is evidence that omalizumab can form cyclic hexamers. Even though this particular molecular complex is relatively stable, at omalizumab concentrations typically found in the sera of treated patients, hexamers represent less than 0.1% of omalizumab molecules. The present assay uses an incubation period of 12–24 h. This is of particular relevance regarding the stability of the IgE-omalizumab complexes, which may be a problem in standardization of the assay. For the performance of the present assay and the applicability of the equation,^[18] it is necessary that the incubation period lasts until all reaction partners are in a stable equilibrium, which is usually after a minimum of 15 h.

The most important advantage of the present recovery ELISA is the general assay concept that is valid for all therapeutic antibodies and corresponding antigens. It allows the measurement of the therapeutic antibody concentration in serum and the percentage of free antigen, and can be modified for every therapeutic antibody.

A potential limitation of the R-ELISA is the range of the assay in particular for free IgE. Sera of patients with very high IgE concentrations must be diluted prior to measurement, and there may be patients with exceptionally high IgE levels that are beyond the scope of the assay even after dilution. However, free IgE and omalizumab serum concentrations of patients treated with omalizumab within the label approved for the drug in most countries should fall into the range for which the assay was optimized.

CONCLUSION

The present report describes a recovery ELISA with performance characteristics that allow us to monitor changes in circulating free IgE concentrations in sera of patients treated with omalizumab. For the implementation of this new assay principle, it is necessary to measure samples in the microplate with different additions of antigen. Therefore, a special microplate layout is used in

the newly developed recovery-ELISA test kits that are meanwhile marked with CE and manufactured in compliance with the directive 98/79/EC regarding In-Vitro-Diagnostic (BioTeZ Kit “RIO”, order No.: R111). Analysis of the concentrations of free serum IgE and omalizumab in patients treated with omalizumab may be a useful analytical measurement for verifying that a sufficient dose was administered and that the concentration of omalizumab is not simply the cause of an apparent lack of drug efficacy. As a next step, the assay has to be validated in a larger clinical setting to confirm that it is able to guide treatment with omalizumab in both the short term and long term.

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