Development of Assay for Determining Free IgE Levels in

Serum from Patients Treated with Omalizumub

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申請年 2018 年

指導教員　權　寧博

ORIGINAL ARTICLE

Development of Assay for Determining Free IgE Levels in Serum from Patients Treated with Omalizumab

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ABSTRACT

Background: Omalizumab, a monoclonal anti-IgE antibody, is currently indicated for the treatment of moderate-to-severe allergic asthma. To measure active IgE levels in sera from patients treated with omalizumab, the IgE subfraction in complex with omalizumab should be eliminated from total IgE, and free IgE levels can then be determined. With the aim of therapeutic monitoring for anti-IgE therapy, we developed a new ELISA for free IgE.
Methods: We used recombinant human soluble FcεRIα as a capture antigen and a biotinylated polyclonal anti-IgE antibody for detection. Using the newly developed ELISA, we measured the serum free IgE levels weekly in four asthmatic patients after their first omalizumab injection. We also measured the serum free IgE levels in 54 patients treated with omalizumab for over 4 weeks.
Results: This assay was technically robust, the mean recovery rate in serum was 93.16% ± 5.34%. For all patients, omalizumab treatment significantly reduced serum free IgE levels prior to the second omalizumab injection. To maintain the benefit of omalizumab, serum free IgE concentrations should be <50 ng/ml. However, in 14 of 54 patients treated with omalizumab for over 4 weeks, serum free IgE concentrations measured by our ELISA were >50 ng/ml.
Conclusions: Our data suggest that the measurement of free IgE levels using our newly developed ELISA would be useful for monitoring serum free IgE levels during omalizumab therapy.

KEY WORDS

asthma, Fc epsilon RI alpha, IgE binding, immunoglobulin E (IgE), omalizumab

INTRODUCTION

Atopic asthma is a chronic airway disease involving the inflammation of the airway resulting from allergic reactions triggered by the binding of an inhaled antigen to antigen-specific IgE. The inhaled antigen binds to IgE, and this antigen-bound IgE cross-links to high-affinity IgE receptors on the surface of mast cells and basophils, thereby inducing the release of mediators that cause airway constriction and inflammation. Allergen immunotherapy, in which a specific antigen is administered over a long period of time, is one of the IgE-targeting approaches for treating bronchial asthma.[1] However, this therapy is ineffective for certain patients such as those who are sensitized to multiple antigens.

Omalizumab, a humanized anti-IgE antibody, is currently the most effective therapy among clinically available IgE-targeting therapies.[2,3] Administered omalizumab is known to bind to the Fc region of serum IgE, inhibiting the binding of IgE to FcεRIα on the surface of mast cells and basophils. This thereby

prevents the degranulation of these cells, suppressing the release of inflammatory mediators.[4] Moreover, preventing IgE from binding to high-affinity IgE receptors for a certain period is considered to suppress their expression on the surface of mast cells in tissues as well as circulating basophils.[5] Consequently, the numbers of eosinophils, T lymphocytes, B lymphocytes, and Th2 cytokine-positive cells decrease in the sputum and airway mucosal tissues;[5] IL-5/IL-13 levels reduce in blood and tissues, and allergic reactions are suppressed.[6,7]

In Japan, omalizumab is considered as a therapeutic option for Step V of the Global Initiative for Asthma (GINA) guideline, which is the treatment step for patients with the most severe and persistent symptoms that cannot be controlled by combination asthma therapy comprising high-dose inhaled steroids and long-acting β2-agonists.[8,9] Among severe asthmatic patients who underwent omalizumab treatment, 60.1% have been reported to show improvements in their symptom scores.[4,9] Furthermore, omalizumab treatment has few side effects. Therefore, it is considered to be safe and effective for treating severe asthmatic patients.[10,11]

However, responders and nonresponders to omalizumab therapy have been observed, even among atopic asthmatic patients.[12,13] Although no known indicator for predicting the therapeutic response is available, even for asthmatic patients having IgE antibodies to inhaled antigens, responders can be identified comprehensively based on, for example, asthmatic attack frequency, QOL, and respiratory function at 16 weeks after administration of the agent. Subsequently, judgments are made as to whether additional administrations are warranted. The recently reported EXTRA study[14] revealed that after 46 weeks of omalizumab treatment, groups with higher blood eosinophil, serum periostin, and exhaled nitric oxide (FeNO) levels responded better to this therapy.

Besides the responder/nonresponder issue, dosage selection is another factor that influences the therapeutic effect of omalizumab. The dose of omalizumab is calculated based on body weight and total blood IgE levels measured before initiating the administration.[15] However, blood IgE levels fluctuate throughout the year,[16] which precludes the assurance of the therapeutically adequate dosage based on the pretreatment IgE levels for the reasons discussed below.

When IgE production increases after the therapy has been initiated, the required dose may be underestimated, and some patients can be misidentified as nonresponders simply because a therapeutically adequate dose was not given. In contrast, overdosing can occur when a patient's IgE production decreases during the treatment. As described above, omalizumab is the only effective molecular-targeted therapeutic agent available for severe asthmatic patients, and

proper monitoring of IgE levels as the therapeutic target is important to avoid underdosing.

Current methods clinically available for measuring serum IgE levels do not distinguish IgE antibodies in complex with omalizunmab from free antibodies. To measure active IgE levels in sera from patients treated with omalizumab, the IgE subfraction in complex with omalizumab should be eliminated from total IgE, and free IgE levels can be then determined. Till date, several methods to measure free IgE levels in sera from patients undergoing omalizumab treatment have been reported.[17,18] In many previous clinical trials, serum free IgE levels have been measured using a combination of rshFcεRIα and an anti-IgE antibody.

However, this method poses some problems, as follows: 1) information on the accuracy and precision of the measurement system is not publicly available, and 2) the measurement cost is high owing to outsourcing.

The recombinant rshFcεRIα protein preparations used in previous studies[17,18] were mostly expressed in *Escherichia coli* or insect cells using baculovirus vectors. Although their amino acid sequences have been shown to be identical to those of the human protein, their sugar chain structures could have been different from the human counterparts. A difference in the sugar chain structure could affect the binding affinity of IgE and its receptor FcεRIα, thereby affecting the measurement results.

In this study, we aimed to develop an original assay system for determining free IgE levels in sera using the recombinant high-affinity IgE receptor FcεRIα with the human form of sugar chains (rshFcεRIα), which was expressed in Chinese hamster ovary (CHO) cells, as the capture antigen. Our ultimate aim was to establish a method to quantify free IgE levels in sera from patients being treated with omalizumab.

METHODS

REAGENTS AND EQUIPMENT

Human IgE (Thermo Fisher Scientific, New York, USA) was used as a reference standard. A mouse anti-human IgE horseradish peroxidase (HRP)-conjugated antibody (Cat#: 61I58A) was obtained from Fitzgerald Industries International (Massachusetts, CA, USA). An anti-human Fc-epsilon Receptor I alpha monoclonal antibody clone AER-37 (CRA1; Cat #: 16-5899-82) was obtained from eBioscience (San Diego, CA, USA). Bovine serum albumin (BSA; Cat#: A-3059) and Tween-20 (Cat#: 170-6531) were from Sigma-Aldrich (St. Louis, MO, USA). A Multiskan GO microplate spectrophotometer was obtained from Thermo Fisher Scientific. The commercial products of omalizumab was obtained from Novartis Pharmaceuticals (Tokyo, Japan).

PREPARATION OF rsFcεRIα

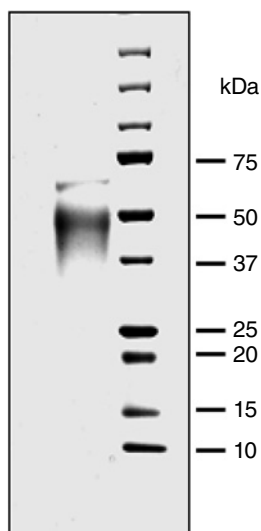rsFcεRIα was prepared in our laboratory as previ-

Fig. 1 Purification of recombinant hsFcεRIαa as a standard protein for quantifying human IgE by ELISA. Standard samples were analyzed for purity using Coomassie Blue staining, which revealed the major band (50 kDa) representing rsFcεRIα.

ously described.[19] In brief, the culture supernatants from CHO cells that secreted rsFcεRIα were collected. This supernatant was concentrated using a Centricon-Plus 70 concentrator (Millipore, Bedford, MA, USA). The rsFcεRIα protein was purified from the supernatant using an affinity column (HiTrap NHS-activated HP column; GE Healthcare Biosciences, Little Chalfont, UK) that was coupled with 5 mg of an anti-human FcεRIα mAb (CRA1). Standard samples were analyzed for purity using Coomassie Blue staining (Fig. 1), which revealed the major band (approximately 50 kDa) representing rsFcεRIα.

IgE ELISA PROCEDURE
Microtiter plates were coated with rshFcεRI (from 0.05 to 0.15 μg/well) and incubated overnight at 4℃. Subsequently, excess rshFcεRI was washed away. For all washing steps, microplates were rinsed three to five times with the washing buffer (PBS/0.05% Tween-20, 300 μl/well). Wells were blocked with PBS/1% BSA (200 μl/well) for 1 h at 25℃. For a standard curve, eight serial dilutions of human IgE ranging from 4.69 ng/ml to 600 ng/ml were prepared in the sample diluent (PBS/0.05% Tween-20 and 1% BSA). Plates were washed and incubated with standards or samples in duplicate (100 μl/well). After 2 h at 25℃, plates were washed, and wells were filled with the secondary detection antibody (mouse anti-human IgE HRP-conjugated antibody, 1/1000 in PBS/1% BSA, 100 μl/well) for 1 h at room temperature. After washing, the TMB substrate solution was added (100 μl/well). After 10 min., the HRP reaction was stopped using 2 N H_2SO_4 (50 μl/well). Optical

density (OD) was then measured at 450 nm using a 96-well microplate reader. The goodness of fit (R^2) of the standard curve was assessed using a four-parameter logistic regression algorithm calculated with Skanit software (Thermo Fisher Scientific). Data were analyzed using GraphPad Prism software (GraphPad, San Diego, CA, USA).

SAMPLES FROM HEALTHY VOLUNTEERS AND ASTHMATIC PATIENTS
To validate the standardized method for measurement of IgE, we obtained serum samples from seven healthy controls, 54 asthmatic patients treated with omalizumab (age range: 19-86 years; median age: 54 years), and seven asthmatic patients not treated with omalizumab. The patients were diagnosed according to the GINA guidelines. Patient's serum samples were stored at -80℃ prior to analysis. Total IgE concentrations were determined using Total IgE ImmunoCap (Thermo Fisher Scientific), with the results of IgE concentration expressed as International Units (kU/l). IgE concentrations in kU/l were converted to ng/ml by multiplication with 2.4. All patients treated with omalizumab had severe asthma according to the GINA guidelines and showed positive reactions for serum-specific IgE tests against inhaled allergens. Serum samples were obtained weekly from five patients after the first administration of omalizumab.

This study was approved by the Institutional Review Board of Nihon University School of Medicine and Juntendo University School of Medicine, and all samples were collected after informed consent was obtained from patients.

DILUTIONAL LINEARITY ASSESSMENT FOR IgE ELISA
The specificity of the assay was evaluated by omitting the detection antibody. Serum IgE concentrations were determined by interpolation from the four-parameter logistic regression algorithm of the standard curve with Skanit software. The dilution linearity of the assay was assessed using diluted samples. Serum samples obtained from 13 healthy controls were diluted with the sample diluent in the range from 1 : 2 to 1 : 32. In addition, the serum samples from five asthmatic patients were diluted with the sample diluent in the range from 1 : 10 to 1 : 20.

SPIKE-RECOVERY ASSAYS FOR IgE ELISA
We performed spike-recovery assays to test for matrix interference.[20] As a standard sample, we used a mixture of pooled serum samples obtained from six male and six female healthy controls with relatively low IgE levels. The final IgE concentration in this pooled serum was 193 ng/ml. The pooled serum was diluted to 1 : 15 and spiked by adding IgE (final concentrations ranging from 4.69 ng/ml to 600 ng/ml) to the standard solution. The recovery rate (%) was cal-

culated by dividing the IgE concentration measured using ELISA by the theoretical concentration of the samples (IgE concentration in the pooled serum + IgE concentration of unspiked IgE). The assay was determined to be at an acceptable level when the recovery rates were between 80% and 120%.[20-22]

PRECISION OF IgE ELISA

The precision of the assay was evaluated by inter- and intra-assay variability tests. A total of 75 ng/ml of IgE was added to the serum samples obtained from seven healthy controls and seven asthmatic patients. For the intra-assay variability test, we analyzed five replicates of individual samples on the same plate. Inter-assay variability was assessed using replicates of a sample on four different plates by two independent operators. The assay was determined to be at an acceptable level when the mean recovery rate was within 20% of the theoretical concentration and the corresponding percentage of coefficient of variation (CV%) was <20%.[20-22]

OMALIZUMAB AND IgE COMPLEX PREPARATION

Human IgE was diluted with the sample diluent at 1200 ng/ml and then mixed with equal volumes of the sample diluent or omalizumab ranging from 2.34 ng/ml to 600 ng/ml. These mixtures were incubated for 30 min at room temperature to enable complex formation prior to the analysis for free IgE level measurements. The final IgE concentration in the absence of omalizumab (buffer control condition) was 600 ng/ml.

RESULTS

PREPARATION OF STANDARDS

The scheme of measurement of free IgE levels in serum samples from patients who were treated with omalizumab is shown in Figure 2A. An eight-point serial dilution of human IgE in the range from 4.69 ng/ml to 600 ng/ml was used to generate the standard curve (Fig. 2B). Curve-fitting analysis was performed using the four-parameter logistic regression algorithm with the measured OD and the absolute IgE concentration. The average goodness of fit (R^2) for eight individual experiments was 0.999. To evaluate the specificity of detecting IgE with ELISA using rsFcεRIα and an anti-IgE antibody, the detection antibody was omitted and resulted in a loss of IgE-derived signals for all concentrations (Fig. 2C).

DETERMINATION OF THE OPTIMAL DILUTION FACTOR FOR SERUM SAMPLES

To determine the optimal dilution factor for serum samples for quantification of IgE in serum, serum samples obtained from six healthy controls with various IgE concentrations were diluted at the indicated dilution factors, and ELISA was performed. As shown

by the results in Figure 3A, the OD values were almost the same at the dilution factors between ×16 and ×32, but the OD values decreased at the dilution factors below ×8. We considered that the binding between IgE and rsFcεRIα was inhibited by serum factor(s) at the dilution factors between ×8 and ×15. As shown in Figure 3B, we measured serum IgE levels for samples diluted by the factors of ×10, ×15, and ×20 and checked the linearity of OD values. In serum from healthy controls and serum from asthmatic patients, the OD values were almost the same at dilution factors between ×15 and ×20, but the OD values decreased at dilution factors below ×10. From these results, we determined that the optimal dilution factor was ×15 for this assay to perform highly sensitive measurement of IgE in serum without interference from serum factors.

ACCURACY OF ELISA

To evaluate the accuracy of the assay, we used a spike recovery assay. We measured eight different spike concentrations in the pooled serum obtained from 12 individual healthy controls (six males and six females). As shown Table 1, the recovery rates were between 80% and 120%, and the CV% values were <20% when we measured spike concentrations ranging from 9.38 ng/ml to 600 ng/ml. According to a guideline on bioanalytical method validation for clinical research by Food and Drug Administration (FDA),[23] we conclude that the working ranges for this assay were as follows: lower limit of quantitation (LLOQ) =9.38 ng/ml and upper limit of quantitation (ULOQ) =600 ng/ml. These are corresponding to 140.7 ng/ml and 9000 ng/ml of serum IgE of concentration respectively.

We next examined the recovery rates of the spikes in each serum sample obtained from seven healthy controls and seven asthmatic patients. The mean recovery rate was 111.3% ± 9.7%, which met our preset acceptance level (Table 2). One sample obtained from a healthy control had a recovery of >120% (125%), suggesting a possible matrix interference in this patient.

Table 3, 4 show the results of our intra-assay and inter-assay variability tests. The mean recovery rate was within 20% of the absolute concentration with a corresponding CV% of <20% for all the three tested concentrations. These results met the acceptance criteria for immunoassay validation.[20-22]

MEASUREMENT OF FREE IgE IN PATIENTS TREATED WITH OMALIZUMAB

To confirm the mutual exclusion of IgE bound with omalizumab at the Cε3 epitope, we performed competition experiments using omalizumab. Samples containing 600 ng/ml of IgE were incubated with the indicated concentrations of omalizumab; thereafter, free IgE levels in the samples were measured using
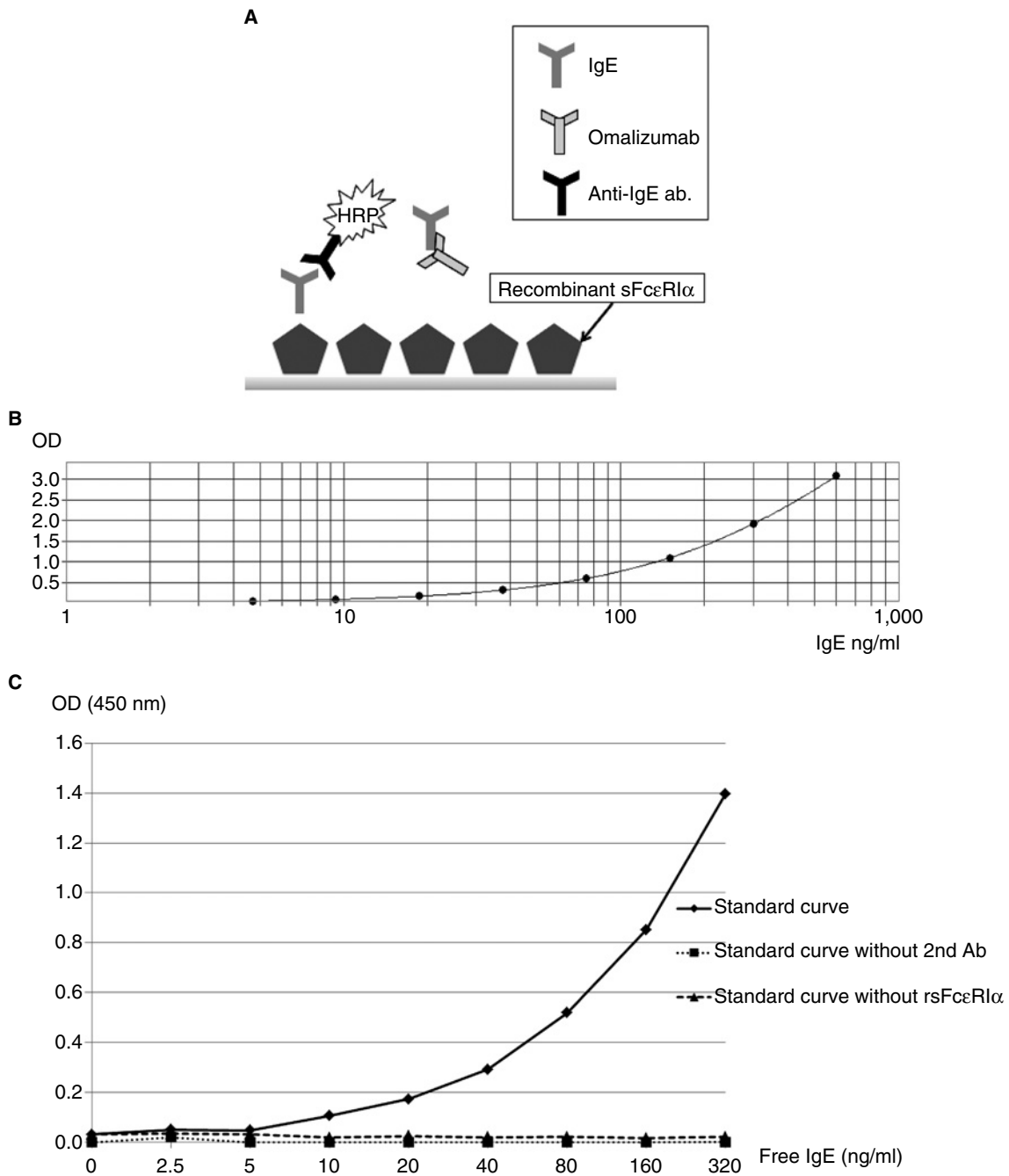
Fig. 2 **A**. Assay scheme for determination of serum IgE levels in the presence of omalizumab. Recombinant high-affinity IgE receptor FcεRIα with the human form of sugar chain (rshFcεRIα) was used as the capture antigen. **B**. Representative standard curve using human IgE. X-axis showing standard IgE concentrations ranging from 4.69 ng/ml to 600 ng/ml. Y-axis showing optical density (OD) values at 450 nm. Four-P Fit: y = (A - D)/(1 + (x/C)B) + D (A: 0.0148; B: 0.999; C: 930; D: 0.9993; R2: 1). **C**. The specificity of detecting free IgE levels with ELISA using rshFcεRIα. Omitting rshFcεRIα or the detection antibody resulted in a loss of free IgE-derived signals over the entire concentration range (0-320 ng/ml).

ELISA. As shown in Figure 4, adding omalizumab reduced free IgE levels in a dose-dependent manner.

Subsequently, we monitored weekly free IgE levels in the serum of four asthmatic patients after the first administration of omalizumab. Free IgE levels in all these patients were significantly reduced after administering omalizumab (Fig. 5). It has been reported that to maintain the benefit of omalizumab treatment
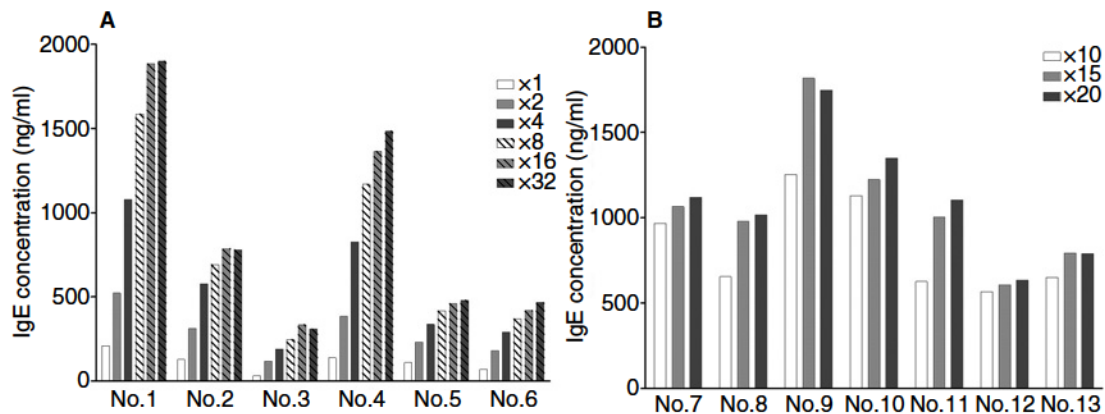
Fig. 3 A. Optimal dilution factor for serum samples. Serum samples obtained from healthy controls (No. 1-6) were diluted by the factors from ×1 to ×32. IgE concentrations were then measured with ELISA using rshFcεRIα. B. Determination of the optimal dilution factor for serum samples. Serum samples obtained from healthy controls (No. 7-13) were diluted by the factors from ×10 to ×20. Subsequently, IgE levels were measured with ELISA using rshFcεRIα.

Table 1 Accuracy of ELISA for measurement of the pooled sample

	IgE Concentration (ng/ml)								
	0	4.69	9.38	18.75	37.5	75	150	300	600
Calculated conc. (ng/ml)		15.23	19.92	29.29	48.04	85.54	160.54	310.54	610.54
Experimentally-found conc. mean(ng/ml)	10.54	14.53	18.92	28.25	48.10	84.59	154.96	289.80	569.41
SD		1.93	3.25	2.47	2.36	5.59	7.69	10.94	19.09
Accuracy (%)		95.40	94.97	96.44	100.11	98.88	96.52	93.32	93.26
%CV		13.3	17.2	8.8	4.9	6.6	5.0	3.8	3.4
Detected spike conc.-mean conc. In serum (ng/ml)	Mean	3.37	7.76	17.09	36.94	73.43	143.80	278.64	558.25
Recovery (%)		71.92	82.74	91.15	98.50	97.91	95.87	92.88	93.04

Spike-recovery assays were used to determine the accuracy and precision of ELISA. Pooled serum from healthy controls was spiked using 4.69 ng/ml to 600 ng/ml of IgE. IgE concentration of the pooled serum was 10.54 ng/ml. Calculated concentration was 10.54 + spiked concentration of IgE. Accuracy is the difference between experimentally determined and calculated concentrations. Recovery (%) is the difference between detected spike and spiked IgE concentrations.

in severe asthmatic patients, serum free IgE concentrations need to remain at <50 ng/ml.[24] Thus, we analyzed trough free IgE levels in 54 asthmatic patients undergoing omalizumab therapy. As shown in Figure 6, in 14 of 54 (25.9%) patients, the trough free IgE concentration was >50 ng/ml. 11 of 54 (20.4%) patients was > 100 ng/ml and 6 of 54 (11.1%) patients was >150 ng/ml. The frequency of appearance of the omalizumab treated patients with over 50 ng/ml free IgE appeared to be independent to the duration of omalizumab therapy.

DISCUSSION

In this study, we developed an original ELISA-based IgE measurement system using immobilized rshFcεRIα with human sugar chains. To explore the feasibility of using this method in clinical settings, we optimized several assay conditions and validated the measurement accuracy. We first studied the effect of serum on the assay's linearity over a range of serum dilutions to determine the optimal serum dilution factor for this assay system. These results indicate that the OD values decreased below a serum dilution factor of ×15 in a concentration-dependent manner. This may be attributed to serum factors that inhibit IgE-FcεRI binding (e.g., autoantibodies to IgE, soluble low-affinity IgE receptors, lipoproteins, or sugar chains). We accordingly selected ×15 as the serum dilution factor for this assay to eliminate interference from inhibitory factors in serum and to enhance the

Table 2 Accuracy of ELISA for IgE measurements in sera from healthy controls and asthmatic patients

| | | IgE (ng/ml) | | | | | | |
| | | Unspiked | | | Spiked (75 ng/ml) | | | |
	Sample	Average	SD	CV (%)	Average	SD	CV (%)	Recovery (%)
Asthma	1	48.93	1.67	3.41	143.29	7.81	5345	125.8
	2	26.9	1.51	5.61	114.87	6.11	5.32	117.3
	3	35.72	2.84	7.94	106.83	13.75	12.87	94.81
	4	2.8	0.75	26.73	83.61	6.57	7.86	107.75
	5	64.79	5.45	8.41	145.62	11.77	8.08	107.78
	6	3.39	0.61	17.99	89.27	8.25	9.25	114.49
	7	17.38	1.55	8.93	107.06	4.64	4.33	119.57
Average							7.59	112.5
SD							2.71	9.35
Healty control	1	58.4	5.13	8.78	143.26	9.94	6.94	113.15
	2	2.63	0.41	15.64	86.84	8.82	10.16	112.28
	3	6.9	0.26	3.7	89.86	6.5	7.23	110.62
	4	35.82	1.28	3.59	110.55	14.42	13.05	99.64
	5	6.61	0.91	13.82	87.72	11	12.54	108.15
	6	22.49	0.62	2.78	94.98	14.21	14.96	96.65
	7	3.76	0.08	2.14	76.04	11.21	14.75	96.37
Average							11.38	105.27
SD							3.33	7.45

Spike-recovery assays were used to determine the accuracy and precision of ELISA regarding the measurement of the levels in sera from seven healthy controls and seven asthmatic patients. Pooled serum from healthy controls was spiked to 75 ng/ml of IgE. Recovery (%) is the difference between detected spike and spiked IgE concentrations.

Table 3 Inter-assay variability of free IgE ELISA

IgE (ng/ml)	Mean $n = 5$	SD	CV (%)	Recovery (%)
600	564.06	20.23	3.56	92.15
300	285.4	8.83	3.09	91.41
150	153.55	8.92	5.81	94.93
75	84.11	6.68	7.94	97.26
37.5	48.58	2.7	5.56	99.78
18.75	28.85	2.7	9.37	94.34
9.38	19.67	3.65	18.56	90.74
4.69	14.83	2.27	15.28	78.4

Inter-assay variability for pooled samples containing from 4.69 ng/ml to 600 ng/ml of IgE, as assessed on four different plates by two independent operators. Recovery (%) is the difference between detected spike and spiked IgE concentrations.

Table 4 Intra-assay variability of free IgE ELISA

IgE (ng/ml)	Mean $n = 4$	SD	CV (%)	Recovery (%)
600	575.14	14.52	2.53	95.86
300	289.27	14.11	4.88	96.42
150	152.66	8.29	5.43	101.77
75	74.72	2.49	3.33	99.63
37.5	36.96	2.25	6.07	98.56
18.75	16.22	1.31	8.05	86.53
9.38	7.2	0.69	9.64	76.84
4.69	3.06	0.51	16.49	65.36

Intra-assay variability for pooled samples containing from 4.69 ng/ml to 600 ng/ml of IgE, as assessed on nine replicates of individual samples on a single plate. Recovery (%) is the difference between detected spike and spiked IgE concentrations.

assay's sensitivity.

Conforming to the acceptance criteria for immunovalidation, the acceptable assay range was defined based on the result from the spike-recovery assay as the concentration range that satisfied the CV% of ≤20% and the recovery rate of 80%-120%. Consequently, LLOQ and ULOQ were determined to be 9.38 ng/ml and 600 ng/ml, respectively. These are corresponding to 140.7 ng/ml and 9000 ng/ml of se-

rum IgE of concentration respectively. Although the LLOQ value was higher compared with that of previously reported assay methods, our method appears to be sufficiently sensitive to determine the level of free IgE remaining in the serum.

In the error assessments where samples from asthmatic patients and healthy controls were tested individually, CV% ranged from 4.33% to 14.96%, with a mean of 7.59% ± 2.71% for asthmatic patients and a
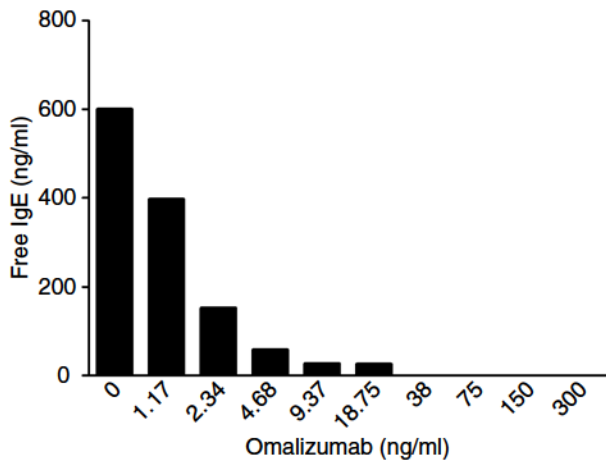
Fig. 4 Reduced serum free IgE levels due to the addition of omalizumab. Pooled serum containing 600 ng/ml of IgE was incubated with the indicated amounts of omalizumab; thereafter, free IgE levels were measured. Data are representative of two independent experiments with similar results.

mean of 11.38% ± 3.33% for healthy controls. This indicated good accuracy in general, although the recovery rate exceeded 120% in one case. The observed difference in assay variability among individual cases may reflect differences in the amounts of serum factors that may inhibit IgE-FcεRIα binding. Nevertheless, the error range was deemed acceptable because no extreme outliers were observed.

Based on these results, this assay method permitted measurement of serum IgE in clinical samples with sufficient accuracy. We measured free IgE levels in sera from asthmatic patients undergoing omalizumab therapy using the aforementioned assay system. To test this method for its applicability to free IgE assays in the presence of omalizumab, we first examined whether omalizumab added to the sample *in vitro* reduced the serum free IgE level in a dose-dependent manner. As shown in Figure 4, adding omalizumab reduced free IgE levels in samples in a concentration-dependent manner.

Subsequently, changes in free IgE levels were monitored over time following implementation of the initial therapy. In each of the four cases assessed, serum free IgE levels decreased immediately after the omalizumab administration (Fig. 5). This result was in accord with that of previous clinical trials on patients with bronchial asthma and allergic rhinitis, in which a rapid decline in serum free IgE levels was documented after the initiation of omalizumab therapy. However, trough free IgE levels were elevated and, in particular, exceeded 50 ng/ml after the second administration in certain cases for which the administration interval was 4 weeks (Cases 3 and 4). For these cases, omalizumab dose selection based on

body weight and pretreatment serum IgE levels may have been insufficient to reduce the serum free IgE level to the targeted level.

The aforementioned study[2,18,25,26] also revealed that free IgE concentrations did not decline to the target value[24] of 50 ng/ml in some cases undergoing short-term therapy after the administration of omalizumab. This led us to suspect that among severe asthmatic patients on long-term omalizumab therapy, some cases may exist for which serum free IgE levels fail to reach the target level. Thus, we measured trough free IgE levels after omalizumab administration in 54 cases that were on omalizumab for more than 4 weeks (Fig. 6). These results indicate that 40 of 54 (74.1%) cases had free IgE levels that reached the therapeutic target for omalizumab treatment (*i.e.*, ≤50 ng/ml). This underruns previously reported outcome 99.2%.[27] A possible reason is that the rshFcεRIα construct used in our assays is different from the recombinant proteins used in other studies, which may have resulted in differences in IgE measurements.

Recombinant rshFcεRIα protein constructs used in most previous studies were mostly expressed in *E. coli* or a baculovirus vector/insect cell expression system[28,29] in which the amino acid sequences of the expressed proteins were identical to those of the human protein; however, the sugar chain structures could have been different from those of the human protein. These structural differences in sugar chains may affect the binding affinity of IgE and its FcεRIα receptor. This difference in binding affinity possibly alters the equilibrium state among IgE, IgE-omalizumab complexes, and IgE-FcεRIα binding in an assay system. As a consequence, measurement systems that use immobilized FcεRIα preparations with different sugar chain structures may give different IgE readings in the presence of omalizumab. We did not address which system could measure free IgE levels more accurately in the presence of omalizumab.

Nevertheless, it is conceivable that our system better approximates the physiological equilibrium that occurs in the human body compared with other assay methods, because our method uses an FcεRIα construct comprising the human form of the sugar chain. Considering that the objective of these measurements is to quantify the IgE subfraction that can bind to FcεRIα in the human body in the presence of omalizumab, it is reasonable that our assay system can detect the effect of omalizumab more accurately. Although our results suggest that our assay system is effective for predicting the responsiveness of certain patients to omalizumab therapy, we could not analyze the relationships between free IgE levels measured by this method and clinical data in the present study. Previously, several methods for measuring free IgE under the presence of omalizumab has been reported,[26] including the recovery-ELISA system.[30] The
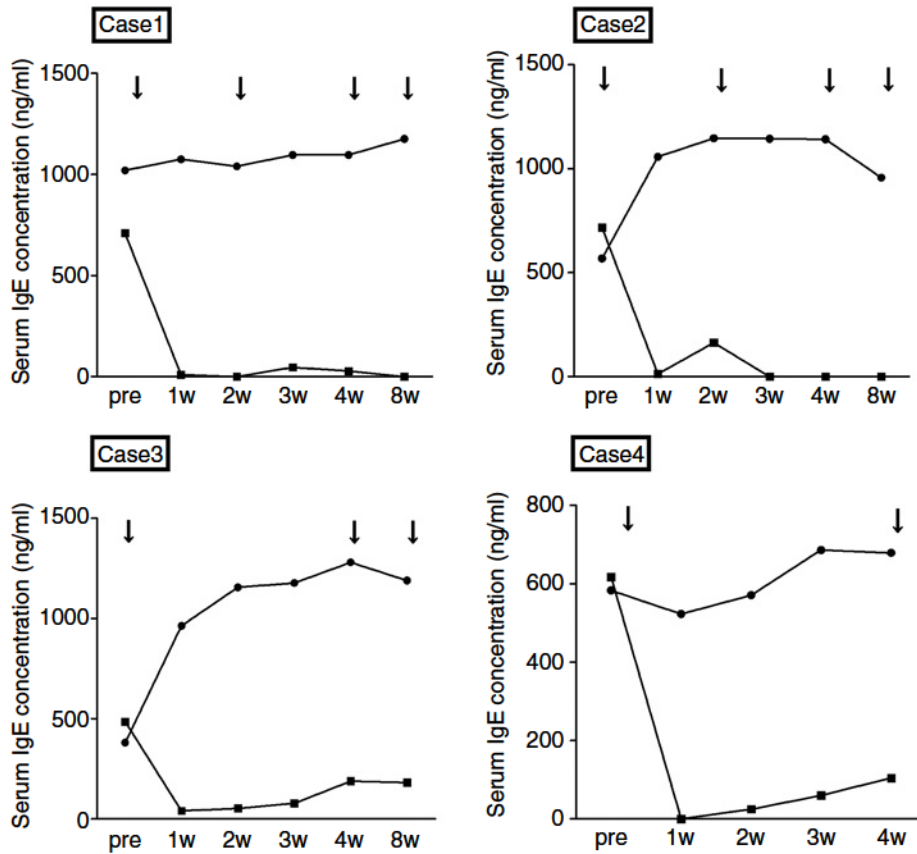
Fig. 5 Measurement of IgE levels in patient sera. Serum free IgE levels (filled squares) in four asthmatic patients after the first administration of omalizumab were determined using ELISA. Total IgE levels (filled circles) were measured by ImmunoCap IgE. Arrows show the time points for omalizumab administration.
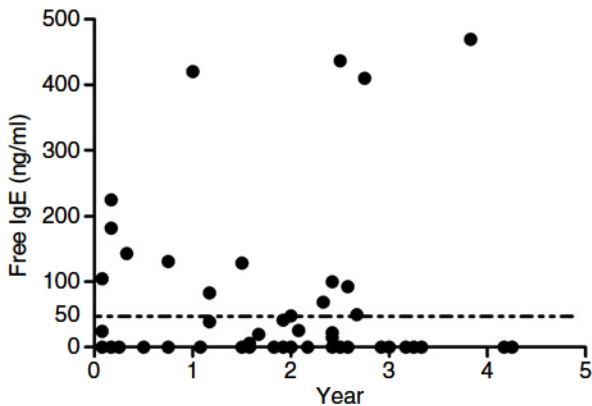

Fig. 6 Free IgE levels in 54 asthmatic patients undergoing omalizumab therapy. Trough free IgE levels in sera from 54 different patients treated with omalizumab for one month to 51 months were measured by ELISA. Dashed line indicates 50 ng/ml of free IgE.

free IgE concentration in a serum specimen from patients administered with omalizumab was measured by the recovery-ELISA system based on (1) creating a calibration curves of various concentration of standard IgE under various concentrations of omalizumab solutions; (2) creating a correlation formula of the relationship between IgE and omalizumab from the calibration curves; (3) spiking the standard IgE into clinical specimens of patients administered with omalizumab and based on the degree of absorbency obtained; (4) calculating the concentrations of both free IgE and free omalizumab using the formula. In the present study, the ratio of the patients who reached the therapeutic target for omalizumab treatment was lower than previously reported outcomes ranging from 73% to 99.2%, based on the values measured by other assays including the recovery-ELISA system. One possible reason of this difference is that the rshFcεRIα construct used in our assays is different from the recombinant proteins used in other studies, which may have resulted in differences in IgE measurements. In addition, although our assay is based on simple ELISA method, the method of recovery-ELISA system is fundamentally different from a simple ELISA. With This method has an ad-

vantage on sensitivity, because undiluted serum is used as a specimen. However, it is difficult to evaluate the accuracy of the recovery-ELISA system, because the recovery rate cannot be directly measured. Furthermore, there are no detailed reports on variability of this method has not been reported. Therefore, we cannot evaluate which assay is closer to the true value. Further analysis with a larger number of patients should be conducted in future studies.

In summary, we developed a new ELISA-based assay system to determine serum free IgE levels using immobilized soluble human FcεRIα that were generated by gene recombination. Using this assay system, we measured free IgE levels in sera from asthmatic patients undergoing omalizumab therapy. This method permitted monitoring of serum free IgE levels. It is expected to realize assessments of appropriate omalizumab doses for nonresponders. In addition, even for responders, it provides for optimization of doses and dosing intervals. Optimized dosing regimens will aid in resolving medical expense issues and benefit a wider range of patients. Although a direct comparison was not performed, our method may be superior to conventional free IgE assay methods based on FcεRIα with nonhuman form of sugar chains in terms of binding affinity to human serum IgE.

ACKNOWLEDGEMENTS

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論文要約

オマリズマブ投与患者における血清遊離 IgE 測定系の構築

背景

抗ヒト IgE モノクローナル抗体であるオマリズマブ（Omalizumab）は、現在、中等度から重症のアレルギー性喘息の治療に適応がある。オマリズマブがアレルゲンに対する IgE を介した気道のアレルギー応答を抑制するためには、血清中の遊離 IgE を極めて低いレベルまで低下させる必要があるが、実臨床においてオマリズマブ使用中の患者で血清遊離IgE レベルを測定することはできない。リコンビナント・ヒト高親和性 IgE 受容体（FcεRIα）を抗原として利用すると、オマリズマブと結合していない遊離 IgE レベルを測定することができることから、オマリズマブ治療の評価に利用可能な ELISA 法を用いた新たな遊離 IgE の測定系を構築した。

方法

可溶性リコンビナント・ヒト FcεRIα（rs hFcεRIα）を捕捉抗原として固相化

し、検出のためにビオチン化ポリクローナル抗 IgE 抗体を使用した。測定系と

しては、測定ごとの変動を排除して安定した測定が行えるように、2 時間、25℃

に設定した。

作製した ELISA 法を用いて、オマリズマブ投与開始前後で 4 人の喘息患者にお

いて 1 週ごとに血清遊離 IgE 値を測定した。また、オマリズマブを 4 週間以上

投与した 54 人の患者の血清遊離 IgE 値を測定した。

本研究は、日本大学附属板橋病院臨床研究審査委員会において 2012 年 4 月 27

日に承認(RK-120413-09)され、実行された。

結果

まず，構築した ELISA の精度評価を行った。低 IgE 標準血清を用いた spike-

recovery assay における血清中の平均回収率は 93.16％±5.34％であった。%CV

が 20%以内を基準とし、Lower Limit of quantification (LLOQ)は 9.38ng／ml、

Upper limit of quantification(ULOQ)は 600ng／ml とした。次に、オマリズマ

ブが存在する血清においても、本測定系が遊離 IgE を測定できるかどうか検証

するため、600ng/ml の IgE を添加した標準血清に濃度の異なるオマリズマブを

添加し、遊離 IgE 濃度がオマリズマブにより濃度依存的に低下することを確認

した。本測定系を用いて経時的なモニタリングを行なった 5 例の重症喘息患者

すべてにおいて、オマリズマブ投与 2 回目投与前から血清遊離 IgE 値は有意に

低下していた。オマリズマブの効果を維持するためには血清遊離 IgE 濃度が

50ng / ml 未満であることが必要とされているが、オマリズマブで 4 週間以上治

療をおこなっている 54 人の喘息患者のうち 14 人は、遊離 IgE 濃度が > 50 ng /

ml であった。

考察

　本測定系では、血清希釈倍率を 15 倍以下とすると固層化したマトリックスに

非特異的な反応がみられるため、血清希釈倍率は 15 倍とした。これが定量限界

の決定因子となり、本測定系の定量限界は 9.38ng/ml となった。これは、オマ

リズマブの薬理効果が得られるための遊離 IgE 濃度の最低が 50ng/ml であるこ

とから十分評価に使用できる検出感度であると考えた。非特異的な反応が高濃

度の血清でみられる要因としては、IgE-FcεRⅠ結合を阻害する血清因子、例え

ば、IgE に対する自己抗体、可溶性低親和性 IgE 受容体、リポタンパク質、また

は糖鎖などに起因すると考えられた。

オマリズマブの臨床治験では血清遊離 IgE を測定しているが、その際に使用された測定方法は現在臨床において使用することができず、また、研究試薬としても入手することができない。測定法についての詳細も明らかにされていないことから、我々の測定法との精度比較をすることはできない。

オマリズマブ治療開始後、全例で 1 回目の投与 1 週間後の血清遊離 IgE は 50ng / ml に低下したが、4 例中 3 例においては、トラフの血清遊離 IgE が 50ng / ml を超えていた。血清 IgE 値は変動するため、初期設定に用いた血清 IgE 値と投与時の血清 IgE 値との間に乖離が生じている可能性や、体重と治療前の血清 IgE 値に基づいて決定されたオマリズマブ投与量が、血清遊離 IgE レベルを目標値まで低下させるのに不十分であった可能性がある。

4 週間を超えてオマリズマブ治療を受けた 54 例のうち 40 例（74.1%）の遊離 IgE 値が治療目標レベルに低下していた。 過去の報告では、遊離 IgE 値が治療目標レベルに達した患者割合は 99.2%と報告されており、我々の検討はそれより低い割合であった。この理由の一つとして考えられるのは、我々が用いた

rshFcεRIα がヒト型糖鎖構造を持っていたのに対して，これまでに使用されていた測定系では、線虫や大腸菌で作製したリコンビナント蛋白を使用していることなどに関係している可能性がある。ヒト Fc レセプターの糖鎖結合部位の糖鎖は、一般には抗体との結合親和性に関連するとされている。よって、使用するリコンビナント蛋白の違いが測定精度に影響している可能性が考えられる。さらに、特にアレルギー疾患の患者において、血中に線虫の糖鎖に対する抗体を持つ場合があることが報告されており(1)、線虫の糖鎖を用いた場合には、測定結果に影響する可能性がある。よって、ヒト型の糖鎖を持つヒトタンパクを抗原に用いることにより、これらの影響を排除して、アトピー型喘息患者の遊離 IgE の測定において、より正確な測定が可能となる。

　オマリズマブ治療の目的は重症喘息患者の増悪回数の抑制であり、投与開始早期の症状と遊離IgE 値の関連についての検討は行っていない。本研究において、遊離 IgE 値と症状の関連について前向きの検討を行っていないが、副論文で行った京都大学との共同研究では、治療効果と遊離 IgE 値に関する前向きの検討を行っており、投与開始後速やかに遊離 IgE 値が低下した群においては、治療開始後 1 年間での高い増悪抑制効果が得られることが確認された(2)。

結論

我々は遺伝子組み変えシステムにより作成した可溶性ヒト $Fc\varepsilon RI\alpha$ を固相化した ELISA による新たな血清遊離 IgE 測定系を構築した。この測定系を用いて、オマリズマブ治療中の重症喘息患者の血清遊離 IgE を測定し、この方法による血清遊離 IgE 値のモニタリングを可能にした。本測定法を用いた検討により、実際オマリズマブ治療を行っている喘息患者において、遊離 IgE が治療目標レベル以上の患者が少なからず存在することが分かった。ノンレスポンダーに対する適切なオマリズマブ用量の評価を可能とすることが期待されている。レスポンダーにとっては遊離 IgE 値のモニタリングは用量および投薬間隔の最適化を可能とする。

　最適化された投与方法は医療費削減に寄与し、より広範囲の患者に利益をもたらす。

本文中の以下について訂正する。

　p38 右 5 行目　omalizunmab　訂正　omalizumab

　p45 右 23 行目　With This method　訂正　With this method

2014 年に本研究掲載後の臨床応用等に関する進展状況のまとめ

本研究で構築した測定系を用いて、遊離 IgE のモニタリングを指標として、オマリズマブの投与量調節を行い、良好な喘息コントロールを得た経験が報告された。(3)

また、オマリズマブ投与下において、遊離 IgE レベルが IgE 産生能を反映しており、IgE 産生の評価に利用可能であることが示された。(4)

京都大学との研究では、治療後の遊離 IgE 低下速度が治療反応性と関連があることを明らかにした。(2)

現在は、本測定系を使った遊離 IgE モニタリングの測定の保険収載を目指し、シノテスト社と共同で開発を行っている。

今後、オマリズマブの治療適応が重症アトピー型喘息以外のアレルギー性鼻炎、食餌性アレルギーといった IgE 依存性疾患に拡大することが予想される。遊離 IgE の測定がこれら疾患を持つより多くの患者の治療モニタリングに活用され、有効な治療につながることが期待されている。

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