Published in final edited form as:

J Immunol Methods. 2011 October 28; 373(1-2): 192–199. doi:10.1016/j.jim.2011.08.018.

Development and validation of a standardized ELISA for the detection of soluble Fc-epsilon-RI in human serum

Willem Lexmond, MD, John van der Mee, Floortje Ruiter, BSc, Barbara Platzer, PhD, Georg Stary, MD¹, Elizabeth H. Yen, MD, Eleonora Dehlink, MD, PhD², Samuel Nurko, MD, MPH, and Edda Fiebiger, PhD

Division of Gastroenterology and Nutrition, Children's Hospital Boston, Department of Pediatrics, Harvard Medical School, Boston, Massachusetts, USA

¹Harvard Medical School, Department of Microbiology and Immunobiology, Boston, Massachusetts, USA

Abstract

The aim of this study was to develop a standardized enzyme-linked immunosorbent assay (ELISA) for detection of human soluble Fc-epsilon-RI (sFcεRI), a serum isoform of the high affinity IgE receptor. A recombinant version of sFcεRI was produced in baculovirus and used as standard. ELISA plates were coated with anti-mouse IgG followed by incubation with the monoclonal capture antibody CRA1. This FcεRI-α-specific antibody binds to the stalk region of the protein and does not inhibit IgE-binding. After incubation with standards or serum samples, plates were incubated with chimeric IgE followed by detection with horseradish peroxidase conjugated anti-human IgE. Enzymatic activity was visualized with (3, 3', 5, 5')-tetramethylbenzidine. Specificity was demonstrated by omission of capture or detection reagents. Units (U) of detection were established and the dynamic range of the assay was defined as 10 640 U/ml for a 1:5 serum dilution. Parameters of linearity ($R^2 > 0.999$), matrix interference test (recovery of 70–110%), intra-assay variability (coefficient of variation (CV) <20%) and inter-assay variability (CV <20%) met acceptance criteria for immunoassay validation. Correlation analysis of serum units of sFcεRI measured with the new ELISA and serum IgE levels confirmed earlier published data describing a weak correlation of the two parameters in patients with elevated serum IgE while no correlation in patients with normal serum IgE or the total patient group was found. In summary, we established and validated a standardized ELISA for the detection of sFcεRI. This novel method now allows for comparative analysis of sFcεRI levels in health and disease.

Keywords

IgE; Fc receptor; allergy; serum biomarker; sFcεRI; soluble IgE receptor

© 2011 Elsevier B.V. All rights reserved.

Address correspondence to: Edda Fiebiger, PhD, 300 Longwood Avenue, EN630, Boston, MA 02115, Tel: +1-617-919-2549 Fax: +1-617-730-0498; edda.fiebiger@childrens.harvard.edu.

²Current address: Department of Pediatrics and Adolescent Medicine, Medical University of Vienna, Waehringer Guertel 18-20, 1090 Vienna, Austria.

Willem Lexmond and John van der Mee contributed equally to this work. Drs. Nurko and Fiebiger are shared senior authors.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

The high affinity IgE receptor, Fc-epsilon-RI (FcεRI), is a multimeric immune activation receptor and a key structure in the pathology of IgE-mediated allergies, the most common disease entity found among allergic patients (Bochner and Busse, 2005; Gould and Sutton, 2008; Holgate and Davies, 2009). Two isoforms of FcεRI are constitutively expressed in humans (Kraft and Kinet, 2007; Dehlink and Fiebiger, 2009). Mast cells and basophils express a tetrameric isoform of FcεRI containing an IgE-binding alpha-chain, a common FcR gamma-chain dimer for signal transduction and a beta-chain, a tetraspanning protein that amplifies ITAM signals of the gamma-chain. In contrast, trimeric FcεRI lacks the beta-chain and is constitutively expressed by human antigen presenting cells, such as dendritic cells in the blood or Langerhans cells in the skin (Maurer et al., 1994; Klubal et al., 1997; Dehlink and Fiebiger, 2009). Monomeric ligation of FcεRI with IgE stabilizes the receptor at the cell surface (Kubota et al., 2006). The natural ligand uses this mechanism to upregulate FcεRI expression levels. Consequently, increased receptor expression levels are found in individuals with elevated serum IgE levels. Additionally, elevated serum IgE induces *de-novo* expression of trimeric FcεRI on monocytes and neutrophils of allergic patients (Kraft and Kinet, 2007; Gould and Sutton, 2008; Dehlink et al., 2010).

Recently, an additional receptor isoform has been described in human serum (Dehlink et al., 2011). Human soluble FcεRI (sFcεRI) is a single chain receptor consisting of a shorter alpha-chain, potentially lacking the transmembrane region and the cytosolic tail. *In vitro*, sFcεRI is generated after IgE-mediated receptor crosslinking from cell lines stably expressing trimeric FcεRI (Dehlink et al., 2011). It has also been shown that sFcεRI can block binding of IgE to cell surface expressed FcεRI (Dehlink et al., 2011). This finding indicates that sFcεRI may be an *in vivo* modulator of IgE-mediated immune responses.

The study describing sFcεRI used a semi-quantitative enzyme-linked immunosorbent assay (ELISA) to analyse serum levels of this protein (Dehlink et al., 2011). Correlation analysis of sFcεRI and serum IgE levels revealed a weak correlation of both parameters in pediatric patients with elevated IgE. The physiologic relevance of this finding is presently unclear and requires further investigation. Only standardized serum measurements can generate data sets that will allow for evaluation of the role of sFcεRI serum levels in health and disease. The goal of this study, therefore, was to develop and validate an ELISA for standardized quantification of sFcεRI in human serum.

2. Materials and methods

2.1. Reagents

- Goat anti-mouse IgG Fc specific (Cat#M3534-1mL) from Sigma-Aldrich (St. Louis, MO);
- Anti-human Fc epsilon Receptor I alpha monoclonal antibody clone AER-37 (CRA1, Cat#16-5899-82) from eBioscience (San Diego, CA);
- Chimeric IgE (cIgE) contains the immunoglobulin heavy chain of human IgE and recognizes the haptens 4-hydroxy-3-nitrophenylacetic acid (NP) and 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) with its murine variable regions. cIgE was derived from Jw 8/5/13 cells as described (Singleton et al., 2009; Dehlink et al., 2010);
- Goat anti-human IgE horseradish peroxidase (HRP) conjugated antibody (Cat#H15707) from Invitrogen (Camarillo, CA);

- Coating buffer (Cat#00-0044-59) from eBioscience, 10 mM phosphate buffer saline (PBS), fetal bovine serum (FBS, Cat#100-106) from Gemini Bio-Products (West-Sacramento, CA), Tween-20 (Cat#P7949-500ML) from Sigma-Aldrich, (3, 3', 5, 5')-tetramethylenbenzidine (TMB, Cat#T0440-1L) from Sigma-Aldrich, 2N Sulfuric acid (Cat#A300SI-212) from Fisher Scientific (Pittsburgh, PA)

2.2. Equipment

- Immuno 96 MicroWell Solid Plates MaxiSorp (Cat#442404) from Thermo Scientific (Rochester, NY);
- BIO-TEK ELISA Microplate washer (Cat#8070-01) from TriContinent Scientific (Grass Valley, CA);
- Spectramax 250 Microplate reader (Cat#BC-MDSMX250) from Molecular Devices (Sunnyvale, CA).

2.3. Production of the reference standard

A sequence-verified plasmid containing the amino acid sequence 1–178 of mature human FcεRI alpha (NM_002001.2) was used to generate a recombinant version of the extracellular portion of the alpha-chain as a standard protein. This standard protein, referred to as recombinant soluble FcεRI (rsFcεRI), was generated in a baculovirus expression system and purified by ProMab Biotechnologies (Richmond, CA). Protein concentrations of standard samples were determined by BCA protein assay (Cat#23227) from Pierce (Rockford, IL). Reducing SDS-PAGE gels were run and purity of the samples was assessed by Coomassie Blue staining.

2.4. Immunoprecipitation and immunoblotting

rsFcεRI was diluted in lysis buffer (0.5% Surfact-Amps NP-40 (Cat#0028324) from Pierce (Rockford, IL), 20 mM Tris, pH 8.2, 20 mM NaCl, 2 mM EDTA, 0.1% NaN₃) at a concentration of 10 µg/ml. Immunoprecipitation was performed as described using cIgE and anti-NIP sepharose (Cat#N-1199-5) from Biosearch Technologies (Novato, CA) (Platzer and Fiebiger, 2010). Precipitated rsFcεRI was eluted in reducing Laemmli buffer, samples were run on 12% SDS-PAGE gels, transferred to PVDF Transfer Membrane (Cat#88518) from Thermo Scientific and probed with 0.5 mg/ml CRA1, followed by detection with goat-anti-mouse IgG HRP conjugated (1:2000, Cat#31430) from Pierce. HRP activity was detected using SuperSignal (Cat#34080) from Thermo Scientific according to the manufacturer's guidelines.

2.5. Sample types

For validation of the standardized method, sera from 66 patients (age range 1.2–17.8 years, median 9.9) were randomly selected from the patient cohort originally used to describe sFcεRI in human serum (Dehlink et al., 2011). Based on diagnosis with upper gastrointestinal (GI) endoscopy, the disease distribution found in the patients was: 28.8% eosinophilic esophagitis (n=19), 59.1% gastroesophageal reflux disease (n=39) and 12.1% with no signs of inflammation in the esophagus (control group, n=8). It is important to note that the control group cannot be considered a healthy population since these children were symptomatic and underwent upper GI endoscopy for diagnostic purposes. Patient sera were stored at –80°C in 1.5 ml Safe-Lock tubes from Eppendorf (Cat#022363204) prior to analysis. Serum IgE levels as assessed for the publication by Dehlink et al. were used for the correlation analysis (Dehlink et al., 2011). This study was approved by the Institutional Review Board of Children's Hospital Boston and all samples were collected after informed consent was obtained from the families.

2.6. Assay procedure

The standardized sFcεRI ELISA was based on the previously published method (Dehlink et al., 2011). Plates were coated with goat-anti-mouse IgG in coating buffer (2.5 µg/ml, 100 µl/well) and incubated overnight at 4°C. Next, excess antibody was washed away. In all washing steps, microplates were rinsed 6 times with washing buffer (PBS/0.05% Tween-20, 300 µl/well). Next, capture antibody CRA1 was added in coating buffer (0.5 µg/ml, 100 µl/well) and incubated overnight at 4°C. Plates were washed and blocked with PBS/10% FBS (300 µl/well) for 4 h at 37°C. For the standard curve, 7 two-fold serial dilutions of rsFcεRI ranging from 62.5 ng/ml to 0.98 ng/ml were prepared in PBS/2% FBS. Plates were washed and incubated with standards or 1:5 dilutions of serum samples (20%) in triplicates (100 µl/well). All samples were pipetted in triplicates. After overnight incubation at 4°C, plates were washed and incubated with the primary detection antibody (200 ng/ml cIgE in PBS/2% FBS, 100 µl/well) for 1 h at 37°C. Plates were washed and incubated with the secondary detection antibody (goat anti-human IgE HRP-conjugated antibody, 1:1000 in PBS/2% FBS, 100 µl/well) for 1 h at room temperature. After washing, TMB substrate solution was added (100 µl/well). The HRP reaction was stopped with 2N H₂SO₄ (50 µl/well) after 10 min. Next, optical density (OD) was measured at 450 nm using a 96-well microplate reader.

2.7. Validation of the sFcεRI ELISA

2.7.1. Linearity of calibration standards, specificity and determination of units of detection—The goodness of fit (R^2) of the standard curve was assessed by using a 4-parameter logistic regression algorithm calculated with GraphPad Prism Version 5.0b (GraphPad Software, La Jolla, CA). The specificity of the assay was assessed by omission of the capture antibody CRA1 or the primary detection antibody cIgE. Serum concentrations of sFcεRI were calculated by interpolation from the 4-parameter logistic regression algorithm of the standard curve with GraphPad Prism. A standard curve was pipetted on each individual plate.

The lowest level of quantification (LLOQ) was determined as the lowest concentration reliably detected (Armbruster et al., 1994). We established that the highest dilution of the standard with a coefficient of variation (CV (%) = mean divided by standard deviation) below 20% was 1:256,000 (equal to BCA-measured concentration of 0.98 ng/ml). Units of detection were established for quantification of sFcεRI in serum. This was necessary because the absolute concentration of the standard protein in the purified standard samples was higher than the amount of properly folded IgE-binding standard detected by the ELISA. For establishing units (U), the LLOQ was set at 2 U/ml.

2.7.2. Spike recovery assays—We next performed spike recovery assays to test for matrix interference (Lee and Hall, 2009). From the group of patients with low (<10 ng/ml) or <LLOQ serum sFcεRI levels, we randomly selected 16 samples that were then diluted to 40% and spiked by adding an equal volume of a 62.5 ng/ml standard solution. Recovery (%) was calculated by dividing the sFcεRI concentration measured by ELISA by the theoretical concentration of the sample (31.25 ng/ml + sFcεRI concentration of the unspiked sample in ng/ml). The assay passed the analysis criteria when recovery rates were within 70% and 110% (Findlay et al., 2000; Lee et al., 2006; Kelley and DeSilva, 2007; Lee and Hall, 2009).

2.7.3. Precision of the ELISA—The precision of the assay was determined by performing inter- and intra-assay variability tests. Sera of three patients with endogenous sFcεRI levels <LLOQ were pooled and rsFcεRI was added to a concentration of 10, 30 or 60 ng/ml. For the intra-assay variability test, we analyzed 9 replicates of individual samples on the same plate. Inter-assay variability was assessed by running samples on 6 different plates by two independent operators. To pass the test, the mean recovery has to fall within 20% of

the theoretical concentration and the corresponding CV has to be <20%. (Findlay et al., 2000; Lee et al., 2006; Kelley and DeSilva, 2007; Lee and Hall, 2009).

2.7.4. Stability of sFcεRI in serum—To assess the stability of sFcεRI in serum, 6 samples were analyzed before and after 2 freeze/thaw cycles. An additional aliquot was stored 4 h at room temperature. Concentration of sFcεRI in serum was assessed by the standardized ELISA and recovery after storage was calculated as percentage of initial concentration.

2.8. Correlation analysis of sFcεRI and IgE

Sera levels of sFcεRI were measured with the newly established standardized ELISA in 66 patients. Age specific IgE limits for the classification of pediatric patients were used as published (Dehlink et al., 2011). We assessed a possible correlation between serum sFcεRI and IgE levels by calculating the Spearman's rank correlation coefficient for the total population (n=66), followed by a sub-analysis of the patient group with normal serum IgE levels (n=44) and the group of patients with elevated serum IgE (n=22). Patients with sFcεRI levels <LLOQ were all scored as 10 U/ml for the purpose of correlation analysis. Means of sFcεRI levels >LLOQ were assessed for normality with Shapiro-Wilk W test and comparison between sub-groups was performed with Mann-Whitney U Test.

2.9. Statistical analysis

Results of the assay validation are presented as CV (%) and recovery (%). Means are presented with standard deviation as the measure of variability. Values were calculated using Microsoft Office Excel (version 14.0.0) from Microsoft Corporation (Redmond, WA). Spearman's rank correlation coefficients, Shapiro-Wilk W test and Mann-Whitney U test were performed with PASW Statistics (version 18.0.3) from IBM Corporation (Somers, NY). Results were considered statistically significant for p values <0.05.

3. Results

3.1. Characterization of the recombinant sFcεRI standard protein (rsFcεRI)

The absolute concentration of rsFcεRI after purification from baculovirus culture supernatants measured as 265.56 µg/ml. Standard samples were analysed for purity by Coomassie Blue staining (figure 1A, representative sample) revealing that the standards contained several protein specimens of different molecular weights. In contrast, immunoprecipitation with cIgE followed by immunoblotting with CRA1 showed a single broad band of 38–43 kDa corresponding to the properly folded IgE-binding protein (figure 1B). This set of experiments showed that not all of the purified standard interacted with cIgE and CRA1. Therefore, the absolute protein concentration of the standard samples does not equal the amount of properly folded standard detected by the ELISA. For this reason, units of detection had to be established for the quantification of sFcεRI serum levels instead of using absolute concentrations in ng/ml.

3.2. Linearity of standards and determination of units

A schematic of the ELISA is shown in figure 2A. A 7-point serial dilution of rsFcεRI in the range of 62.5–0.98 ng/ml was used for generating the standard curve (figure 2B). Curve fitting analysis was performed with a 4-parameter logistic regression algorithm using the measured OD and the absolute concentration of rsFcεRI in ng/ml. The average goodness of fit (R^2) of 8 individual experiments was 0.999.

A 20% serum dilution was determined empirically as the minimal required dilution for the assay. This dilution allows for detection of patients with low sFcεRI levels that might not otherwise be detected.

The lower level of quantification (LLOQ) is the lowest concentration that can still be quantified accurately (Armbruster et al., 1994). The LLOQ for the sFcεRI ELISA was found to be at the absolute standard concentration of 0.98 ng/ml (table 1). The LLOQ was set at 2 units/ml. After multiplication by the serum dilution factor (1:5), the dynamic range for the ELISA was 10 – 640 units/ml. Serum samples with OD values of equal or greater value than our highest standard dilution were considered above the upper limit of quantification and re-analysed at higher dilutions (i.e., 1:10 or 1:50). Samples ≤10 units/ml were defined as <LLOQ of the test. Figure 2C shows that concentrations sFcεRI above 62.5 ng/ml did not show a significant increase in OD (Standard curve plate B, figure 2C).

3.3. Specificity of the ELISA

Specificity of an immunoassay can be jeopardized by both specific nonspecificity as well as nonspecific nonspecificity. Specific nonspecificity is defined as cross-reactivity of detection reagents with substrates other than the analyte of interest (Kelley and DeSilva, 2007). By omission of the capture antibody CRA1 or the primary detection antibody, we confirmed specificity of the ELISA for sFcεRI, since both test modifications resulted in a loss of the signal over the whole range of concentrations (figure 2C). Nonspecific nonspecificity is the result of interference of unrelated matrix compounds with analyte detection (Kelley and DeSilva, 2007). Table 2 shows the results of the matrix interference assays with spiked serum samples. The mean recovery of 16 test samples was $81.3\% \pm 11.7\%$ and thereby meets our pre-set acceptance criteria. Two samples had recoveries <70%, suggesting a possible matrix interference in these patients. In the literature, hemolysis, lipidemia, hyperbilirubinemia, heterophilic antibodies and rheumatoid factor have been mentioned as potential interfering factors in immunoassays (Lee et al., 2006; Kelley and DeSilva, 2007). The two patients had different ages (3 vs. 7 years) and diagnosis (reflux esophagitis vs. gastroesophageal reflux with allergy) and at this time we have insufficient additional information available for further analysis of potentially interfering factors. We wish to point out that no cases of positive interference were detected. This is clinically relevant, since it limits the possibility of falsely elevated sFcεRI levels due to matrix interference.

3.4. Intra-assay and inter-assay variability

Table 3 and table 4 show the results of the intra-assay and inter-assay variability tests. Mean recovery was within 20% of the absolute concentration with a corresponding CV <20% for all 3 tested concentrations. These results met the acceptance criteria for immunoassay validation (Lee et al., 2006; Kelley and DeSilva, 2007; Lee and Hall, 2009).

3.5. Stability of sFcεRI in serum

Table 5 summarizes the effects of different storage conditions on the stability of sFcεRI in serum. Samples were subjected to two freeze/thaw cycles or stored 4 h at room temperature. Concentration of sFcεRI in all samples was assessed by the standardized ELISA and compared with the concentration of the original samples. Neither storage condition led to a substantial decrease in the detected levels of sFcεRI with an average recovery of 99% after the second freeze/thaw cycle and 104% after storage at room temperature. Additionally, we were able to measure two patient samples prior to the initial freezing step (storage at -80°). Analysis before and after the first freeze showed 119% and 76%. These stability tests show that the % of recovery at the three different storage conditions tested is within the intra-assay variability established for the test. We conclude that sFcεRI is a fairly stable serum protein

and that storage of frozen samples or two freeze/thaw cycles prior to measuring serum concentrations of sFcεRI does not significantly influence the outcome of the ELISA.

3.6. Correlation of sFcεRI and IgE levels in serum

Analyzing 66 patient sera from an established cohort (Dehlink et al., 2011), we found that 28 patients (42.4%) had sFcεRI serum levels <LLOQ. The average expression in patients with sFcεRI serum levels >LLOQ was 27.0 ± 23.4 units/ml (figure 3A). Subgroup analysis in patients with normal serum IgE levels and in those with elevated serum IgE showed that 20 patients (45.5%) had sFcεRI levels <LLOQ in the normal serum IgE group versus 8 patients (36.4%) in the elevated IgE group. sFcεRI levels were not normally distributed (significance of Shapiro-Wilk W test 0.00). Patients with normal serum IgE had significantly higher levels of sFcεRI (32.8 ± 27.7 units/ml) compared to patients with elevated IgE (17.1 ± 5.8 units/ml, $p=0.01$; figure 3A). The skewed population used for this analysis, however, hampers conclusions about the prevalence of sFcεRI or the clinical relevance of different serum levels of sFcεRI at this point.

In agreement with published data from this patient cohort (Dehlink et al., 2011), no correlation of sFcεRI and IgE levels was found in the total population ($r=0.093$, figure 3B) or in the patient sub-group with normal IgE levels ($r=0.161$, figure 3C). In the sub-group with elevated serum IgE, a weak correlation of both parameters was shown ($r=0.381$, figure 3D). In summary this set of data shows that correlation analysis performed for serum sFcεRI levels as quantified by the new standardized ELISA and IgE yields data that are comparable to the earlier study from our laboratory in which serum levels were assessed with a semi-quantitative, nonstandardized method (Dehlink et al., 2011).

Discussion

The goal of this study was to develop and validate a standardized ELISA for the detection of sFcεRI, as this is an indispensable step for the evaluation of serum levels of this protein in large patient cohorts with multiple diseases. The generation of such data sets is critical for performing studies that aim to understand the physiological relevance of this serum IgE receptor.

One of the initial hurdles for establishing a standardized method was the generation of a recombinant standard protein. The standard protein needed to be comparable to the endogenous sFcεRI containing an intact IgE-binding site as well as the epitope of CRA1 (Dehlink et al., 2011). IgE-precipitations of the rsFcεRI yielded a single band in Western Blot experiments, while Coomassie Blue stained gels of the same samples showed several proteins of different molecular weights. Aggregation of the protein standard during purification and protein degradation could be responsible for the higher and lower protein specimens respectively. This phenomenon is common during the generation of recombinant proteins; it does imply, however, the necessity for generating units of detection for quantification rather than using the measured concentration of the recombinant standards. Displaying serum sFcεRI in SI units as determined with absolute standard concentrations measured with BCA would result in the display of incorrectly high serum concentration, because the protein amount detected by ELISA does not equal the total amount of the standard in the purified fractions. An additional advantage of using units of detection defined with the LLOQ for each standard preparation individually is that inter-assay variability of the baculovirus expression and purification system can be taken into account. In summary, the use of units of detection allows for a reliable standardized evaluation of sFcεRI levels in large patient numbers.

The paper originally describing sFcεRI, correlated serum levels of the protein with serum IgE levels (Dehlink et al., 2011). We repeated the correlation analysis with serum sFcεRI quantified by the standardized ELISA as a validation of the test. Correlation analysis with sFcεRI levels determined by either ELISA methods yielded comparable results. In both cases, a weak correlation of sFcεRI and IgE was found in the group of patients with elevated serum IgE. This second correlation analysis with sFcεRI quantified by the standardized ELISA confirmed the findings of the original publication and therefore can be considered a validation of the newly established standardized method.

Conclusion

In summary we here developed and validated a standardized ELISA for the detection of sFcεRI in human serum. This new assay now allows for reliable detection of serum levels of sFcεRI in large patient cohorts. Consequently, studies addressing the question of whether sFcεRI is a biomarker for allergy or other diseases (such as cancer or autoimmune diseases) can be initiated. Such studies are the next most important step towards a better understanding of the physiological role of sFcεRI.

Acknowledgments

This work was supported by the Gerber Foundation (to S.N. and E.F.). Further support came from National Institutes of Health (NIH) grants R01AI075037 (to E.F.), K24DK82792-1 (to S.N.) and the Harvard Digestive Diseases Center (NIH Grant DK34854). W.L. is supported by grants from the Ter Meulen Fund, the Royal Netherlands Academy of Arts and Sciences and the Banning de Jong Fund. J.M. was supported by the Jo Keur Fonds and Stichting de Fundatie van de Vrijvrouwe van Renswoude te 's-Gravenhage. B.P. was supported by NIH grant DK081256. G.S. acknowledges support by Intendis Austria and the Max Kade-Foundation. We thank Michael Pardo for critically reading the manuscript, Hans Stry for helpful advice with regards to the statistical analysis of our data and Jessica LaRosa for her support with collecting patient material. A patent is pending for the ELISA method.

Role of the funding source:

None of the funding sources had a role in the design of the experiments.

Abbreviations

ELISA	Enzyme-Linked Immunosorbent Assay
sFcεRI	soluble Fc-epsilon-RI
rsFcεRI	recombinant soluble Fc-epsilon-RI
IgE	Immunoglobulin epsilon
IgG	Immunoglobulin gamma
cIgE	chimeric IgE
CV	coefficient of variation
HRP	horseradish peroxidase
PBS	phosphate-buffered saline
FBS	fetal bovine serum
TMB	(3, 3', 5, 5')-tetramethylenbenzidine
LLOQ	lower limit of quantification
BCA	bicinchoninic acid assay

OD	optical density
NP	4-hydroxy-3-nitrophenylacetic acid
NIP	4-hydroxy-3-iodo-5-nitrophenylacetic acid
kDa	kilodaltons
mAb	monoclonal antibody
IP	immunoprecipitation
SD	standard deviation

References

- Armbruster DA, Tillman MD, Hubbs LM. Limit of detection (LQD)/limit of quantitation (LOQ): comparison of the empirical and the statistical methods exemplified with GC-MS assays of abused drugs. *Clin Chem*. 1994; 40:1233–8. [PubMed: 8013092]
- Bochner BS, Busse WW. Allergy and asthma. *J Allergy Clin Immunol*. 2005; 115:953–9. [PubMed: 15867851]
- Dehlink E, Baker AH, Yen E, Nurko S, Fiebiger E. Relationships between levels of serum IgE, cell-bound IgE, and IgE-receptors on peripheral blood cells in a pediatric population. *PLoS One*. 2010; 5:e12204. [PubMed: 20808937]
- Dehlink E, Fiebiger E. The role of the high-affinity IgE receptor, FcepsilonRI, in eosinophilic gastrointestinal diseases. *Immunol Allergy Clin North Am*. 2009; 29:159–70. xii. [PubMed: 19141351]
- Dehlink E, Platzer B, Baker AH, Larosa J, Pardo M, Dwyer P, Yen EH, Szepefalusi Z, Nurko S, Fiebiger E. A Soluble Form of the High Affinity IgE Receptor, Fc-Epsilon-RI, Circulates in Human Serum. *PLoS One*. 2011; 6:e19098. [PubMed: 21544204]
- Gould HJ, Sutton BJ. IgE in allergy and asthma today. *Nat Rev Immunol*. 2008; 8:205–17. [PubMed: 18301424]
- Holgate ST, Davies DE. Rethinking the pathogenesis of asthma. *Immunity*. 2009; 31:362–7. [PubMed: 19766079]
- Kelley M, DeSilva B. Key elements of bioanalytical method validation for macromolecules. *Aaps J*. 2007; 9:E156–63. [PubMed: 17614356]
- Klupal R, Osterhoff B, Wang B, Kinet JP, Maurer D, Stingl G. The high-affinity receptor for IgE is the predominant IgE-binding structure in lesional skin of atopic dermatitis patients. *J Invest Dermatol*. 1997; 108:336–42. [PubMed: 9036935]
- Kraft S, Kinet JP. New developments in FcepsilonRI regulation, function and inhibition. *Nat Rev Immunol*. 2007; 7:365–78. [PubMed: 17438574]
- Kubota T, Mukai K, Minegishi Y, Karasuyama H. Different stabilities of the structurally related receptors for IgE and IgG on the cell surface are determined by length of the stalk region in their alpha-chains. *J Immunol*. 2006; 176:7008–14. [PubMed: 16709862]
- Lee JW, Devanarayan V, Barrett YC, Weiner R, Allinson J, Fountain S, Keller S, Weinryb I, Green M, Duan L, Rogers JA, Millham R, O'Brien PJ, Sailstad J, Khan M, Ray C, Wagner JA. Fit-for-purpose method development and validation for successful biomarker measurement. *Pharm Res*. 2006; 23:312–28. [PubMed: 16397743]
- Lee JW, Hall M. Method validation of protein biomarkers in support of drug development or clinical diagnosis/prognosis. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009; 877:1259–71.
- Maurer D, Fiebiger E, Reininger B, Wolff-Winiski B, Jouvin MH, Kilgus O, Kinet JP, Stingl G. Expression of functional high affinity immunoglobulin E receptors (FcεRI) on monocytes of atopic individuals. *J Exp Med*. 1994; 179:745–750. [PubMed: 8294882]
- Platzer B, Fiebiger E. The signal peptide of the IgE receptor alpha-chain prevents surface expression of an immunoreceptor tyrosine-based activation motif-free receptor pool. *J Biol Chem*. 2010; 285:15314–23. [PubMed: 20304923]

Singleton TE, Platzer B, Dehlink E, Fiebiger E. The first transmembrane region of the beta-chain stabilizes the tetrameric Fc epsilon RI complex. *Mol Immunol.* 2009; 46:2333–9. [PubMed: 19406478]

HIGHLIGHTS

- We developed a standardized ELISA to quantify soluble FcεRI in human serum. (75)
- Specificity and precision tests met acceptance criteria for immunoassay validation. (83)
- We further validated our ELISA in an established pediatric patient cohort. (74)
- Serum soluble FcεRI correlates with IgE in patients with elevated serum IgE. (76)
- This ELISA can now be used to study the role of soluble FcεRI in health and disease. (84)

Figure 1. Generation and characterization of a standard protein for the quantification of human sFcεRI by ELISA. A recombinant protein (rsFcεRI) was generated in a baculovirus expression system using a plasmid containing the extracellular domain of human FcεRI-alpha

A) Coomassie staining of rsFcεRI purified from baculovirus supernatants. Purified standard (lane I) is compared to a protein standard (lane II). Purified rsFcεRI contains multiple protein specimens of various sizes with a prominent band at ~40kD.

B) Immunoprecipitation of rsFcεRI with IgE (left lane) and IgG as a negative control (Ctrl, right lane) was followed by Western blot analysis with FcεRI-alpha specific mAb CRA1. CRA1 detects an IgE-precipitate of ~40kD.

Figure 2. Establishing a standardized ELISA for the detection of sFcεRI

A) Schematic model of the procedure. Plates were coated with anti-mouse IgG (step 1). mAb CRA1, which binds to an epitope in the stalk region of human FcεRI- α , was used as the capture antibody (step 2). After blocking, standard protein or diluted serum samples were added to the wells (step 3). For detection, wells were incubated with cIgE (primary detection Ab, step 4), followed by horseradish peroxidase conjugated (HRP) anti-human IgE (secondary detection Ab, step 5).

B) Representative standard curve using rsFcεRI. X-axis shows a standard concentration ranging from 0.98–62.5 ng/ml. Y-axis shows the optical density (OD) measured at 450 nm. Means of triplicates $\pm$ SD are shown for each measurement. C) Specificity for the detection of rsFcεRI by ELISA. Omission of the capture antibody (step 2, filled squares) or the first detection antibody (step 4, open circles) resulted in a loss of the rsFcεRI-derived signal over the entire concentration range (0–125 ng/ml).

Figure 3.

Correlation analysis of sFcεRI levels determined by the standardized ELISA and serum IgE.

(A) Analysis of serum levels of sFcεRI in a patient population. Three different patient groups are displayed (y-axis): the total patient population (circles), a subgroup of patients with IgE levels within their age-specific reference range (squares, IgE ≤ reference range) and a subgroup of patients with elevated IgE levels for their age (triangles, IgE > reference range). Serum sFcεRI levels are shown as units/ml on the x-axis. Serum sFcεRI levels of 28 out of 66 patients (42.4%) were <LLOQ. Patients in the subgroup with normal serum IgE levels (32.8 ± 27.7 units/ml) had significantly higher serum sFcεRI levels compared to the subgroup of patients with elevated IgE (17.1 ± 5.8 units/ml, $*p=0.01$).

The Spearman's rank correlation coefficient was calculated for sFcεRI levels (x-axis) and serum IgE levels (y-axis) for the total patient population (3B), the patient subgroup with normal IgE levels (3C) and patients with elevated serum IgE (3D). Patients with sFcεRI levels <LLOQ were scored as 10 U/ml for this purpose. No correlation was found in the total population ($r=0.093$) or in the population with normal IgE levels ($r=0.161$). A weak positive correlation ($r=0.381$) was found in the population with elevated IgE levels.

Table 1

Establishing units of detection for serum sFcεRI concentration

Dilution Factor ($\times 10^3$)	rsFcεRI (ng/ml)	Mean CV% (n=8)	sFcεRI (units/ml)	sFcεRI with 1:5 dilution (units/ml)
1:4	62.50	3.53	128	640
1:8	31.25	2.64	64	320
1:16	15.63	4.51	32	160
1:32	7.81	4.16	16	80
1:64	3.91	5.08	8	40
1:128	1.95	8.27	4	20
1:256	0.98	12.68	2	10
1:512	0.49	52.81	<LLOQ	<LLOQ

Establishing units for the quantification of serum sFcεRI. For every dilution factor, the imprecision (CV%) of 8 replicate plates was calculated. 1:256,000 was the highest dilution meeting the criteria of CV <20% and was therefore determined as the lowest level of quantification (LLOQ). The LLOQ was set at 2 units/ml. For establishing and validating the ELISA we used 1:5 serum dilutions and the resulting range of detection was therefore 10–640 units/ml.

Table 2

Matrix interference test of the sFcεRI ELISA

Sample	Unspiked	Spiked (31.25 ng/ml)	
	sFcεRI (ng/ml)	rsFcεRI (ng/ml)	Recovery %
1	<LLOQ	28.0	89.6
2	<LLOQ	27.6	88.3
3	<LLOQ	30.0	96.0
4	<LLOQ	31.3	100.2
5	<LLOQ	24.9	79.7
6	<LLOQ	25.5	81.6
7	<LLOQ	29.0	92.8
8	<LLOQ	23.6	75.5
9	<LLOQ	24.2	77.4
10	<LLOQ	29.6	94.7
11	2.34	24.9	74.1
12	2.65	27.2	80.2
13	2.91	21.9	64.1
14	2.14	24.9	74.6
15	6.98	22.1	57.8
16	3.55	26.0	74.7

Accuracy of the sFcεRI ELISA. Spike-recovery assays were used to determine the accuracy and precision of the immunoassay. Sera from 16 different patients were spiked to 31.25 ng/ml of rsFcεRI. The difference between detected and theoretical concentration (Recovery %) was measured for every patient. Mean recovery was 81.3% ±11.7%.

Table 3

Intra-assay variability of the sFcεRI ELISA

rsFcεRI (ng/ml)	Mean	SD	CV %	Recovery %
60	63.85	3.69	5.78	106.42
30	28.02	1.13	4.02	93.39
10	10.77	1.37	12.75	107.73

Precision of the sFcεRI ELISA. Intra-assay variability is shown with nine replicates of test samples containing low (10 ng/ml), medium (30 ng/ml) and high (60 ng/ml) concentrations of rsFcεRI on a single plate. Average calculated concentrations and percentage of coefficient of variation (% CV) are shown for each concentration (n=9). SD = standard deviation. Recovery % is the difference between detected and theoretical concentration.

Table 4

Inter-assay variability of the sFcεRI ELISA

rsFcεRI (ng/ml)	Mean	SD	CV %	Recovery %
60	59.05	5.13	8.68	98.41
30	28.06	3.01	10.73	93.54
10	9.81	1.28	13.00	98.08

Precision of the sFcεRI ELISA. Inter-assay variability is shown with test samples, containing low (10 ng/ml), medium (30 ng/ml) and high (60 ng/ml) concentrations of rsFcεRI on 6 different plates pipetted by two independent operators. Average calculated concentrations and percentage of coefficient of variation (% CV) are shown. SD= standard deviation. Recovery % is the difference between detected and theoretical concentration.

Table 5

Stability of sFcεRI in serum samples

Sample	Initial measurement sFcεRI (U/ml)	+ 1 cycle	+ 2 cycles	+ 4 h at RT
1	127.4	129.2 (101%)	133.1 (104%)	146.1 (114%)
2	117.8	115.3 (98%)	114.5 (97%)	109.3 (93%)
3	108.3	107.1 (99%)	114.8 (106%)	124.9 (115%)
4	120.6	117.8 (98%)	92.6 (77%)	104.8 (87%)
5	46.1	44.8 (97%)	48.7 (106%)	47.6 (103%)
6	62.9	68.4 (109%)	65.8 (105%)	68.2 (108%)

Stability of sFcεRI in serum samples was tested after repetitive freeze/thaw cycles and after storage of the serum at RT. Serum levels of sFcεRI were measured in 6 individual samples. Concentration is given in sFcεRI in U/ml. Serum samples were reevaluated after one or two freeze/thaw cycles. Another aliquot was left at room temperature (RT) for 4 h prior to analysis. Recovery is given in brackets as % of the initially measured sFcεRI concentration.