Original Article

Evaluation of FcεRI-bindable human IgE with an enzyme-linked immunosorbent assay using a recombinant soluble form of the human FcεRIα ectodomain

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ABSTRACT
To estimate the levels of serum IgE that is actually able to be bound to FcεRI, we developed a novel enzyme-linked immunosorbent assay (ELISA) using a recombinant soluble form of the human FcεRIα ectodomain (soluble α-ELISA). We evaluated the levels of FcεRI-bindable serum IgE in normal volunteers and in patients with atopic dermatitis and bronchial asthma, and compared them with those of total IgE measured by a conventional sandwich-ELISA. There was a striking difference between the IgE values evaluated with these two ELISA systems. In most of the sera, the IgE values evaluated with the soluble α-ELISA were substantially lower than those evaluated with the sandwich-ELISA. Our results indicate that all serum IgE (especially in hyper-IgE sera) does not necessarily bind to FcεRI and that it will be useful to evaluate FcεRI-bindable IgE for further analysis of allergic states.

Key words: allergy, enzyme-linked immunosorbent assay, FcεRI, FcεRI-bindable IgE, immunoglobulin E, soluble human FcεRIα ectodomain.

INTRODUCTION
Immunoglobulin E (IgE) is the critical antibody that elicits the type I allergic reaction when it binds to the high affinity receptor for IgE (FcεRI) on the surface of mast cells and basophils and cross-linked with multivalent allergens. Therefore, it would be useful to estimate the actual IgE able to bind to FcεRI for a detailed analysis of type I allergy.

There was one report published 20 years ago documenting that the number of cell-bindable IgE molecules did not always correlate with serum IgE levels in atopic individuals.1 A few other reports have presented further evidence that serum IgE molecules may be heterogenous with respect to cell-binding and histamine-releasing properties in passive cutaneous anaphylaxis (PCA) reactions.2-5 Taking these reports into consideration, all serum IgE molecules may not always bind to FcεRI on the cell surface and may not always evoke an allergic reaction. This heterogeneity of serum IgE may be explained by the possible occurrence of FcεRI-bindable and non-bindable IgE molecules in sera. Anti-IgE autoantibodies,6-9 anti-FcεRIα chain autoantibodies10 and/or other IgE-binding molecules, such as soluble CD23 (FcεRII, the low-affinity receptor for IgE)11 and 60 kDa IgE-binding component,12 were also detected in the sera of allergic patients. In addition, epsilon binding protein/macrophage differentiation antigen-2 (eBP/Mac-2), which can bind to both IgE and FcεRI, has been detected in the sera in a soluble form.13-16 These autoantibodies and IgE-binding proteins in the sera may block IgE-binding to FcεRI and may modulate allergic reactions.

As one of the useful means for the clinical diagnosis of allergy, enzyme-linked immunosorbent assay (ELISA) systems to evaluate serum IgE levels are widely used. However, present ELISA systems do not indicate whether the IgE measured by these systems is able to bind to FcεRI.
or not. It is important to evaluate the levels of serum IgE that can bind to FcεRI for the further estimation of the allergic state, with regard to factors that block IgE-binding to FcεRI. For this purpose we have developed a novel ELISA system to detect FcεRI-bindable IgE using a recombinant soluble form of the α-subunit of human FcεRI (soluble α-ELISA).

FcεRI has a tetrameric structure composed of one α-, one β- and two disulfide-linked γ-subunits, of which the α-subunit binds IgE with high affinity. A recombinant soluble form of the ectodomain of the human FcεRIα subunit (soluble α) was recently generated by gene engineering and was produced by transfected mammalian cells, insect cells and yeast. Soluble α is shown to bind IgE with an affinity as high as that of native FcεRI and the binding stoichiometry is confirmed to be 1:1.

Serum IgE bound onto the soluble α (the product of CHO transfectants) was detected by an anti-IgE polyclonal antibody. As, in this assay system, IgE binds directly to its specific binding site on the FcεRIα, we could, for the first time, estimate FcεRI-bindable IgE. We evaluated levels of FcεRI-bindable IgE in sera from normal subjects and in sera from patients with atopic dermatitis and bronchial asthma and compared the values of FcεRI-bindable IgE with those of total IgE measured by a conventional sandwich-ELISA using two different antibodies against IgE.

**METHODS**

**Serum samples**

Sera were obtained from peripheral blood samples taken from 127 individuals who had given their informed consent. Serum samples were stored at −20°C until use. Five serum samples from normal subjects and in sera from patients with atopic dermatitis and bronchial asthma were analyzed in the present study.

Briefly, peripheral venous blood was taken and left on ice for 1 h and was then centrifuged at 300 g at 4°C for 20 min to separate the serum.

**ELISA to detect FcεRI-bindable IgE using soluble α**

Ninety-six-well microtiter plates (Immulon 2; Dynatech Laboratories, Chantilly, VA, USA) were coated with 90 ng/100 μL per well soluble α in coating buffer at 4°C overnight. This soluble α was purified from the culture supernatant of CHO transfectants secreting soluble α, as previously described. Briefly, soluble α was purified by a mouse IgE-Affigel-10 column (Bio-Rad Laboratories, Richmond, CA, USA) and gel filtration through a Sephacryl S-200 column (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The wells were washed five times with the washing buffer, blocked with the blocking buffer at 37°C for 1 h and washed again five times with the washing buffer. The serially diluted IgE standard (100 μL/well) or diluted sera in dilution buffer were added to the wells and were incubated at 37°C for 3 h. Briefly, this standard IgE was purified from the serum of IgE-myeloma by a combination of ammonium sulfate precipitation, gel and ion-exchange chromatography and immuno-adsorbents, as described previously. Wells were washed five times with the washing buffer and were then incubated with horseradish-peroxidase (HRP)-labeled goat anti-human IgE polyclonal antibody (Organon Teknika Corp., Durham, NC, USA; diluted 1/1000 with dilution buffer) at 37°C for 1 h. After washing, the substrate solution (100 μL/well) was finally added and the plates were incubated at room temperature for 10 min. To stop the reaction, 25 μL/well of 2 mol/L H2SO4 (25 μL/well) was added. Optical absorbance of the samples was measured at a wavelength of 450 nm using a Bio-Rad microplate reader (model 450; Bio-Rad Laboratories, Richmond, CA, USA) with a 450 nm filter. All assays were performed in triplicate and the standard deviation was below 5%.

The buffers used in this assay are as follows: (i) coating buffer: 50 mmol/L carbonate buffer, pH 9.4; (ii) phosphate-buffered saline (PBS): 10 mmol/L phosphate buffer containing 0.15 mol/L NaCl, pH 7.2; (iii) washing buffer: PBS containing 0.05% (v/v) Tween-20 (Wako Pure Chemical Industries Ltd, Osaka, Japan); (iv) dilution buffer: PBS containing 0.1% (w/v) casein (skimmed milk; Snow Brand Industries Co. Ltd, Sapporo, Japan); (v) blocking buffer: PBS containing 1% (w/v) casein and 0.02% (w/v) NaN3; (vi) substrate buffer: 10 mmol/L sodium acetate buffer, pH 5.8; and (vi) substrate solution: 3,3′,5,5′-tetramethyl-benzidine (TMBZ; Wako Pure Chemical Industries Ltd) 0.1 mg/mL in substrate buffer containing 0.0075% (w/v) H2O2.

**Conventional sandwich-ELISA to detect total IgE**

Sandwich-ELISA was performed using an ELISA*-IgE kit (International Reagents Corp., Kobe, Japan) following the manufacturer’s instructions. Serially diluted standard
EVALUATION OF FcεRI-BINDABLE IgE

IgE or the diluted sera in dilution buffer (200 μL/well) were added to the wells coated with a mouse anti-human IgE monoclonal antibody. The plates were incubated at room temperature for 1 h, washed five times with washing buffer and then further incubated with a HRP-labeled anti-human IgE polyclonal antibody (Ab) at room temperature for 30 min. After washing, 100 μL/well substrate solution (ortho-phenylene diamine (OPD); 2 mg/mL in substrate buffer) was added and the plates were incubated in the dark at room temperature for 30 min; 2 mol/L H₂SO₄ was added to stop the reaction. Optical absorbance was measured at a wavelength of 490nm using a Bio-Rad microplate reader (model 450) with a 490 nm filter. All assays were performed in triplicate and the standard deviation was below 5%.

Effect of soluble FcεRII/CD23 on the evaluation of IgE by soluble α-ELISA

The effect of soluble CD23 was examined by adding soluble CD23 (T Cell Diagnostics Inc., Cambridge, MA, USA) to sample sera at various concentrations when evaluating IgE by the soluble α-ELISA.

Soluble CD23 dilutions were mixed with the sample sera, incubated at 37°C for 2 h and the mixture was added to the soluble α-coated plate.

The following steps of the soluble α-ELISA were the same as those described above. The levels of serum FcεRII/CD23 were determined using the CD23 test kit (T Cell Diagnostics, Inc.) following the manufacturer’s instructions.

Statistical analysis

The data of the calibration curves, shown in Fig. 2, and of the stability of the serum IgE, shown in Fig. 3, were statistically analyzed by the Student’s t-test. A probability of 5% or less was considered significant. The data of comparisons of serum IgE values, shown in Fig. 4, were statistically analyzed by Fisher’s Z-transformation. A probability of 1% or less was considered significant. These analyses were performed by using Stat View II⁹ (Abacus Concepts Inc., Berkeley, CA, USA).

RESULTS

Compatibility of soluble α- and conventional sandwich-ELISA

Representative calibration curves of the standard IgE generated by soluble α- and conventional sandwich-ELISA are presented in Fig. 1a,b, respectively. The sensitivity of the soluble α-ELISA was 300 pg and that of the sandwich-ELISA was 1.5 ng.

In the beginning, we had plotted 10 doses of the diluted standard IgE for generating the calibration curves by soluble α-ELISA and compared these calibration curves with those of five doses, as shown in Fig. 1. As

![Graph](image)

Fig. 1 Calibration curves of the standard IgE generated by (a) soluble α-ELISA and (b) conventional sandwich-ELISA. Calibration curves were generated each time when IgE measurements were performed.
there had been almost no difference between them, we subsequently used five doses of standard IgE for generating standard curves each time.

Standard IgE, HRP-labeled anti-human IgE Ab and enzyme substrate applied in these two ELISA systems were different from each other, as described in Methods; therefore, we first tried to confirm the compatibility of these two ELISA systems by comparing the calibration curves of both ELISA when the reagents were exchanged between them. The calibration curves shown in Fig. 2a were generated by the sandwich-ELISA using two different IgE, the first was a myeloma IgE originally used in the soluble α-ELISA and the second was the IgE supplied in the sandwich-ELISA kit. The concentration of the latter IgE was originally shown as international units (IU), but we converted it to represent an absolute amount (ng) with the following equation:

\[ 1 \text{ IU IgE} = 2.4 \text{ ng IgE} \]

As shown in Fig. 2a, there was almost no difference between the two standard curves using two different IgE with all other reagents of the kit as mentioned above. For the sandwich-ELISA, we next used the HRP-labeled anti-human IgE Ab (Organon Teknica Corp.), which was originally used in the soluble α-ELISA, and almost the same standard curves were obtained with the two different standard IgE (data not shown). We subsequently generated calibration curves using the same HRP-labeled anti-human IgE Ab and the same substrate for HRP (TMBZ) in both soluble α- and sandwich-ELISA. As shown in Fig. 2b,c (soluble α-ELISA and sandwich-ELISA, respectively), there was almost no difference between each calibration curve, indicating that we can compare the IgE values estimated by each ELISA system despite using different reagents.

Based on these results, we evaluated serum IgE levels with these two ELISA systems using different reagents and compared those IgE values with each other.

**Stability of serum IgE for binding to FcεRIα**

As it is well known that the binding activity of IgE to the cell surface is easily lost under some conditions, such as high temperature (56°C, 30 min: heat inactivation), we examined the stability of serum IgE for the soluble α-ELISA after at least 35 days storage at 4 or -20°C. Serum IgE levels of five samples from normal subjects were evaluated by the both ELISA systems every 7 days as shown in Fig. 3. The IgE values in each serum sample estimated by both ELISA systems remained almost constant during the indicated preservation periods at both temperatures (4 or -20°C), although the IgE values estimated by the soluble α-ELISA were substantially lower than those obtained with the sandwich-ELISA. These results indicate that serum IgE was stable for binding to the FcεRIα chain under these conditions for at least 35 days.
Evaluation of FcεRI-bindable IgE in sera by soluble α-ELISA

We estimated serum IgE levels by both soluble α- and conventional sandwich-ELISA and compared each IgE value between these two different ELISA systems. We observed a positive correlation between the IgE values estimated by each ELISA system ($r = 0.581, P < 0.01$). However, as already mentioned, serum IgE values estimated by the soluble α-ELISA were significantly lower than those measured by the conventional sandwich-ELISA (Fig. 4). This discrepancy between the two ELISA systems was strikingly larger when we evaluated hyper-IgE sera. In hyper-IgE sera, in which total IgE values were over 3000 ng/mL, the FcεRI-bindable fractions were less than 10%. In most patients' sera, this ratio was less than 50% (the largest population had a distribution of 20–40%). In only two cases was the FcεRI-bindable fraction more than 80%.

Effect of soluble FcεRII/CD23 on IgE binding to FcεRI

The levels of the soluble FcεRII/CD23 in the sample sera ranged from 7 to 289 ng/mL and did not correlate with either the levels of total or FcεRI-bindable IgE. We also examined the dose-dependent effect of soluble
FceRII/CD23 on IgE-binding to FcεRI, as described in Methods. Even 100-fold the molar excess of soluble CD23 over the coated-soluble α could not inhibit IgE-binding.

**DISCUSSION**

As functional IgE that can elicit type I allergy should be the IgE that can bind to the cell surface FcεRI, it would be useful to estimate FcεRI-bindable IgE levels in sera for a more detailed analysis of the allergic state. We therefore developed a novel ELISA to detect FcεRI-bindable IgE using soluble α, which is a recombinant soluble form of the human FcεRIα ectodomain produced by CHO transfectants. Soluble α can bind IgE with the same high affinity as that of native FcεRI on the cell surface. We determined the binding affinity of soluble α for IgE using surface plasmon resonance (BLA core system; Pharmacia, Uppsala, Sweden). For several myeloma IgE, α value of Kₐ (soluble α) = 1.2-1.6×10⁻⁹/mol per L was obtained (C Ra et al., unpubl. obs., 1996). Furthermore, the stoichiometry of IgE-binding of soluble α in the liquid phase has been confirmed to be 1:1. The capacity of IgE-binding of soluble α in the solid phase is smaller than that of soluble α in the liquid phase because soluble α is randomly coated on the plate. However, for our purposes, randomly coated soluble α (90 ng (1.8 pmol)/well) is enough to evaluate serum IgE bound to soluble α (soluble α-ELISA).

Calibration curves of standard IgE (human myeloma IgE) generated by the soluble α-ELISA correlated well with standard curves determined by a conventional sandwich-ELISA using two different anti-human IgE antibodies, despite the fact that the reagents used in the two ELISA systems were different, and we confirmed the compatibility of the soluble α-ELISA with the sandwich-ELISA by exchanging the reagents used between them. As IgE had been considered to easily degenerate and to lose its binding activity for the receptor, we examined the stability of the binding activity of serum IgE to the soluble α-coated plate. The binding of the IgE in sera stored at 4 or −20°C did not change for at least 35 days in the soluble α-ELISA system. Using this newly developed soluble α-ELISA to detect FcεRI-bindable IgE, we evaluated the fraction of FcεRI-bindable IgE in patients’ sera. When we compared the IgE values obtained with the soluble α-ELISA system with the values obtained with the sandwich-ELISA (total serum IgE), there was a large discrepancy in the IgE values between the two ELISA systems. In most patients’ sera, the proportion of soluble α-bindable IgE was less than 50% of the total IgE, although the absolute IgE values showed a positive correlation in these two ELISA systems.

It is known that the binding of IgE to the receptor is easily lost at high temperatures. We therefore estimated IgE values in heat-inactivated sera by both ELISA systems. In all of sera, approximately 90% of IgE binding to soluble α was lost by treatment at 56°C for 30 min and almost 100% was lost by treatment at the same temperature for 2 h. By sandwich-ELISA, almost the same reduction in IgE values after heat-inactivation was obtained, except for some hyper-IgE sera in which some IgE was still detected. Therefore, it is unlikely that the differences obtained in IgE values between the two ELISA systems are due to the degeneration of IgE molecules.

These results suggest that there may be some factors in these sera (especially in hyper-IgE sera), such as anti-IgE and/or anti-FcεRI autoantibodies, that may block IgE binding to FcεRI and/or structurally FcεRI-non-bindable IgE. Soluble FcεRII/CD23 seems to be one of the candidates for a factor that inhibits IgE-binding to FcεRI. However, even a 100-fold molar excess of soluble FcεRI/CD23 over soluble α could not block IgE binding to the soluble α-coated plate (data not shown) and the level of soluble FcεRII/CD23 in the patients’ sera ranged from 7 to 289 ng/mL. Anti-IgE autoantibodies have been reported to significantly increase in the sera of patients with bronchial asthma and atopic dermatitis and to occur as immune complexes with IgE in these sera. If the recognition sites of these anti-IgE autoantibodies localize within the Cε3 domain of the Fcε chain, which is the binding site of IgE to FcεRI, these anti-IgE autoantibodies will be able to block IgE binding to FcεRI.

In contrast, anti-FcεRIα chain autoantibodies were detected in the sera of chronic urticaria patients at a high frequency and were elucidated to trigger basophil degranulation. We also detected anti-FcεRIα autoantibodies in the sera of patients with atopic dermatitis, which inhibited IgE binding to FcεRIα (S Hayashi et al., unpubl. obs., 1996). As the IgE binding site on the FcεRIα ectodomain is mainly localized to the second immunoglobulin-homologous domain, if these autoantibodies recognize around this site, they will block IgE binding. If soluble forms of the FcεRI chain occur, which would maintain the IgE-binding capacity of the sera, they may also contribute to the blocking of IgE binding to the receptor. There are several other molecules reported that can also interfere with IgE binding to...
the receptor, such as the 60 kDa IgE-binding component in the sera of patients with atopic dermatitis and eBP/Mac-2 secreted from macrophages. Serum IgE values estimated by our soluble α-ELISA may reflect the influences of such serum factors, including as yet undefined factors. If there are some structural changes in serum IgE of some patients, these will also have an effect on the IgE values measured by the soluble α-ELISA system. In particular, structural changes in the Ce3 domain (the binding site for FceRIα) in the primary sequence and/or in carbohydrate modification may exert some effect on the binding of IgE to the receptor. Whatever the factors are that affect the binding of IgE, we can expect to acquire a further insight into the allergic states by estimating FceRI-bindable serum IgE.

In the present study we have reported on a trial to establish a soluble α-ELISA system to estimate FceRI-bindable serum IgE without regard to antigen specificity. Estimation of FceRI bindable-IgE by our soluble α-ELISA system revealed that all serum IgE does not necessarily bind to FceRI. With regard to the aforementioned factors, further investigations of a molecular nature will have to be performed in future. In the meantime, we are trying to develop other systems to evaluate allergen-specific and FceRI-bindable IgE that is most critical for the activation of mast cells in allergic reactions (C Ra et al., unpubl. obs., 1996).

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