

Direct Expression of the Extracellular Portion of Human FcεRIα Chain as Inclusion Bodies in *Escherichia coli*

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The extracellular portion of the α chain of the human high-affinity IgE receptor (FcεRIα) was expressed as inclusion bodies in *Escherichia coli*. In immunoblot analysis, two bands were reactive to human IgE and mouse anti-human FcεRIα monoclonal antibodies. N-terminal sequencing showed that the two bands were equivalent to the soluble FcεRIα with a methionine residue at the N-terminus (Met-1-172) and 23-172, in which the N-terminal 22 residues of the soluble FcεRIα have been removed, possibly by degradation in *E. coli* cells. IgE-binding to CHO cells expressing FcεRI was inhibited by the addition of the recombinant products prepared by the refolding procedure from inclusion bodies. The system for the expression of soluble human FcεRIα in *E. coli* presented in this study and its further improvement would be useful for the production of the protein as a potent therapeutic and for analysis of the IgE-FcεRIα interaction.

Key words: allergy; high-affinity IgE receptor; *Escherichia coli*; expression system; inclusion body

The high-affinity receptor for IgE (FcεRI) is the key molecule in the triggering of the IgE-mediated allergic reaction. Multivalent allergens bridge the receptor-bound IgE and induce the aggregation of FcεRI on the surface of mast cells and basophils, triggering the subsequent release of mediators responsible for allergic symptoms.^{1,2} FcεRI consists of non-covalently associated subunits: one α chain (FcεRIα), one β chain, and two disulfide-linked γ chains.^{3,4} The binding site for the Fcε portion of IgE

is located in the extracellular portion of FcεRIα.^{5,6} Interfering with the binding of IgE to FcεRIα is considered a straightforward strategy for preventing the IgE-mediated allergic reaction specifically.⁷ Various systems for the expression of FcεRIα^{6,8–11} and synthetic peptides designed based on the sequence of FcεRIα¹² have been developed. Recently, the crystal structure of the extracellular portion of the human FcεRIα, which was expressed in insect cells using the baculovirus expression system, was analyzed by Garman *et al.*¹³

Robertson detected the production of a soluble FcεRIα, which was fused with a signal peptide for secretion and *c-myc* tag for affinity purification, using an anti-*c-myc* monoclonal antibody (mAb), in the medium and periplasmic space of *E. coli*.⁹ In this study, we examined expression of a soluble human FcεRIα with an additional methionine at the N-terminal residue as inclusion bodies within *E. coli* cells because overexpression of recombinant proteins as insoluble inclusion bodies within *E. coli* cells is generally recognized as another efficient system in terms of productivity and simplicity of purification.

Materials and Methods

Construction of the expression vector of the soluble human FcεRIα. The DNA fragment encoding the extracellular portion of the human FcεRIα with an initiation codon and a stop codon was generated by polymerase chain reaction (PCR) incorporating unique restriction sites to assist with subcloning. PCR products with an *Eco*RI site and a *Nde*I site at the 5'

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Abbreviations: FcεRI, high affinity IgE receptor; FcεRIα, the α subunit of FcεRI; D1, the first domain; D2, the second domain; mAb, monoclonal antibody; CHO, Chinese hamster ovary; CHO/αβγ, CHO transfectant expressing human FcεRI; PBS, phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; PCR, polymerase chain reaction

end and with a stop codon and a *SalI* site at the 3' end were generated as described.¹⁴⁾ An initiation codon was included within the *NdeI* site. The amplified fragments were digested with *NdeI* and *SalI*, and ligated to the pGEMEX1- Δ *NdeI* vector¹⁵⁾ which was digested with *NdeI* and *SalI*. The DNA sequence of the inserted fragment was analyzed using a 373A DNA sequencer and Dye-deoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.) and confirmed to be the same as the corresponding region reported in a previous report.¹⁶⁾

Construction of deletion mutants of the soluble human Fc ϵ RI α . The DNA fragments encoding the deleted peptides of the extracellular portion of human Fc ϵ RI α were generated by PCR and subcloned into the pGEMEX1- Δ *NdeI* vector as was the DNA fragment encoding the whole extracellular portion. The inserted fragments were sequenced and confirmed to have the same sequences as the corresponding regions.

Expression of the soluble human Fc ϵ RI α and its deletion mutants in *E. coli*. Soluble human Fc ϵ RI α and its deletion mutants were expressed in *E. coli* BL21 (DE3) transformed with each expression vector.¹⁵⁾ The cells were harvested by centrifugation.

N-terminal amino acid sequencing. The N-terminal amino acid sequences of the proteins electroblotted onto a polyvinylidene difluoride membrane (Immobilon: Millipore Co., Bedford, MA, U.S.A.) were analyzed with a 473A protein sequencer (Applied Biosystems).

Mouse anti-Fc ϵ RI α mAbs. Mice were immunized with a recombinant extracellular portion of human Fc ϵ RI α expressed in Chinese hamster ovary (CHO) cells.⁸⁾ Hybridoma cell lines secreting anti-human Fc ϵ RI α mAbs were established. The five mouse anti-human Fc ϵ RI α mAbs, CRA1, CRA2, CRA3, CRA4, and CRA5, were purified from ascitic fluid.¹⁴⁾

Immunoblotting. The harvested *E. coli* cells were suspended in 500 μ l of a buffer containing 10% (v/v) glycerol, 5% (w/v) SDS, 62.5 mM Tris/HCl (pH 6.8), and 5% (v/v) 2-mercaptoethanol, and boiled for 5 min. The cell lysates were electrophoresed on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (18%). After electrophoresis, the gel was used for immunoblotting as described previously but with some modifications.^{17,18)} Proteins transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, U.S.A.) were incubated with the mouse anti-human Fc ϵ RI α mAbs, or human myeloma IgE (Yamasa Co, Choshi, Japan), washed and detected by a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody (Ab) (Bio-Rad), or an

HRP-conjugated goat anti-human IgE Ab (ϵ -specific, Biosource International, Camarillo, CA, U.S.A.) respectively, with diaminobenzidine (Wako, Osaka, Japan) and H₂O₂.

Recovery of inclusion bodies accumulated in *E. coli* cells and refolding of the recombinant products. The method used has been described previously.¹⁵⁾ Briefly, cells were harvested by centrifugation, suspended with buffer, and disrupted by sonication. The cell lysate was centrifuged and the precipitate of insoluble inclusion bodies recovered, washed with buffer, and solubilized with buffer containing urea. The recombinant products denatured with urea were refolded by dialysis against 20 mM Tris-HCl (pH 8.5).

Flow cytometry. Activity of the recombinant products prepared by the refolding procedure to inhibit the binding of human IgE to the CHO transfectant cells expressing human Fc ϵ RI (CHO/ $\alpha\beta\gamma$) was analyzed as follows. Various volumes of the solution of the inclusion bodies after the refolding procedure were mixed with 0.25 μ g of a fluorescein isothiocyanate (FITC)-conjugated human myeloma IgE and incubated at 37°C for 1 hr (the total volume: 100 μ l). Then, 2×10^5 CHO/ $\alpha\beta\gamma$ cells were added to the mixture. After incubation at 4°C for 1 hr (the total volume: 200 μ l), the cells were washed three times with FACS buffer (PBS, 0.1% bovine serum albumin, and 10 mM sodium azide, pH 7.4) and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.).

Results and Discussion

*Expression of the soluble human Fc ϵ RI α in *E. coli**

The extracellular portion of human Fc ϵ RI α with an additional methionine at the N-terminal residue was expressed in *E. coli* BL21 (DE3) transformed with the expression vector. Insoluble inclusion bodies were accumulated in *E. coli* cells. The cells were harvested by centrifugation. Expression of the recombinant products was confirmed by SDS-PAGE under reducing conditions (Fig. 1. Amido Black staining). Two bands of approximately 22 kDa (a) and 17 kDa (b) indicated with arrows were detected as different from those in the lane of the control cells expressing a control protein, Der f 2,¹⁵⁾ on SDS-PAGE.

Binding of human IgE and five mouse mAbs to the recombinant products

Reactivities of the recombinant products to human IgE and five mouse anti-human Fc ϵ RI α mAbs, CRA1, CRA2, CRA3, CRA4 and CRA5, were analyzed by immunoblotting after SDS-PAGE under reducing conditions (Fig. 1). IgE and three mAbs inhibitory for IgE-binding to Fc ϵ RI expressing cells,

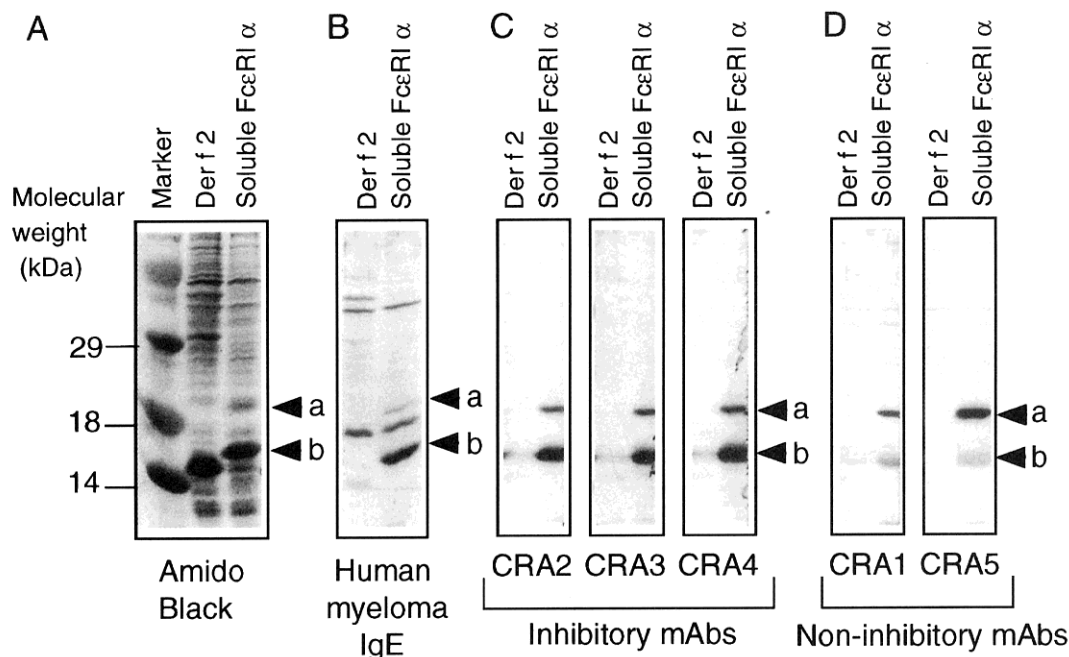


Fig. 1. Expression of the Soluble Human FcεRIα in *E. coli* and Immunoblot Analysis of the Recombinant Products.

Total cellular proteins of *E. coli* BL21 (DE3) transformed with the expression vector of the extracellular portion of FcεRIα with an additional methionine at the N-terminal residue (Met-1-172) were run on SDS-PAGE (18%) under reducing conditions and electroblotted onto nitrocellulose membranes. The membranes were stained with Amido Black (A) or probed with a combination of human myeloma IgE and an HRP-conjugated goat anti-human IgE Ab (B), with that of inhibitory mAbs (CRA2, CRA3, and CRA4) and an HRP-conjugated goat anti-mouse IgG Ab (C), or with that of non-inhibitory mAbs (CRA1 and CRA5) and an HRP-conjugated anti-mouse IgG Ab (D). Total cellular proteins expressed in the same expression system for the recombinant major house dust mite allergen Der f 2¹⁵ were also analyzed as negative controls. The bands indicated with arrows ((a) and (b)) are proteins differentially expressed in the cells transformed with the expression vector of the soluble FcεRIα.

CRA2, CRA3, and CRA4, reacted to both of the bands of (a) and (b) (Fig. 1B and C). Two mAbs non-inhibitory for IgE-binding, CRA1 and CRA5, were more strongly reactive to band (a) than to band (b) (Fig. 1D).

Analysis of N-terminal sequences of the recombinant products

The N-terminal sequencing showed that bands (a) and (b) were equivalent to soluble human FcεRIα with a methionine residue at the N-terminus (Met-1-172) and 23-172 in which the N-terminal 22 residues of the soluble FcεRIα were removed, respectively (Fig. 2A).

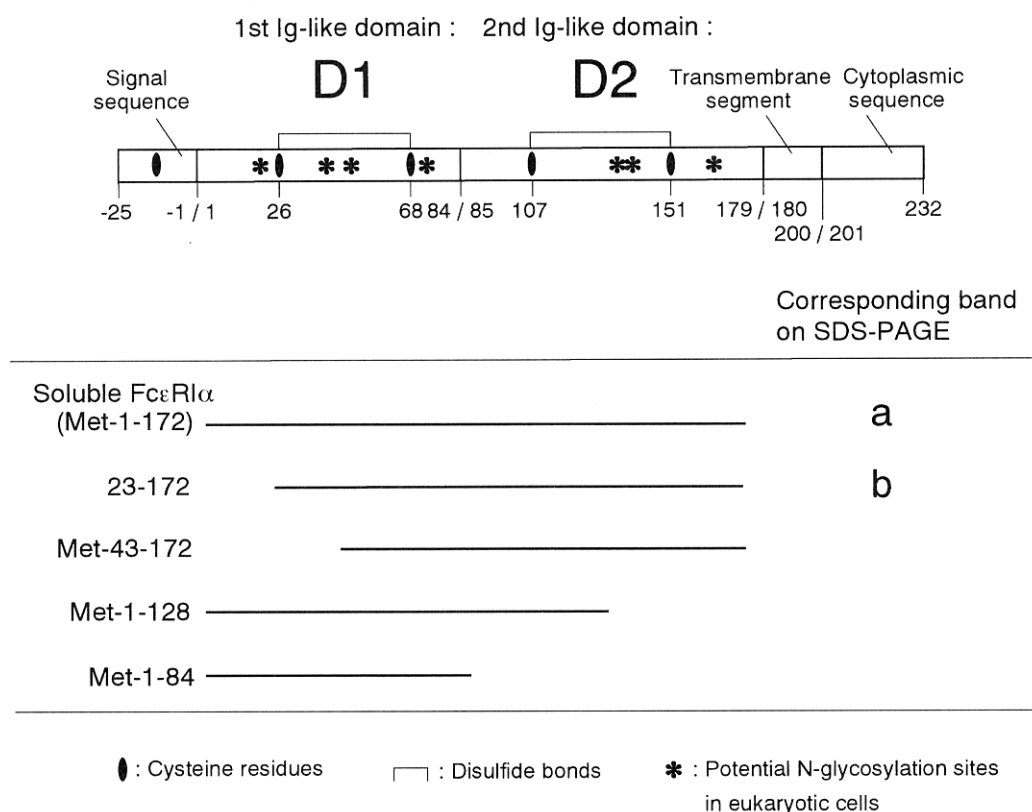
Binding of human IgE and five mouse mAbs to deleted polypeptides of soluble human FcεRIα

Three deletion mutants, Met-43-172, Met-1-128, and Met-1-84, were constructed and analyzed by immunoblotting to compare their apparent molecular masses on SDS-PAGE and their profiles of binding to inhibitory or non-inhibitory mAbs with those of Met-1-172 and 23-172. Apparent molecular masses of Met-1-172 and 23-172 on SDS-PAGE compared well with those of Met-1-128 and Met-1-84 (Fig. 2(B)). Differences between apparent molecular masses and those expected based on the number of amino acid residues, for example, observed in Met-43-172 might

be attributable to differences between the peptides and the molecular masses standards in the conformations or distributions of charge in the buffer for SDS-PAGE under reducing conditions. A considerable proportion of Met-1-172 was converted to 23-172 lacking the N-terminal region (Fig. 1 and Fig. 2). We considered that this was the result of degradation of Met-1-172 in *E. coli* cells. Both Met-1-172 and 23-172 were reactive to human IgE and three mAbs inhibitory for IgE-binding while two non-inhibitory mAbs were more strongly reactive to Met-1-172 than to 23-172 (Fig. 1).

These results support our recent observation that human IgE and the three inhibitory mAbs recognize the second immunoglobulin-like domain (D2, 85-172) of human FcεRIα while the two non-inhibitory mAbs recognize the first immunoglobulin-like domain (D1, 1-84) made on the basis of the experiments using deletion mutants of human FcεRIα expressed as fusion proteins with maltose binding protein in *E. coli*. The domain structure of D2 was essential to the binding of human IgE and inhibitory mAbs.¹⁴ Previously, Robertson definitively located the binding site for IgE to D2 by using a phage display method.⁹ Therefore, degradation of the C-terminal region of Met-1-172 and 23-172 that could disrupt the domain structure of D2 did not seem to occur in the expression form of protein.

A



B

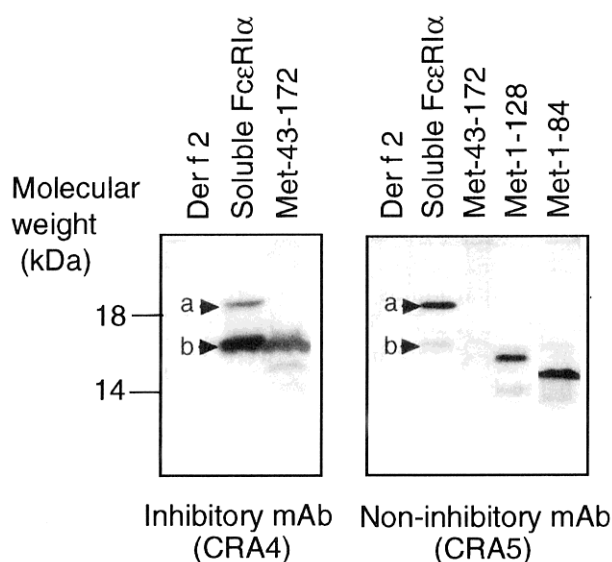


Fig. 2. N-terminal Sequences of the Recombinant Products from the Soluble Human FcεRIα with an Additional Methionine at the N-terminal Residue and Comparison of Their Apparent Molecular Masses with Those of Deletion Mutants of FcεRIα.

(A) Schematic representation of N-terminal sequences of the recombinant products from the soluble FcεRIα with an additional methionine at the N-terminal residue (Soluble FcεRIα (Met-1-172) and 23-172) and the deletion mutants (Met-43-172, Met-1-128, Met-1-84). A schematic view of the whole human FcεRIα protein encoded by the cDNA is also shown.¹⁶⁾ (B) Comparison of apparent molecular mass between the recombinant products from the soluble FcεRIα with an additional methionine at the N-terminal residue and its deletion mutants. Total cellular proteins of *E. coli* BL21 (DE3) transformed with the expression vector of the extracellular portion of FcεRIα with an additional methionine at the N-terminal residue (Soluble FcεRIα, Met-1-172) or its deletion mutants (Met-43-172, Met-1-128, Met-1-84) were run on SDS-PAGE (18%) under reducing conditions and analyzed by immunoblotting. Total cellular proteins expressed in the equivalent expression system for the recombinant major house dust mite allergen Der f 2¹⁵⁾ were also analyzed as negative controls. The bands indicated with arrows (a) and (b) are proteins differentially expressed in the cells transformed with the expression vector of the soluble FcεRIα as shown in Fig. 1.

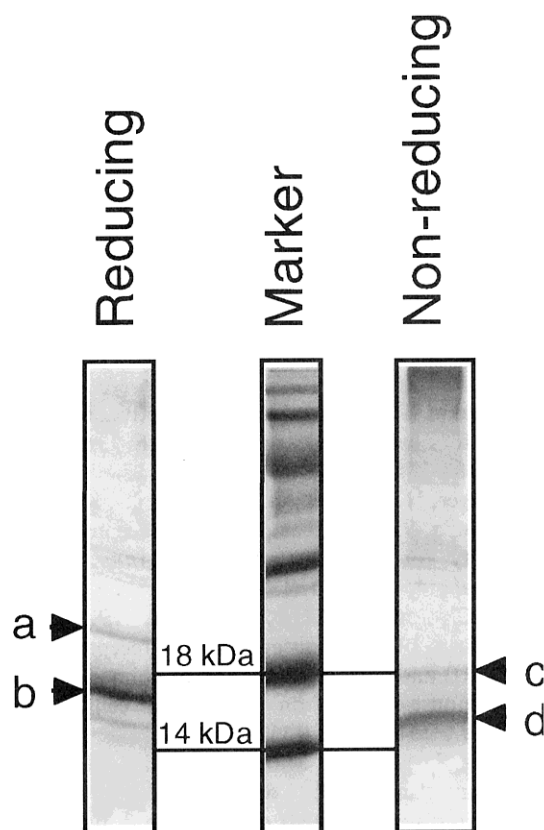


Fig. 3. Recombinant Products in Inclusion Bodies Accumulated in *E. coli* Cells Transformed with the Expression Vector of the Soluble Human FcεRIα.

Inclusion bodies were recovered, solubilized, and dialyzed for refolding. The solution was run on SDS-PAGE (18%) in the presence (Reducing) or absence (Non-reducing) of 2-mercaptoethanol. The gels were stained with Coomassie Brilliant Blue. The bands (a) and (c) correspond to Met-1-172. The bands (b) and (d) correspond to 23-172.

Analysis of the recombinant products included in inclusion bodies

The recombinant products were recovered in the precipitated inclusion bodies but not in the supernatant of the cell lysate (data not shown). The recombinant products after the refolding procedure were analyzed by SDS-PAGE under reducing or non-reducing conditions (Fig. 3). The purity of the recombinant products recovered as inclusion bodies was increased compared with that in the whole cell lysate (Fig. 3 and Fig. 1A). Apparent molecular masses of the recombinant products, Met-1-172 and 23-172, under the non-reducing conditions (Fig. 3, band (c): 18 kDa and (d): 15 kDa, respectively) were lower than those under the reducing conditions (Fig. 3, band (a): 22 kDa and (b): 17 kDa, respectively). The increase in the apparent molecular mass under the reducing condition is considered a result of disruption of intramolecular disulfide bonds in D1 and D2.

Inhibition of IgE binding to CHO transfectant

cells expressing human FcεRI by the recombinant products included in inclusion bodies

Activity of the recombinant products prepared by the refolding procedure to inhibit the binding of human IgE to the CHO transfectant cells expressing human FcεRI (CHO/αβγ) was analyzed by flow cytometry. Incubation of FITC-conjugated human IgE with a small amount of the recombinant products prepared by the refolding procedure from inclusion bodies from *E. coli* cells transformed with expression vectors of the soluble human FcεRIα or a control protein, Der f 2, made increase of the fluorescence intensity of the cells (Fig. 4B and C, +2.5 μl, +5 μl). However, incubation of FITC-conjugated human IgE with a larger amount of the recombinant products prepared by the refolding procedure from inclusion bodies significantly made decrease of the once-increased fluorescence intensity (Fig. 4B, +10 μl, +20 μl). No such inhibitory effect on the IgE-binding to cells was observed using the recombinant products prepared by the refolding procedure from inclusion bodies from cells transformed with the expression vector of a control protein, Der f 2 (Fig. 4C, +10 μl, +20 μl). The materials from inclusion bodies prepared by the refolding procedure including Met-1-172 and 23-172 have significant activity to inhibit the binding of human IgE to the CHO/αβγ cells expressing human FcεRI on their surfaces (Fig. 4B). The transient increase in the fluorescence intensity during titration of the addition of the recombinant products is considered to be an artifact of some other substance from inclusion bodies or *E. coli* cells or the buffer used for solubilization or dialysis (Fig. 4B and C).

Human FcεRIα contains seven potential N-glycosylation motifs. the native FcεRIα and the recombinants expressed in mammalian cells are actually glycosylated.^{6,19,20} Robertson reported that human soluble FcεRIα without the glycochain expressed in *E. coli* as a secreted form maintain the high affinity for IgE equivalent to that of FcεRIα with the glycochain expressed in mammalian cells, and that the binding site for IgE is located in D2.⁹ Moreover, he suggested that structural elements in D1 are necessary to impart the high affinity IgE binding activity to the soluble FcεRIα. Therefore, measurement for IgE binding activity of Met-1-172 with an additional methionine at the N-terminus of D1 and 23-172 with partial deletion of D1 should be addressed using purified proteins for further development of our expression system, although we showed that Met-1-172 and/or 23-173 restore IgE binding activity by dialysis even though once denatured by urea.

In this study, we tried to establish an efficient expression system for soluble FcεRIα with an additional methionine at the N-terminal residue (Met-1-172) as inclusion bodies within *E. coli* cells. The recom-

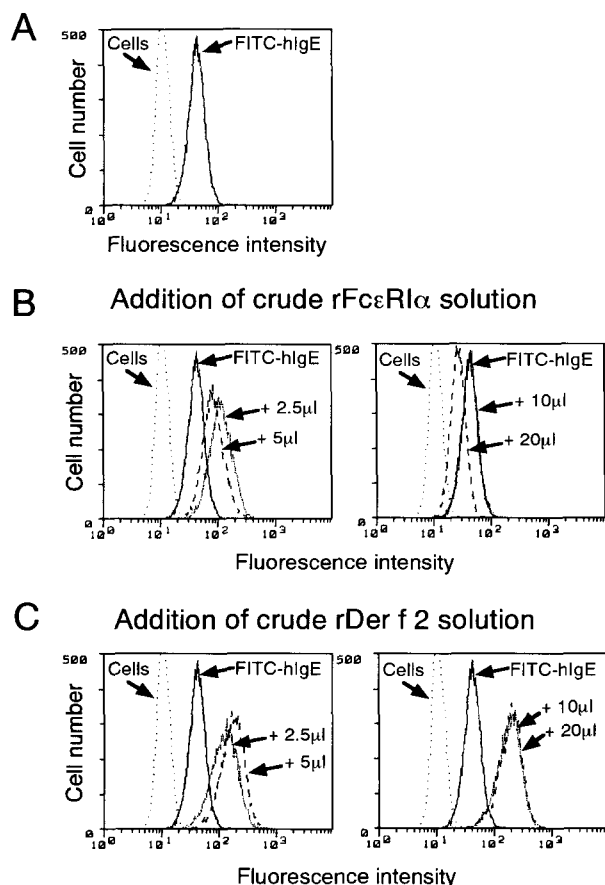


Fig. 4. Inhibition of Human Myeloma IgE Binding to CHO Transfectant Cells Expressing Human Fc ϵ RI by Addition of the Recombinant Products Prepared by the Refolding Procedure from Inclusion Bodies Accumulated in *E. coli* Cells Transformed with the Expression Vector of the Soluble Human Fc ϵ R- α .

(A) Binding of FITC-conjugated human IgE to CHO/ $\alpha\beta\gamma$ cells (FITC-hIgE). The histogram for CHO/ $\alpha\beta\gamma$ cells only is also shown as a negative control (Cells). (B) Effects of incubation of FITC-conjugated human IgE with the recombinant products prepared by the refolding procedure from inclusion bodies from *E. coli* cells transformed with the expression vector of the soluble human Fc ϵ R α (+ 2.5 μ l, + 5 μ l, + 10 μ l, and + 20 μ l). The histograms for CHO/ $\alpha\beta\gamma$ cells incubated with FITC-conjugated human IgE and CHO/ $\alpha\beta\gamma$ cells only are also shown as positive and negative controls, respectively. The indicated volumes are those of the inclusion body-solution (approximately 0.5 mg/ml) added to the solution of FITC-conjugated human IgE. (C) Effects of incubation of FITC-conjugated human IgE with the recombinant products prepared by the refolding procedure from inclusion bodies from *E. coli* cells transformed with the expression vector of the house dust mite major allergen Der f 2¹⁵ (+ 2.5 μ l, + 5 μ l, + 10 μ l, and + 20 μ l).

binant products prepared by the refolding procedure from inclusion bodies have IgE-binding activity, although the conversion of a considerable proportion of Met-1-172 to 23-172 was observed. Expression of recombinant products as insoluble inclusion bodies within *E. coli* cells is generally considered to have a benefit for productivity and simplicity of purification. Total productivity of Met-1-172 and 23-172 is

very high in our system. The use of other types of host *E. coli* cells or introduction of a mutation to the N-terminal region to protect against proteolysis might improve the yield of Met-1-172. The system for the expression of soluble human Fc ϵ R α in *E. coli* presented in this study and its further improvement would be useful for the production of protein as a potent therapeutic and for analysis of IgE-Fc ϵ R α interaction.

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