

Application of Human Fc ϵ RI α -Chain-Transfected RBL-2H3 Cells for Estimation of Active Serum IgE

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We transfected the α -chain of human Fc ϵ RI into rat basophilic leukemia cell line RBL-2H3, established several stable transfected cells, and screened them by β -hexosaminidase release induced by sensitization with human IgE and stimulation with anti-human IgE antibody. A cloned cell line RBL-hE1a-2B12 was the strongest responder among the transfected cell clones. The concentrations of cytosolic free Ca²⁺ concentration in the human IgE-sensitized cells increased after stimulation with anti-human IgE antibody. Thus, it is suggested that the α -chain of human Fc ϵ RI is associated with the β -chain and/or γ -chain of rat Fc ϵ RI, and that they form functional high affinity IgE receptor complexes. The total IgE concentrations of the sera from allergic patients were determined by using the β -hexosaminidase release assay, where the transfected cells were sensitized with diluted and heat-inactivated (at 56 °C for 30 min) serum and stimulated with anti-human IgE antibody. The IgE concentration obtained correlated with those measured by an enzyme immunoassay method. β -Hexosaminidase release induced by stimulation with 5 times diluted serum was sometimes less than the release induced by the same serum; diluted 25 times or 125 times, suggesting that these serum contained factors that blocked IgE binding to Fc ϵ RI or cross-linking by anti-human IgE antibody. The results suggested that our system will be useful for detecting Fc ϵ RI α -bindable IgE in human serum.

Key words Fc ϵ RI α ; basophile; transfectant; human serum; IgE; degranulation

The binding of serum IgE antibodies to high-affinity IgE receptors (Fc ϵ RI) on the surface of mast cells and basophils, and the cross-linking of the receptors by multivalent allergens are the first step in the degranulation where the pharmacologically active substances that cause type I allergy are released. Serum IgE level has been determined by using enzyme-linked immunoassay method, however, it has been reported that some factors are present in serum (especially in hyper-IgE sera), such as anti-IgE and/or anti-Fc ϵ RI antibodies.¹⁾ And many persons are patch-test-negative even though their serum contains a high level of allergen-specific IgE.²⁾ As above, not all IgE antibodies in allergy patients' serum contribute to activation of allergic reactions. Thus, stable human cell lines of mast cells or basophils are essential to investigation of both the activation mechanisms and the evaluation of Fc ϵ RI-bindable serum IgE levels of allergic patients. However, since no useful cell lines are available we must every time prepare cells from freshly collected peripheral blood or develop mast cells from human cord blood CD34⁺ progenitor cells.³⁾

Fc ϵ RI is expressed in tetrameric form ($\alpha\beta\gamma_2$) on mast cells and basophils^{4,5)} and in trimeric form ($\alpha\gamma_2$) on eosinophils⁶⁾ and antigen-presenting cells.^{7,8)} The α -chain binds IgE, and the γ -chain transduces signals. The β -chain is an amplifier of signal strength and enhances the expression of unoccupied receptor at the cell surface.^{9–11)} Earlier studies showed that the transfected human α -chain of Fc ϵ RI associates with endogenously expressed β - and γ -chains in rat RBL-2H3 cells and that the human-rat complexes act as functional IgE receptors.^{12–14)} We therefore attempted to prepare a stable human Fc ϵ RI α -transfected RBL-2H3 cell line which can show human IgE-dependent degranulation, and evaluate Fc ϵ RI-bindable IgE antibody in human serum.

MATERIALS AND METHODS

Serum Specimens Sera of allergic patients and healthy volunteers were collected from 47 individuals from whom their informed consent was obtained and the ethical aspects of our experiments were approved by Institutional Review Board of National Institute of Health Sciences. The symptoms of the patients were as follows, bronchial asthma (39.1%), atopic dermatitis, urticaria, or eczema (32.6%), allergic rhinitis including pollenosis (30.5%), and so on. Total IgE antibodies in their sera determined by EIA method had been already known and some antigen-specific IgE antibodies were detected in their sera by radioallergosorbent test (RAST), they were pollen (67.4%), house dust (56.5%), mite (56.5%), food (17.4%), fungi (10.9%) and so on.

Allergen-specific sera were purchased from Plasma Lab International (Everett, WA, U.S.A.). The sera were stored at –80 °C, and they were heat-treated at 56 °C for 30 min before use.

Reagents Human myeloma IgE purified by dialysis against deionized water and gel filtration was obtained from Chemicon International, Inc. (Temecula, CA, U.S.A.), and affinity-purified anti-human IgE (Fc) antibody was purchased from American Qualex Antibodies (San Clemente, CA, U.S.A.). Murine anti-dinitrophenyl (DNP) monoclonal IgE antibody (IgE-53-569) and dinitrophenylated bovine serum albumin (BSA) (DNP γ /BSA) were prepared as described previously.¹⁵⁾ *Dermatophagoides farinae* (Df) extract was obtained from LSL Co., Ltd. (Tokyo, Japan).

Plasmid Construction pGEM-3-110B-1 plasmid containing human α -chain cDNA was purchased from ATCC (Manassas, VA, U.S.A.). The human α -chain cDNA was amplified using the pGEM-3-110B-1 plasmid as a template by PCR. The used primers were GAATTCGAAGAAGATG-GCTCCTGC and GTCGACTAAATCCTTGAGCACAGAC. The amplified cDNA was cloned into pCR2.1-TOPO vector

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(TOPO TA cloning kit, Invitrogen), and its sequence was confirmed. The pCR2.1-TOPO-human α -chain cDNA was exposed to *EcoRI* and *SalI* and cloned into *EcoRI*–*SalI* site of pCI-neo plasmid (Promega, Madison, WI, U.S.A.).

Cell Culture and Electroporation RBL-2H3 cells¹⁶⁾ were maintained in Dulbecco's modified Eagle Medium (Nissui Co., Tokyo, Japan) containing 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, U.S.A.) and 60 μ g/ml kanamycin. The transfection procedure was performed as described previously.¹⁷⁾ The cells were washed twice with HEPES buffered saline (50 mM HEPES buffer pH 7.4 containing 180 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4 and 6 mM glucose) and suspended in K^+ PBS (10 mM PBS containing 30 mM NaCl, 120 mM KCl, and 10 mM MgCl_2) at 2×10^7 cells/ml. A total of 20 μ g of human α -chain DNA in the pCI-neo vector was incubated with 1×10^7 RBL-2H3 cells in a gene pulse cuvette and transfected at 250 V, 950 μ F with Gene Pulser (Bio-rad, Richmond, CA, U.S.A.). The cells were then incubated in the medium, and the next day the culture medium was replaced with fresh medium containing 500 μ g/ml of G418 (Geneticin, Life Technologies, Rockville, MD, U.S.A.). The cells were cloned by limited dilution and the clones, which released β -hexosaminidase in response to the stimulation with anti-human IgE antibody after sensitization with human myeloma IgE, were selected.

Measurement of Degranulation β -Hexosaminidase is known as a component of the basophil and mast cell specific granule and the release by degranulation was parallel with histamine release in RBL-2H3 cells.¹⁸⁾ Therefore, degranulation of the transfectants was monitored by measuring released β -hexosaminidase activity.¹⁹⁾ The transfectants were sensitized with various concentrations of human myeloma IgE for 3 h or with diluted human serum for 18 h, and the sensitized cells were stimulated with anti-human IgE antibody (10 μ g/ml) in PIPES buffer (140 mM NaCl, 5 mM KCl, 0.6 mM MgCl_2 , 1.0 mM CaCl_2 , 5.5 mM glucose, 0.1% BSA and 10 mM PIPES, pH 7.4) for 30 min. And the medium was then collected, and 0.2% Triton X-100 was added to the cells. β -Hexosaminidase activities released into the medium and within the cells (Triton X-100 extract) were determined by a colorimetric assay using *p*-nitrophenyl-2-deoxy- β -glucopyranoside as a substrate. The β -hexosaminidase activity released into the medium is expressed as a percentage of total activity. For estimation of serum IgE, the RBL-hEla-2B12 cells were cultured in 96-well plates, and the medium was replaced with diluted human serum. After incubation for 18 h at 37 °C, the cells were stimulated with anti-human IgE, and the β -hexosaminidase release was determined.

Measurement of Cytosolic Free Calcium Concentration ($[\text{Ca}^{2+}]_i$) The transfectants (6×10^5 cells/ml) were sensitized with 1 μ g/ml human IgE or diluted human serum and loaded with 6 μ M fura-2 AM (a fluorescent dye reagent for calcium ions, Dojindo, Kumamoto, Japan). After removing the free dye and unbound IgE by centrifugation, the cells were suspended in PIPES buffer (6×10^5 cells/ml) and stimulated with 100 μ g/ml antigen or 10 μ g/ml anti-human IgE antibodies. Fluorescence measurements were made in a 1 cm quartz cuvette with a Shimadzu RF-5000 spectrofluorophotometer (Shimadzu, Kyoto, Japan) as previously reported.¹⁹⁾

Measurement of Total IgE Level by EIA Total IgE levels in human serum were determined by fluorescence enzyme

immunoassay (Uni CAP system, Pharmacia Corporation, Peapack, NJ, U.S.A.) or colorimetric enzyme-linked immunosorbent assay (Mesacup IgE Test, MBL Co., Ltd, Aichi, Japan).

RESULTS AND DISCUSSION

Preparation of the Human Fc ϵ RI α -Expressing RBL Cells The Fc ϵ RI α -transfected RBL-2H3 cells were cloned by the limited dilution method, and the human-Fc ϵ RI α -expressing clones were selected by their β -hexosaminidase release following sensitization with human IgE and stimulation with anti-human IgE. Some of the clones released β -hexosaminidase in a human-IgE-dependent manner, and the results for three highly responsive clones, RBL-hEla-2B12, RBL-hEla-1G4, and RBL-hEla-1C10, are shown in Fig. 1. Empty-vector-transfected RBL-2H3 cells were also cloned, but none of them showed β -hexosaminidase release in a human-IgE-mediated manner. The results for one of them (RBL-pCI-1C5) are also shown in Fig. 1. And Table 1 shows the β -hexosaminidase release by the three α -chain-transfected clones, an empty-vector-transfected clone, and wild-type RBL-2H3 cells in a human IgE/anti-human IgE mediated manner or a mouse anti-DNP-IgE/DNP-BSA mediated

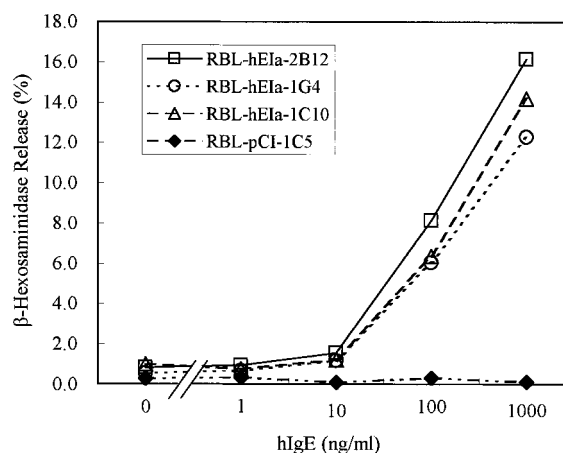


Fig. 1. Human IgE Dependent β -Hexosaminidase Release by Cloned Transfectants

After sensitization with various concentration of human myeloma IgE (0–1000 ng/ml) for 3 h at 37 °C, three clones of human-Fc ϵ RI α -transfected RBL-2H3 cells (RBL-hEla-2B12, RBL-hEla-1G4, and RBL-hEla-1C10) and one clone of empty-vector-transfected cells (RBL-pCI-1C5) were stimulated with anti-human IgE (10 μ g/ml). Values are the mean of the duplicate analysis. Similar results were observed in two other experiments.

Table 1. β -Hexosaminidase release (%) by transfectants and wild-type RBL-2H3 cells

Cell line	Control	Human IgE/ anti-human IgE	Mouse anti-DNP IgE/ DNP-BSA
RBL-hEla-2B12 ^{a)}	0.85	16.16	10.11
RBL-hEla-1G4 ^{a)}	0.56	12.29	20.15
RBL-hEla-1C10 ^{a)}	1.01	14.19	14.31
RBL-pCI-1C5 ^{b)}	0.45	0.31	11.56
Wild-type RBL-2H3	0.77	0.54	21.08

^{a)} Human α -chain cDNA transfected clone. ^{b)} Empty-vector transfected clone. After sensitization with 1.0 μ g/ml human myeloma IgE or mouse monoclonal anti-DNP IgE for 3 h at 37 °C, cells were stimulated with 10 μ g/ml anti-human IgE or DNP-BSA. Values are averages of duplicate analyses.

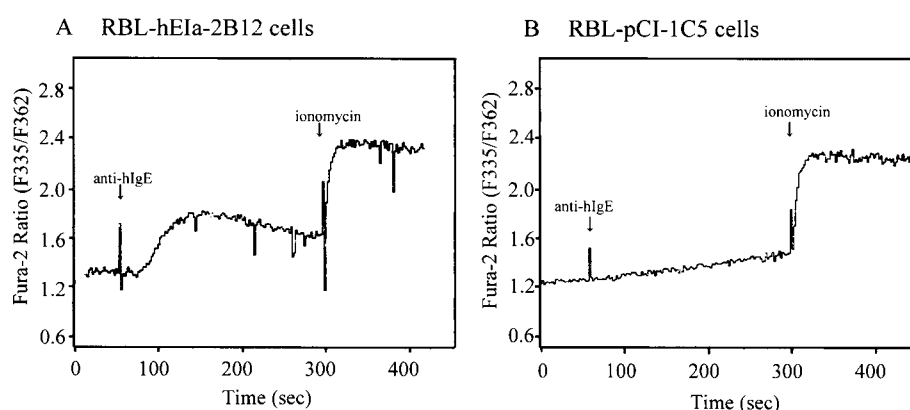


Fig. 2. Human IgE Dependent $[Ca^{2+}]_i$ Changes in the Transfectants

RBL-hEla-2B12 cells (A) and RBL-pCI-1C5 cells (B) were sensitized with human myeloma IgE ($1 \mu\text{g/ml}$) for 3 h at 37°C and suspended in PIPES buffer. Anti-human IgE ($10 \mu\text{g/ml}$) was added to the suspension followed by $0.5 \mu\text{M}$ ionomycin at the times indicated. Similar results were observed in two other experiments.

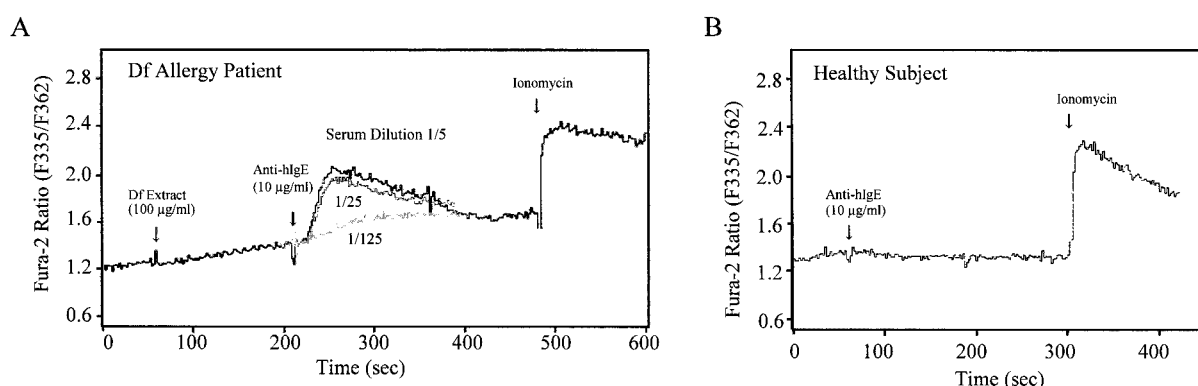


Fig. 3. $[Ca^{2+}]_i$ Changes in RBL-hEla-2B12 Cells Sensitized with Human Serum

RBL-hEla-2B12 cells were sensitized with serum from a Df allergy patient (5, 25, or 125 times diluted, A), or a healthy volunteer (50 times diluted, B) for 18 h at 37°C and suspended in PIPES buffer. Df extract ($100 \mu\text{g/ml}$, A), anti-human IgE ($10 \mu\text{g/ml}$) and ionomycin ($0.5 \mu\text{M}$) were added to the suspension at the times indicated.

manner. Following sensitization with mouse IgE, both transfectants released β -hexosaminidase in response to the specific antigen, similar to the wild-type RBL-2H3 cells (Table 1). Mouse anti-DNP-IgE antibodies bind rat α -chain of Fc ϵ RI, however they do not bind human α -chain, so both transfectants seem to be expressing intrinsic rat Fc ϵ RI.

Human IgE-Induced Ca^{2+} Response in the Transfectants Then, intracellular $[Ca^{2+}]_i$ in the human-IgE-sensitized transfectants was determined after stimulation with anti-human IgE. The increase in $[Ca^{2+}]_i$ in the transfectants (6×10^5 cells) loaded with fura-2 AM was monitored with a spectrophotometer, and the results showed that anti-human IgE antibody increase $[Ca^{2+}]_i$ in RBL-hEla-2B12 cells (Fig. 2A). The empty-vector transfectant RBL-pCI-1C5 did not show Ca^{2+} signaling (Fig. 2B).

Sensitization with mouse monoclonal anti-DNP IgE antibody and stimulation with the specific antigen DNP-BSA increased $[Ca^{2+}]_i$ in both the RBL-hEla-2B12 cells and RBL-pCI-1C5 cells (data not shown). Thus, endogenous rat Fc ϵ RI is not lost by transfection.

Ca^{2+} Response to Using IgE in Allergic Patient Sera

Instead of human myeloma IgE, we examined for IgE in human sera from allergic patients and healthy volunteers. A representative data was shown in Fig. 3, where the patient was allergic to mites (*Dermatophagoides farinae*, Df). The serum total IgE level of the patient measured by EIA was

6960 ng/ml, while that of normal serum was 624 ng/ml. The stimulation with anti-human IgE antibody induced Ca^{2+} response in RBL-hEla-2B12 cells sensitized with diluted patient serum for 18 h at 37°C (Fig. 3A). However, a longer sensitization period (18 h) is required for the human serum IgE than the myeloma IgE (3 h). The Ca^{2+} response for the normal serum was very weak (Fig. 3B).

In contrast to stimulation with anti-human IgE antibody, stimulation with the specific allergen Df extract, did not induce Ca^{2+} signaling in the sensitized cells (Fig. 3A).

The fact that both the Ca^{2+} response and β -hexosaminidase release were induced by sensitization with human myeloma and allergic patient IgE and stimulation with anti-human IgE suggests that the transfected human α -chains form a functional human IgE receptors on RBL-hEla-2B12 cells. However, specific-allergen-induced response was not detectable. It is very likely that the cell surface density of human α -chain is still low to be cross-linked by specific allergens, and the ratio of specific IgE antibodies to the total IgE antibodies in a patient's serum seems to be very small and most of the expressed IgE receptors must be occupied with non-specific IgE antibodies, even if the patient shows allergic symptom to the allergen that is different with the case of the mouse monoclonal anti-DNP IgE.

Relationship between β -Hexosaminidase Release and Total IgE Concentration Measured by EIA Since β -hex-

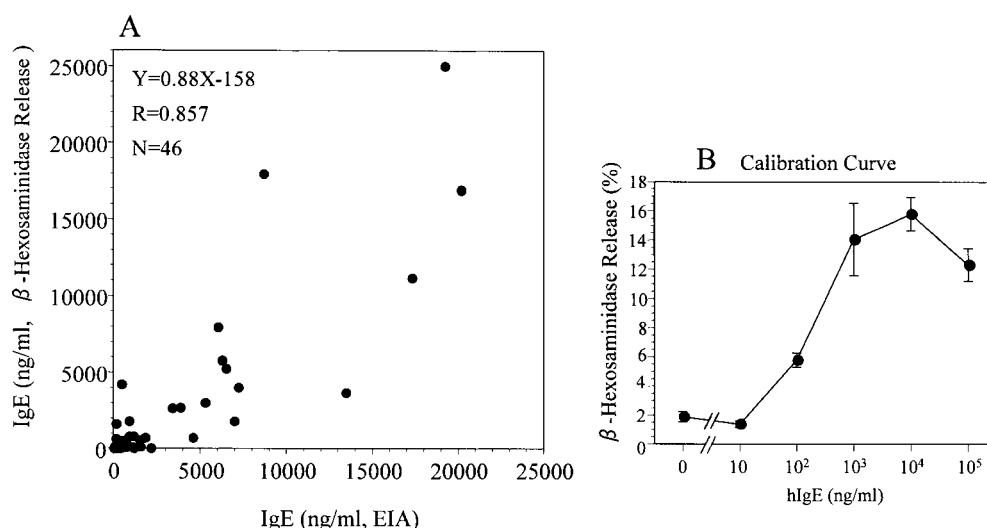


Fig. 4. Comparison of Serum IgE Levels Determined by Degranulation of RBL-hEla-2B12 Cells and by the EIA Method

RBL-hEla-2B12 cells were cultured in 96-well plates and sensitized with diluted human serum from 46 allergy patients for 18 h at 37°C. The cells were then stimulated with 10 μ g/ml anti-human IgE, and the β -hexosaminidase release was determined. Each IgE value was calculated based on the calibration curve of β -hexosaminidase release induced by human myeloma IgE sensitization followed by 10 μ g/ml anti-human IgE stimulation (B), and compared with the values determined by the EIA method (A). Each value in the calibration curve represents the mean $\pm$ S.D. of triplicate analysis and similar results were observed in two other experiments.

osaminidase release in the IgE/anti-IgE system is dependent on the IgE concentration as shown in Fig. 1, we calculated the IgE concentrations of patient sera using RBL-hEla-2B12 cells and myeloma IgE as a standard.

When diluted patient sera (25-times or 125-times dilutions) was used, the release of β -hexosaminidase correlated with the IgE levels measured by EIA method. However, for 5-times diluted sera of some patients, enzyme release did not correlate with the IgE level determined by the EIA method. Even if IgE level of serum by EIA was less than 1000 ng/ml (linear part of calibration curve), β -hexosaminidase release was lower than the expected level. For this reason, we measured that β -hexosaminidase release from cells sensitized with serum diluted 5 times, 25 times, and 125 times, and the value at the highest release was used for calculation using calibration curve in Fig. 4B. As shown in Fig. 4A, β -hexosaminidase release correlated with the IgE concentrations determined by the EIA method ($R=0.857$).

As described above, a part of IgE antibodies in allergy patients' serum contribute to activation of allergic reactions and some of human sera were growth-inhibitory during the sensitization period at high concentrations.

In conclusion, our stable transfectant system made it possible to detect the level of the IgE in patient sera that binds receptors and causes degranulation under the conditions similar to physiological.

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