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Expression of the Alpha Chain of Human FcεRI in Transfected Rat Basophilic Leukemia Cells: Functional Activation after Sensitization with Human Mite-Specific IgE

Key Words

Human FcεI receptor
 RBL-2H3 transfectants
 Mast cell activation
 IgE triggering

Abstract

The actual dilemma in studying the binding and triggering capacity of IgE from allergic patients is the lack of cultured basophils or mast cell analogs of human origin. Human IgE binds with exquisite species specificity to the high affinity IgE receptor (FcεRI) expressed on the surface of these cells. In rodents this receptor has been characterized as a tetrameric plasma membrane protein composed of an IgE-binding α chain, a β chain and two disulfide-linked γ chains. In order to establish a cell line expressing the α chain of human FcεRI which can be triggered with IgE from human patients and specific allergen, we transfected the cDNA coding for the human α subunit into rat basophilic leukemia cells. The resulting transfectants express the human α chain on the cell surface in the form of a hybrid complex associated with endogenous rat γ chains. After sensitization with human IgE from mite-specific patients, the transfectant produces a calcium response upon incubation with allergen. The established cell line can be used as a model system to study the mechanism of mast cell triggering through IgE from allergic patients.

Introduction

Degranulation of blood basophils and tissue mast cells in an allergic reaction is triggered upon aggregation of allergen-specific IgE bound with high affinity to the type-I Fcε receptor (FcεRI) [1] on their cell surface. Most of the information on the structure of the receptor and the biochemical events taking place upon aggregation have been obtained using animal model systems, such as rat baso-

philic leukemia (RBL) cells, a cultured analog of rat mucosal mast cells [2]. The receptor is a tetramer (αβγ₂) containing an α chain [3], a β chain [4] and two disulfide-linked γ chains [5]. The ectodomain of the α chain is responsible for ligand binding [6, 7]. Recent studies have shown that *src* protein-related tyrosine kinases associate with the receptor [8] and that the β and γ chains are structurally modified by phosphorylation at tyrosine residues only a few seconds after receptor aggregation [9], suggest-

ing an important role of these chains in signal transduction mechanisms.

Studies of receptor binding and activation in the human system have been hampered by the lack of an established cell line of basophilic lineage and species specificity of IgE for the human FcεRI. Purification of human basophils and tissue mast cells requires relatively laborious procedures and human blood or tissue as a source [10, 11]. In addition, the depletion of cell-bound IgE by a brief incubation at acidic pH, preliminary to any sensitization, may subsequently alter cell viability and response [12].

The availability of the cDNA coding for the α chain of human FcεRI raised the possibility of generating human IgE-binding specificity by its transfection into adequate cell lines. Thus, human receptors can be expressed in Cos 7 cells as a hybrid α human γ rat/mouse receptor or αγ human receptor [13, 14]. In contrast to rodent receptors the human α chain can be expressed on the cell surface without requirement of the β subunit. However, when the β chain is present, a proportion of the receptors forms complexes with β and γ chains [13]. Transfection of human receptors into mast cell-derived P815 cells has shown that β-less receptor complexes are capable of triggering early biochemical events upon receptor aggregation [15].

Our goal in the present study was to reconstitute a receptor for human IgE into RBL cells which could be activated when sensitized with IgE from allergic patients followed by aggregation with allergen. We show that, upon transfection of the cDNA coding for the human α chain of FcεRI, this chain is expressed on the cell surface of RBL cells in the form of a hybrid receptor complex associated with endogenous rat γ chains. The hybrid receptors exhibit a calcium response when stimulated with IgE from mite-sensitive patients and purified mite allergen *Der f I* [16].

Material and Methods

RBL Cells and Transfections

The RBL-2H3 subline was maintained at 37 °C in Eagle's minimal essential medium (MEM) containing 16% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin. For transfection the RBL-2H3 line was trypsinized, washed twice in ice-cold phosphate-buffered saline (PBS) and resuspended at 1×10^7 cells/ml in PBS. A total of 30 μg of human αDNA in the pCMV-4 neo vector was incubated with 8×10^6 RBL cells in gene pulse cuvettes (Biorad) and transfected at 250 V, 960 μF using a Biorad electroporation apparatus. The cells were then incubated in normal medium. Twenty-four hours after transfection, the cells were placed in selective medium containing 1–2 mg/ml G-418 (Geneticin, Gibco) and 20 mM HEPES (pH 7.3) and plated at a density of $1\text{--}2 \times 10^4$ cells/well in flat-bottom 96-well plates.

Twenty-four independent transfectants were tested for human FcεRI expression.

Immunoglobulins and Reagents

Anti-dinitrophenyl (DNP) monoclonal mouse IgE was from the hybridoma Hi-DNPε 26.82 [17]. Rat IgE of unknown specificity was from the immunocytoma IR 162 [18].

Human IgE of unknown specificity was from the serum of an IgE myeloma patient (PS). Human IgE with specificity for Dermatophagoides allergen was obtained from the serum of mite-sensitive patients. Serum IgE was purified by passing it directly over an anti-human IgE affinity column. Elution was performed in 0.2 N acetic acid containing 1% FCS and immediate neutralization with 2 M Tris (pH 7). After dialysis against PBS and concentration, the purified IgE was aliquoted and stored at –20 °C before use.

Anti-human IgE was obtained by immunizing rabbits with human IgE according to standard methods [19]. The anti-human IgE was further purified by precipitation in 50% ammonium sulfate and affinity chromatography using human IgE Sepharose.

When necessary, the immunoglobulins were radioiodinated using the chloramine T method [20]. For FACS analysis, the antibodies were FITC-labeled according to standard techniques [21].

DNP₄₃-BSA was a gift of A. Prouvost-Danon.

The purified allergen from *Dermatophagoides farinae*, *Der f I*, was obtained as described earlier [16].

Cytofluorometric Analysis

About 5×10^5 cells were resuspended in 100 μl of ice-cold PBS containing 1% FCS and preincubated for 30 min with or without 50 μg/ml unlabeled IgE. Then 2 μg/ml of FITC-labeled IgE were added and incubation was continued for 1 h. After two washes in PBS/FCS, the cells were resuspended in PBS/FCS and 10^4 viable cells were analyzed using a FACScan flow cytometer (Beckton Dickinson).

Plasmid Constructions and Northern Blot Analysis

Basic cloning procedures were performed as described [22]. The expression plasmid pCMV-4 neo was constructed by introduction into the unique Hpa I site of pCMV-4 [23], a neomycin resistance gene cassette obtained from the pFRSVneo vector [24].

The human α full-length [25] cDNA was excised with *Eco R I*, filled in with Klenow and cloned into the blunt-ended *Cla I* site of pCMV-4 neo downstream of the cytomegalovirus promoter.

Northern blot analysis was basically carried out as described [22]. Briefly, 2×25 μg of RBL transfectant 8 (T8) total RNA were size fractionated in parallel by electrophoresis in 1% formaldehyde/1% agarose gels. Following transfer to Hybond nylon membranes (Amersham) one of the lanes was hybridized with 2×10^7 cpm of a rat α probe (specific activity 6.9×10^{12} cpm/nmol) and the second lane with 2×10^7 cpm of a human α probe (specific activity 6.1×10^{12} cpm/nmol). Final washes were performed in $0.5 \times$ SSC at 65 °C before autoradiography.

Measurement of the FcεRI Number

Suspensions of RBL cells and transfectants (T8 and T22) at 3×10^6 cells/ml were incubated in duplicate with or without 50 μg/ml of unlabeled human or rat myeloma IgE for 30 min with constant shaking at room temperature. Human ¹²⁵I-IgE (specific activity 6,031 cpm/ng) or rat ¹²⁵I-myeloma IgE (3,483 cpm/ng) at a final concentration of 5 μg/ml was added and incubation was continued for 1 h; 200-μl aliquots of cell suspensions were then deposited onto a 200-μl cushion made

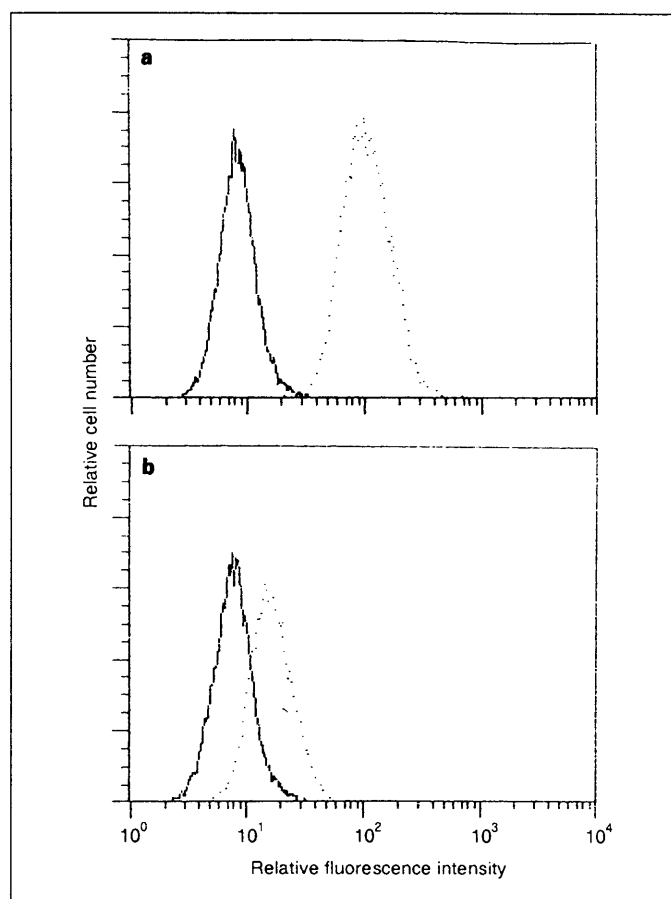


Fig. 1. Surface expression of rat (a) and human (b) α chains of Fc γ RI on transfected RBL cells T8. Cells were analyzed by direct immunofluorescence using FITC-conjugated rat or human IgE. For measurement of rat receptors, human receptors were blocked using unlabeled human IgE. In negative controls the cells have been preincubated with an excess of the corresponding unlabeled IgE.

of a mixture of silicone oil (85%) and vaseline oil (15%) in polyethylene Eppendorf Beckman type ETT₂₃ tubes.

Cell-bound counts were determined by pelleting the cells through rapid centrifugation (2 min) in a microfuge. The tubes were put in dry ice and the pelleted cells were cut using a razor blade. Cell-bound counts were determined in a γ counter and specific binding was determined by subtracting counts from samples which had been preincubated with cold IgE. The receptor number was calculated from the specific activity. Since rat IgE binds both to rat and human receptors, the number of rat-specific receptors was obtained by subtracting the number of human receptors.

Coimmunoprecipitation of IgE-Fc ϵ RI Receptor Complexes

Transfected RBL T8 cells were loaded with rat ¹²⁵I-IgE (3 μ g/ml) or human ¹²⁵I-IgE (300 ng/ml) for 45 min at 4 °C, washed once in PBS and solubilized in 10 mM CHAPS/borate buffer saline (BBS) on ice (10 min) at 5×10^7 cells/ml. The extracts were centrifuged in an Eppendorf microfuge and equivalents of 1.8×10^7 cells (human IgE-Fc ϵ RI complexes) or 1.7×10^6 cells (rat IgE-Fc ϵ RI complexes) were

then incubated for 30 min with varying concentrations of anti- γ antibody prebound to protein A-Sepharose beads. The beads were washed once in 2 mM CHAPS/BBS and the fraction of counts bound to beads was determined.

Measurement of Intracellular Calcium Level

Changes in the intracellular calcium levels in transfected RBL cells were monitored using flow cytometry and the calcium-binding dye indo-1 [26]. Transfected cells, previously cultured for 3 days without G-418, in MEM supplemented with 16% FCS (medium A) were trypsinized. Cells were washed with medium A and suspended in MEM containing 5% FCS and 25 mM HEPES (pH 7.3; medium B).

Samples of 2.5×10^6 transfected cells in 1 ml medium B containing human IgE and 4 μ M of indo-1 acetoxymethyl ester were incubated for 40 min at 37 °C. Transfected cells were sensitized either by 4 μ g/ml of purified human myeloma IgE or by 1.4 μ g/ml of purified IgE from a pool of sera from mite-sensitive patients.

Fluorescence measurements were obtained using a Facstar Plus dual laser system (Becton-Dickinson), equipped with a COHERENT 90-S Argon-ion laser tuned to 150 mW at 320 nm. Two fluorescence emission peaks at 405 (violet) and 485 (blue) nm were monitored using 405/20 and 485/22 band pass filters in front of the respective photomultipliers. Data were collected at the rate of approximately 4,000 cells per time point after stimulation, at 30-second intervals. All data were collected in the linear mode to allow calculation of mean violet/blue fluorescence ratios (V/B ratio) reflective of the intracellular calcium concentration. Data were analyzed using standard chronys software.

Calcium measurements were also made using Quin 2 as calcium-binding dye and a Perkin-Elmer LS-5 spectrofluorometer as previously described [15].

Results

Stable Transfection of the Human Fc ϵ RI α Chain in RBL Cells

The cDNA coding for the human Fc ϵ RI α chain was introduced into the expression vector pCMV-4 neo and transfected into the RBL-2H3 cell line. After selection in G-418-containing medium and subsequent FACS analysis using human FITC-IgE, 14 of 24 clones expressed the α chain of the human IgE receptor. Figure 1 shows the analysis of the expression of rat and human receptors on a representative transfectant T8 generally used in this study. This clone was selected by four rounds of cell sorting to obtain higher expression of human Fc ϵ RI, although without success. As seen in figure 1, expression of the transfected α chain was considerably lower than those of the endogenous rat receptors. By direct binding of iodinated human and rat IgE, we determined the number of human and rat receptors expressed by two of the transfectants. The results are shown in table 1. The number of endogenous rat Fc ϵ RI observed is similar to the one previously described [27], for Fc ϵ RI with specificity for human IgE the number of receptors was only about 4×10^3 /cell.

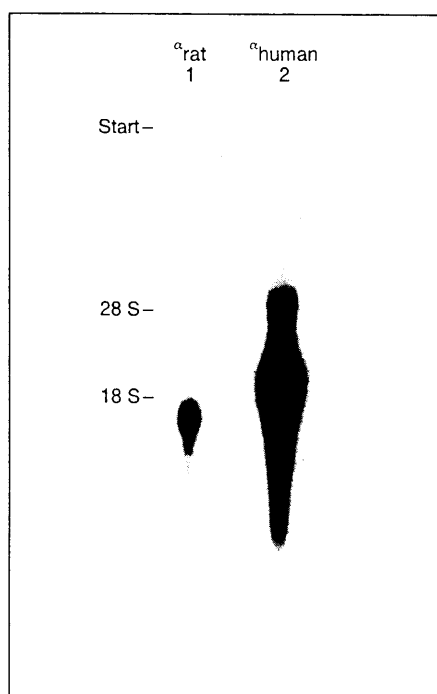


Fig. 2. Northern blot analysis of rat and human α mRNA in RBL T8 transfectants. Twenty-five micrograms of total RNA were electrophoresed and blotted. Blots were hybridized with rat and human α -specific probes of about the same specific activity (see Materials and Methods).

Table 1. Measurement of the Fc ϵ RI number

	Number of human Fc ϵ RI per cell	Number of rat Fc ϵ RI per cell
RBL	ND	186×10^3
T8	4.1×10^3	88×10^3
T22	3.7×10^3	160×10^3

The low surface expression of human receptors is not due to inefficient transcription of transfected α DNA, since Northern blot analysis of RNA levels revealed a much higher expression of human α mRNA than endogenous rat α mRNA (fig. 2).

Analysis of Human Receptor Complexes

We wished to verify whether the transfected α chain is expressed as a hybrid receptor complex associated with endogenous rat subunits. However, low expression of human receptors prevented us from using the classical biochemical approach of immunoprecipitating biosyntheti-

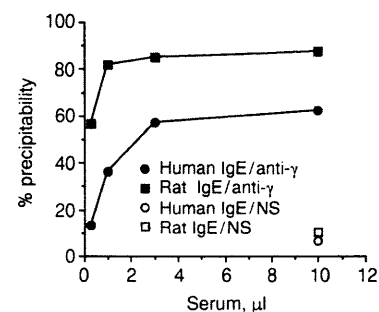


Fig. 3. Coimmunoprecipitation of rat or human IgE-receptor complexes expressed on RBL T8 transfectants using an anti- γ antibody. The fraction of precipitated counts obtained from duplicate samples was plotted as a function of anti- γ concentration. Control precipitation was performed using 10 μ l of normal rabbit serum (NS).

cally labeled receptor complexes. We therefore chose to immunoprecipitate transfected human receptor complexes to which human ^{125} I-IgE had been prebound with an anti- γ antibody.

As can be seen in figure 3, in a control experiment more than 80% of endogenous rat IgE-Fc ϵ RI complexes can be precipitated using 1 μ l of rabbit serum containing the anti- γ antibody, demonstrating the association of rat α and γ subunits. Precipitation of human IgE receptor complexes is slightly less at about 60% at 10 μ l of rabbit anti-serum. These results clearly show that the human α chain is associated with the endogenous rat γ chains. Background precipitation using normal rabbit antiserum is at about 7%.

Calcium Response in Transfected Cells

To examine whether the cell can be activated through these receptors, the increase in internal calcium concentration, one of the most extensively characterized early responses of RBL cells, has been chosen.

Changes in intracellular calcium levels in transfected RBL cells were monitored using flow cytometry and the calcium binding dye indo-1. The mean V/B ratio (reflective of the intracellular calcium concentration) was measured.

Rabbit anti-human IgE stimulation of indo-1-loaded transfected cells, sensitized with human myeloma IgE, elicited an increase in the mean V/B ratio to reach a maximum at 100 s (fig. 4a). The addition of an equivalent dose of rabbit anti-human IgE to nonsensitized transfected cells did not induce any change (fig. 4b). We have also verified that the addition of anti-human IgE to nontrans-

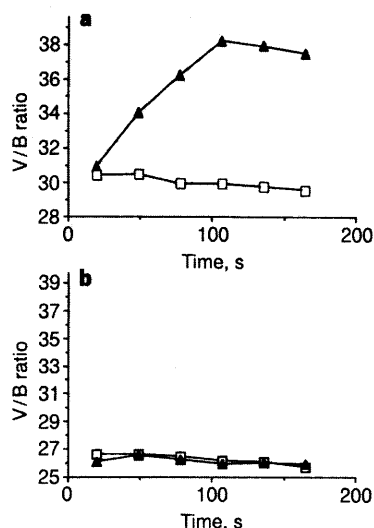


Fig. 4. Variation of $(Ca^{2+})_i$ upon triggering of RBL T8 transfectants. Mean indo-1 violet (405 nm) to blue (485 nm) fluorescence ratios (V/B fluorescence ratio) were determined as a function of time using flow cytometry after addition of anti-human IgE. Transfected RBL cells T8 sensitized with human myeloma IgE (**a**) or not-sensitized (**b**) were challenged with rabbit anti-human IgE at time zero. $\square$ = Unchallenged; $\blacktriangle$ = challenged with anti-human IgE at 25 μ g/ml.

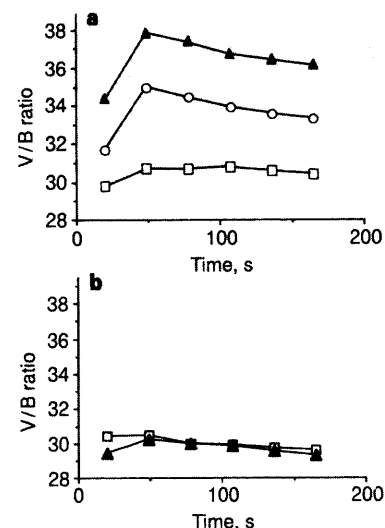


Fig. 5. Variation of $(Ca^{2+})_i$ upon triggering of RBL T8 transfectants. Mean indo-1 violet to blue fluorescence ratios (V/B ratio) were determined as a function of time by flow cytometry after addition of mite allergen (*Der f* I). Transfected RBL cells sensitized with human mite-specific IgE (**a**) or with human myeloma IgE (**b**) were challenged with mite allergen at time zero. $\square$ = Unchallenged; $\circ$ = challenged with *Der f* I at 20 ng/ml; $\blacktriangle$ = challenged with *Der f* I at 100 ng/ml.

fected cells previously treated with human IgE did not induce any response (data not shown).

In figure 5a the response of transfected cells sensitized with human IgE, purified from a pool of sera from mite-sensitive patients, is shown. Triggering with 20 ng/ml of purified mite antigen induced a significant increase in the ratio level reaching the maximal level at 50 s. The addition of 100 ng/ml of mite antigen (*Der f* I) elicited a higher response similar to the anti-IgE-induced response shown in figure 4a. The specificity of the mite antigen-induced response was demonstrated, since the addition of the mite antigen on cells sensitized with human myeloma IgE did not induce any change in the calcium level (fig. 5b). The calcium response measured by flow cytometry cannot easily be calibrated for the actual calcium concentration due to the inability to obtain an accurate flow cytometric determination with calcium-free dye (V/B ratio in the absence of calcium) [28]. We therefore measured the calcium response of transfected RBL cells after stimulation with anti-human IgE using spectrofluometry and the calcium-dye Quin 2. We obtained a response of 546 ± 351 nm ($n = 2$) which is of about the same order of magnitude for

published values using stimulation through DNP-BSA of endogenous receptors sensitized with mouse anti-DNP IgE [17] and our own estimate with the Quin 2 system (410 nm).

Discussion

In this study we obtained transfected RBL cell lines expressing the α chain of the human Fc ϵ RI. This chain is expressed on the cell surface in the form of a hybrid receptor associated with endogenous rat γ subunits. Work is in progress to evaluate whether the α subunit is also associated with endogenous rat β subunits. The number of human receptors present at the cell surface was, however, considerably lower than that of endogenous rat receptors and could not be increased even by repeated rounds of cell sorting. Comparison of RNA levels revealed expression of human α mRNA exceeding those of endogenous rat α mRNA. Assuming these mRNAs are translated efficiently, a possible explanation for the low number of human receptors expressed might be that human α chains

are degraded more rapidly than endogenous α chains. Endogenous α chains might also assemble with higher efficiency to species-identical β and γ subunits than the heterologous human α chain.

The transfected RBL cells could be triggered to produce an increase in intracellular calcium with anti-human IgE upon sensitization with human myeloma IgE. This allowed us to examine whether the established transfectant could be used as a test system of the sensitization by IgE derived from allergic patients. When IgE derived from the serum of mite-sensitive allergic patients was incubated with RBL T8 cells, they could be triggered with *Derf*I antigen in a dose-dependent manner. So far, we had to purify the IgE from human serum by affinity chromatography. In calcium assays we observed substantial toxicity of human serum on RBL cells at the high concentrations necessary to get sufficient IgE binding. We are currently exploring culture conditions in which these cells could be sensitized with diluted human serum, obviating the need to purify IgE.

In spite of the low surface expression of the hybrid receptors, the actual increase in intracellular calcium was in the same order as when triggering was performed through endogenous rat receptors, thus confirming previous data showing that only a small number of aggregated receptors is necessary to produce a maximal response [29]. The calcium response of transfected cells shows kinetic differences when sensitized cells are triggered either by antigen or by anti-IgE. The response of transfectant cells sensi-

tized by human IgE from mite-sensitive patients and triggered by the cognate antigen begins earlier and is more rapid than the response of human IgE-sensitized cells triggered by anti-human IgE. These different kinetics in calcium response may reflect a more effective cross-linking of surface antibody IgE by a multivalent ligand (mite allergen) than by the functionally bivalent anti-human IgE. These results may be compared to the difference in the rate of release of histamine induced either by antigen or anti-IgE in rat mast cells [30] or in human basophils [31]. Indeed, the release of histamine by antigen occurs with a shorter lag period and a more rapid rate than seen with anti-IgE.

The experimental results show that RBL cells upon transfection of the human α chain provide a useful system to investigate binding of human IgE and activation through IgE from patient serum. In future the introduction of mutant receptors into the RBL cells could increase the present knowledge on the IgE binding-site and thus contribute to the screening and the development of substances inhibiting the human IgE-receptor interaction.

Acknowledgements

The authors thank Dr. J.P. Kinet who kindly provided us with the anti- γ antibody; Dr. Tom Huff for some of the human IgE used in this study; Dr. C. Rougeot for expert iodination of human and rat IgE, and J. Weyer for secretarial assistance. We would like to thank Dr. A. Prouvost for the generous gift of DNP₄₃-BSA.

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