ORIGINAL ARTICLE

High Affinity IgE-Fc Receptor α and γ Subunit Interactions

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ABSTRACT

Objective: To explore the relationships between the subunits (α, β and γ) of the high affinity IgE receptor (FcεRI) and its ability to mediate transmembrane signaling.

Study Design: Experimental study.

Place and Duration of Study: Department of Molecular Biology and Biotechnology, University of Sheffield, UK, from 2008 to 2009.

Methodology: The approach employed was to create a chimera (human αγγ) using the extracellular (EC) domain of the human high affinity IgE receptor. The alpha subunit (huFcεRIα) of IgE receptor was spliced onto the rodent gamma TM and cytoplasmic domain (CD). This was transfected into the Rat Basophilic Leukemia cell line in order to assess the possibility of selectively activating cells transfected with this single pass construct for antigen induced mediator release.

Results: The RBLs cell lines transfected with the huFcεRIα/γ/γ cDNA constructs were assessed for the cell surface expression of the huFcεRIα subunit and the response to the antigenic stimulus by looking for degranulation and intracellular Ca^{2+} mobilisation. The results obtained showed the absence of huFcεRIα subunit expression on the surface of transfected cells as seen by flowcytometric studies, β-hexosaminidase assays and intracellular calcium mobilisation studies.

Conclusion: In the present study the grounds for non-expression of huFcεRIα/γ/γ cDNA remains elusive but may be due to the fact that the human-rodent chimeric receptors are assembled differently than the endogenous rodent receptors as seen in study in which COS 7 cells were transfected with human/rat chimeric complexes.

Key Words: The high affinity IgE receptor (FceRI). Rat basophilic leukemia cells (RBLs). *Immunoglobulin E (IgE).*

INTRODUCTION

The High Affinity IgE Receptor, FcεRI has the highest affinity of all immunoglobulin receptors with a binding constant in the 10^{-9} to 10^{-10} M range for its ligand, IgE.[1,2] FcεRI exists as a tetramer on the surface of human mast cells and basophils consisting of a α-chain, a β-chain and a disulphide-linked dimer of γ-chains, while on Langerhans cells, dendritic cells and monocytes the FcεRI is present as a trimer.[3] The subunits (α, β and γ) of FcεRI are composed of three domains: extracellular (EC), transmembrane (TM) and cytoplasmic (CT) domains.[4,5] Our group is exploring the relationships between the structure of the FcεRI and its ability to mediate transmembrane signaling.[6,7] The approach employed was to create a chimera (humanαγγ) using the extracellular (EC) domain of the human high affinity IgE receptor alpha subunit (huFcεRIα) spliced onto the rodent gamma TM and cytoplasmic domain (CD) and transfect them into the rat basophilic leukemia cell line in order to assess the possibility of selectively activating cells transfected with this single pass construct for antigen induced mediator release. This strategy might facilitate assessment of the surface expression of the huFcεRIα ligand binding domain independent of the endogenous rodent huFcεRIα.

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METHODOLOGY

The study was carried out from 2008 to 2009 at the Department of Molecular Biology and Biotechnology, Sheffield, United Kingdom. Splice Over Extension (SOE) PCR strategy was used to construct the αγγ chimaeric receptor, where the α chain is of human origin[8] and the γ chain is of rat.[9]

Primer A - Forward Primer for Human FcεRIα:

5'-TCCTGCTAGCATGAAGAAGATGGCTCCT-'3 with a NheI restriction site.

Primer B - Reverse Primer for Human FcεRIα:

5'-AGGCGTCGACGTACTTCTCACGCGGA-'3 with a SalI restriction site.

Primer C - Forward Rat FcεRIγ Primer:

5'-CTCACTCGAGGGAGAGCCGCAGCTC-'3 with a XhoI restriction site.

Primer D - Reverse Rat FcεRIγ Primer:

5'-AAGACTCGAGCTATTGGGGTGGTTT-'3 with a XhoI restriction site.

Primer E - Forward Human FcεRIα Rat FcεRIγ Linker Primer:

5'-CCGCGTGAGAAGTACGGAGAGCCGCAGCTC-'3.

Primer F - Reverse Human FcεRIα Rat FcεRIγ Linker Primer:

5'-GAGCTGCGGCTCTCCGTACTTCTCACGCGG-'3.

Primer G - Reverse Human TM Region Internal Primer:

5'-TCCTGTCGACTGAGATAAATAATCCT-'3 with a SalI restriction site.

Primer H - Forward Rat CD Region Internal Primer:

5'-GGACGTCGACCGACTCAAGATCCAGG-'3 with a SalI restriction site.

The huFcεRIα gene cDNA was provided in the vector pGEM3Zf, (kindly provided by U. Blank and J-P. Kinet), and was used as the PCR template. The pUC18 plasmid was used as the cloning vector, and the pEE6 plasmid (Celltech) was used as the transfection vector. Miniprep plasmid DNA samples were sent for sequencing to the company Cogenics, using the M13-48REV and M13For-40 primers, after which the results were analysed using ALIGN, CHROMA and CLONE computer programs. The confirmed, non-mutated, clones were used to subclone the cDNA construct (huFcεRIα/γ/γ) into expression vector pEE6 for subsequent transfection into mammalian cells (RBL-2H3.1). Vectors pEE6 and pUC18 with huFcεRIα/γ/γ were digested using the EcoRI and Hind III restriction enzymes (Figure 1). The desired bands were excised from the agarose gel and purified using the Quantum Prep™ Freeze N Squeeze DNA gel extraction spin columns kit. The purified bands were ligated together and a negative (water) control was also set up to eliminate self-ligations. The ligation reactions were transformed into XLI-Blue supercompetent cells. Qiagen/Wizard DNA miniprep kit was employed for small scale DNA extraction. Test digestion of 10µl miniprep plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes followed by resolving on TAE gel to confirm the presence of the desired fragment. The miniprep plasmid DNA was transformed into XLI-Blue competent cells. QIAGEN Plasmid Midi Kit was employed for large scale DNA extraction.

Expression of the αγγ chimeric receptor: The RBL-2H3.1 cell line[10] was employed as the host for the huFcεRIα/γ/γ construct. Stable transfections were carried out using electroporation. In each case, a negative control using water in place of DNA was setup. The transfected cells were then used to carry out flow cytometric studies to ascertain the presence of the desired huFcεRIα receptors on the cell surface. Following flowcytometric studies stocks of the transfected cells were prepared by cryogenic preservation and employed for release assays and intracellular calcium mobilisation studies. The RBL-2H3.1 transfected cell line with the huFcεRIα/γ/γ cDNA constructs was monitored for β-hexosaminidase release through endogenous and huFcεRIα transfected receptors by challenging them with mouse IgE with DNP-HSA specificity and human IgE with NIP-HSA specificity.[11,12] The RBL-2H3.1 cell lines transfected with the huFcεRIα/γ/γ cDNA constructs were assessed for surface expression of huFcεRIα by employing the flow cytometry technique. Cells were labelled in series with a combination of human IgE (JW8), biotinylated anti-human IgE and streptavidin R-phycoerythrin antibodies. Intracellular Ca^{2+} mobilization in RBL-2H3.1 transfected with huFcεRIα/γ/γ cDNA constructs brought about by antigenic stimulus (NIP/DNP) was assessed using the Ca^{2+} indicator Fluo-3AM.[11,12] IgE sensitized cells loaded with Fluo-3AM were analysed using a FACSort flow cytometer preset for Fluo-3AM studies. Data was recorded in the form of a density plot of mean fluorescence against time. After an initial background reading of 30-50 seconds, samples were activated with the appropriate cross-linking agent (NIP/DNP 100 ng/ml) and in the event of no calcium mobilization, the samples were challenged with a calcium ionophore (ionomycin) as a positive control to ensure that the cells have been loaded with Fluo-3AM.

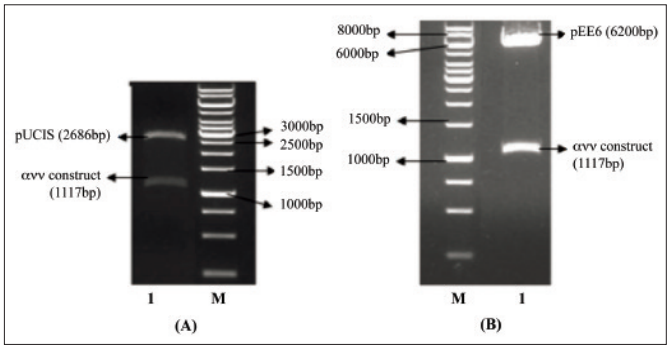

Figure 1: Test Digestion of Miniprep and Midiprep Plasmid DNA was carried out using the EcoRI and Hind III restriction enzymes followed by resolving on TAE gel to confirm the presence of the desired fragment.
In Lane 1 (Figure A) bands visible are 2686bp (pUC18), 6200bp (pEE6) and Lane 1 (Figure B) 1117bp (huFcεRIα/γ/γ construct) while lane M is the 1Kbp DNA marker.

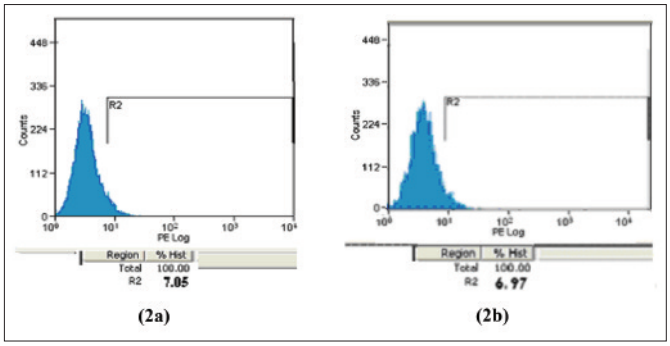

Figure 2: Assessment of cell surface expression of huFcεRIα transfected receptors in RBL-2H3 cells transfected with huFcεRIα/γ/γ construct by flow cytometry.
Figure 2a shows the control histogram while Figure 2b shows the cell population histograms of sample of huFcεRIα/γ/γ transfected cell lines which were assessed for huFcεRIα receptor expression.

RESULTS

Data from the FACS analysis of the transfected RBLs is shown in Figure 2. The data demonstrates the absence of huFcεRIα receptor subunit on the cell surface of transfected RBLs (Figure 2b). The RBLs transfected with huFcεRIα/γ/γ cDNA constructs when sensitised with human IgE and subsequently challenged with human IgE specific NIP-HSA model antigen failed to release above background readings (Figure 3). RBL-2H3 transfected with huFcεRIα/γ/γ cDNA constructs sensitized with mIgE exhibited an increase in mean fluorescence peaking at 26 seconds after being activated by mIgE-specific DNP-HSA (Figure 4a) and no calcium mobilization was observed on being sensitized with huIgE and activated by huIgE-specific NIP-HSA (Figure 4b). The same transfected RBLs on failure to elicit calcium mobilization with huIgE-specific NIP-HSA were activated by ionomycin as a positive control and exhibited an immediate increase in mean fluorescence which was maintained until a slow decrease occurred relating to a depletion of intracellular Ca^{2+} (Figure 4b).

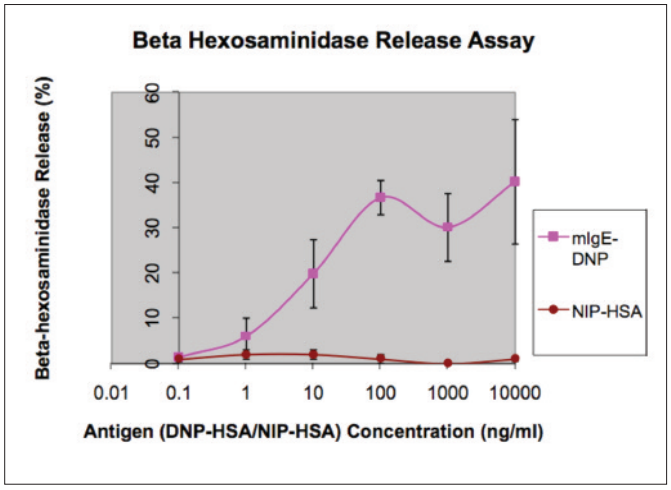

Figure 3: Release of β-hexosaminidase through rodent FcεRIα and huFcεRIα transfected receptors in RBL-2H3 transfected with human huFcεRIα/γ/γ construct in response to antigenic stimulus.
Cells were cultured, harvested, resuspended at 0.5 x 10^6/ml in appropriate media with NIP-specific huIgE (SPE, 1/500)/ DNP-specific mIgE and plated into 96 well plates. Next days, cells were washed and activated with DNP-HSA/NIP-HSA cross-linking agent (0.1-10000 ng/ml) for 20 minutes prior to incubation with β-hexosaminidase substrate for 2 hours. β-hexosaminidase release was assessed as described in material and methods. Data is presented as mean ± S.D. from three separate experiments performed in triplicate. Released β-hexosaminidase is expressed as a percentage of total β-hexosaminidase.

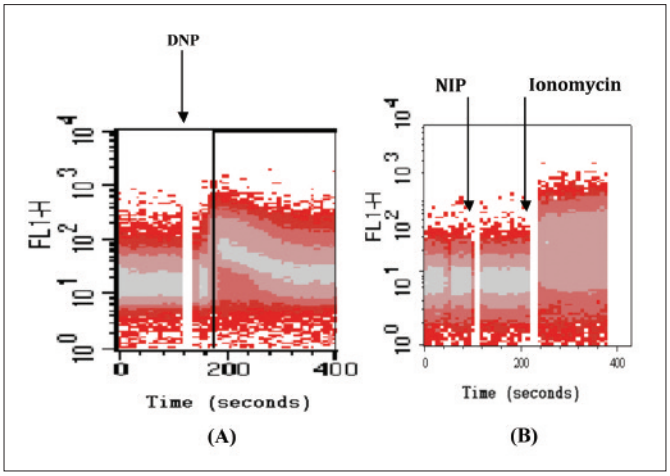

Figure 4: Assessment of Intracellular calcium level of RBLs transfected with huFcεRIα/**γ/γ** cDNA constructs following activation in the absence of extracellular calcium.
RBL-2H3 cells transfected with the huFcεRIα/**γ/γ** cDNA constructs were sensitised with (Figure A) DNP-specific mIgE (SPE-7, 1/500) or (Figure B) NIP-specific huIgE (JW8, 1/500) for 16 hours. After an initial background reading the cells were activated with appropriate cross-linking agent DNP (Figure A) and NIP (Figure B) at a concentration of 100 ng/ml. Following excitation at 488 nm emitted fluorescence was recorded at 525 nm using a FACSort flow cytometer. The cells were then activated with ionomycin (10 μM, Figure B).

DISCUSSION

The technique of constructing novel chimera has provided insight into the understanding the phenomena of allergy.[8] In addition, research is being done in treatment of cancers by constructing chimeric antigen receptor-engineered T-cells.[9] Various studies have been conducted using chimera made up of the CD of the γ subunit FcεRI or TCRζ and the EC domain of CD4[10] or Tac, the interleukin 2 receptor.[11] In the study by Letourneur *et al*. (1991) a chimeric receptor consisting of EC domain of α chain of the interleukin receptor (Tac) and CT domain of either ζ or γ. When they are expressed in T-cells or RBLs could be activated leading to release of interleukin 2 in T-cells and serotonin in RBLs. stably transfected into RBL-2H3 cells. The two constructs prepared by Repetto *et al*. (1996) using the EC and cytoplasmic (CT) domains of the FcεRI and the IL-2R p55 subunit[1] were α/γ/γ and I/γ/γ and results demonstrated that FcεRIα EC and FcεRγ CT domains are mandatory for signalling process leading to the response characteristically seen after the receptor aggregation.[12] The RBLs cell lines transfected with the huFcεRIα/γ/γ cDNA constructs were assessed for the cell surface expression of the huFcεRIα subunit and the response to the antigenic stimulus by looking for degranulation and intracellular Ca^{2+} mobilisation. The results obtained showed the absence of huFcεRIα subunit expression on the surface of transfected cells as seen by flowcytometric studies, β-hexosaminidase assays and intracellular calcium mobilisation studies. Similar results were obtained previously by Higginbottom in 1996 (PhD thesis). In contrast, a study by Repetto *et al*. a α/γ/γ construct when stably transfected into RBL-2H3 cells lead to expression of the receptor which was able to exhibit all the signalling events similar to the native rat FcεRI thus demonstrating the mandatory role of both the FcεRIα EC domain and FcR-γ CT domains.[12] It was expected that similar expression if achieved in the present study could lead to mutations being introduced in TM domains of the γ that would provide insight into the intersubunit interactions. The huFcεRIα/γ/γ cDNA construct had the problem of presence of spurious point mutations, which needed

correction. Similar problems were encountered in the generation and transfection of α/γ/γ cDNA constructs into RBL cells in a study by Higginbottom (1996). It is, therefore, surprising that the results published by Repetto and collaborators (1996) could not be reproduced in the present and previous study conducted by Higginbottom (1996). In the study by Repetto *et al*. VIS expression vector (unavailable), which possesses a visna virus promoter for constitutive expression, was used for transfection into COS and RBL cell lines,[12] in addition in the study by Higginbottom pMAMneo vector, which is an inducible vector, was employed for transfection of α/γ/γ cDNA constructs into RBL cells.[13] In the present study, constitutive expression pEE6 vector, which has been successfully used for the expression of wild-type and mutant huFcεRIα constructs into RBL-2H3 cell line, was used and the reason for the non-expression of huFcεRIα/γ/γ cDNA constructs remains uncertain. The human-rodent chimeric receptors are assembled differently than the endogenous rodent receptors as seen in study in which COS 7 cells were transfected with human/rat chimeric complexes.[14]

CONCLUSION

In the present study, the grounds for non-expression of huFcεRIα/γ/γ cDNA remains elusive but may be due to the fact that the human-rodent chimeric receptors are assembled differently than the endogenous rodent receptors as seen in study in which COS 7 cells were transfected with human/rat chimeric complexes.

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