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A novel human immunoglobulin Fc γ –Fc ϵ bifunctional fusion protein inhibits Fc ϵ RI-mediated degranulation

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Abstract

Human mast cells and basophils that express the high-affinity immunoglobulin E (IgE) receptor, Fc ϵ receptor 1 (Fc ϵ RI), have key roles in allergic diseases. Fc ϵ RI cross-linking stimulates the release of allergic mediators¹. Mast cells and basophils co-express Fc γ RIIb, a low affinity receptor containing an immunoreceptor tyrosine-based inhibitory motif and whose co-aggregation with Fc ϵ RI can block Fc ϵ RI-mediated reactivity^{2–4}. Here we designed, expressed and tested the human basophil and mast-cell inhibitory function of a novel chimeric fusion protein, whose structure is γ Hinge-CH γ 2-CH γ 3-15aa linker-CH ϵ 2-CH ϵ 3-CH ϵ 4. This Fc γ –Fc ϵ fusion protein was expressed as the predicted 140-kD dimer that reacted with anti-human ϵ - and γ -chain specific antibodies. Fc γ –Fc ϵ bound to both human Fc ϵ RI and Fc γ RII. It also showed dose- and time-dependent inhibition of antigen-driven IgE-mediated histamine release from fresh human basophils sensitized with IgE directed against NIP (4-hydroxy-3-iodo-5-nitrophenylacetyl). This was associated with altered Syk signaling. The fusion protein also showed increased inhibition of human anti-NP (4-hydroxy-3-nitrophenyl-lacetyl) and anti-dansyl IgE-mediated passive cutaneous anaphylaxis in transgenic mice expressing human Fc ϵ RI α . Our results show that this chimeric protein is able to form complexes with both Fc ϵ RI and Fc γ RII, and inhibit mast-cell and basophil function. This approach, using a Fc γ –Fc ϵ fusion protein to co-aggregate Fc ϵ RI with a receptor containing an immunoreceptor tyrosine-based inhibition motif, has therapeutic potential in IgE- and Fc ϵ RI-mediated diseases.

Allergen aggregation of IgE bound to Fc ϵ RI induces release of preformed mediators and synthesis of later acting leukotrienes and chemokines from mast cells and basophils⁵. This process has a major role in many of human diseases such as asthma, allergic rhinitis, chronic urticaria, angioedema and anaphylaxis. Fc ϵ RI aggregation induces release of preformed mediators and synthesis of later-acting leukotrienes, chemokines and cytokines⁵. The Fc ϵ RI is a heterotetramer consisting of a single IgE-binding α -subunit, a β -subunit and two disulfide-linked γ -subunits. The β and γ -subunit cytoplasmic tails each contain a conserved immunoreceptor tyrosine-based activation motif (ITAM). Cross-linking Fc ϵ RI via IgE bound to multivalent antigen activates tyrosine phosphorylation of ITAMs, thereby initiating downstream signaling⁶. Mast cells and basophils co-express Fc γ RIIb, which contains two extracellular immunoglobulin-like loops and a single conserved immunoreceptor tyrosine-based inhibition motif (ITIM) within its cytoplasmic tail⁷. Fc γ RIIb may co-aggregate with Fc ϵ RI under physiologic conditions. It is hypothesized that when Fc γ RIIb aggregates, it

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Competing interests statement

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induces inhibitory signaling via SH2-domain-containing inositol 5-phosphatase (SHIP) and phosphorylation of FcγRIIb requires the co-aggregation with FcεRI (refs. 8⁹). Aggregating FcγRII to FcεRI leads to the rapid tyrosine phosphorylation of the FcγRIIb ITIM tyrosine by FcεRI-associated Lyn and inhibition of FcεRI signaling^{10–13}. To take advantage of this FcγRIIb-negative signaling in human basophils and mast cells, we constructed a single chimeric human bifunctional protein (GE2) engineered by fusing the human Fcγ1 and Fcε. Here we show that this chimeric protein is able to inhibit IgE-mediated *in vitro* activation of human basophils and mast cells and *in vivo* activation of human FcεRIα-bearing mast cells in FcεRIα transgenic mice. This approach using chimeric Fcγ proteins provides a platform for non-specific as well as future antigen-specific inhibition of IgE–FcεRI-mediated reactivity in a host of human diseases.

Human genomic DNA encoding the IgG γ1 constant region extends from the hinge through the CH3 domain. This sequence was initially cloned into a mammalian expression vector with a cytomegalovirus promoter and a murine immunoglobulin κ-chain leader sequence. Genomic DNA of the human ε heavy chain CH2 through CH4 domains was placed after the CHγ3. Between Fcγ1 and Fcε, we placed a 15 amino-acid linker (Gly₄Ser)₃, which has been used in single-chain Fv fragment expression¹⁴. This flexible peptide linker facilitates chain pairing and minimizes refolding and aggregation problems encountered when the two chains are expressed individually. The fusion protein contained the binding sites for FcεRI (CHε2–CHε3) and for FcγRII (CHγ2–CHγ3)^{15,16}. The first constant region domains (CHγ1 or CHε1) were deleted as this domain associates with BiP (immunoglobulin heavy-chain binding protein) and a fusion protein containing these regions was not expressed¹⁷. SDS–PAGE demonstrated the Fcγ1–Fcε fusion protein was expressed as the predicted ~140-kD dimer. Western-blot analysis and ELISA testing demonstrated that the GE2 protein was recognized by antibodies specific for human ε and γ chains.

Binding of GE2 to both the human FcεRI and FcγRII was demonstrated using CHO3D10, a human FcεRIα-transfected cell line and HMC-1, which expresses human FcγRII but not FcεR (ref. 18). GE2 protein bound to both FcεRI and FcγRII in a fashion equivalent to human IgE and IgG, respectively, as assessed by flow cytometry (data not shown). These results demonstrate that expressed GE2 protein is properly folded so as to preserve bifunctional heterotypic FcR binding.

Fresh human basophils expressing both FcεRI and FcγRII can be passively sensitized with chimeric human IgE specific for NIP (4-hydroxy-3-iodo-5-nitrophenylacetyl) and then histamine release induced by cross-linking the anti-NIP IgE–FcεRI complex with NIP-BSA (ref. 19). We sensitized basophils with 1 μg/ml human anti-NIP IgE, plus doses of GE2 ranging from 0.01 to 10 μg/ml for 1 hour before activation with 50 ng/ml NIP-BSA (Fig. 1a). Myeloma IgE (PS myeloma) was used as a control. We found that 1 μg GE2 at 1 μg per ml inhibited almost half of histamine release, whereas at 10 μg/ml GE2 gave an average of 84% inhibition. 10 μg of non-specific PS IgE only decreased histamine release by 19%. Adding the GE2 at the same time as the IgE anti-NIP gave optimal inhibition. The longer the delay in GE2 addition following sensitization with IgE anti-NIP, the less the inhibition (Fig. 1b). These results show that the inhibition of antigen-driven histamine release induced by GE2 is dependent on time and dosage. We obtained similar results with cultured human mast cells; a dose of 1 μg caused 50% inhibition of IgE-mediated degranulation (data not shown). Cross-linking of GE2 by adding an anti-IgG antibody *in vitro* along with GE2 caused enhanced inhibition of IgE-mediated release (data not shown). In performing this experiment, we sought to mimic what may occur *in vivo* should subjects given GE2 make an antibody response against it. The actual fate of antibody bound GE2, should it occur *in vivo*, is not possible to predict.

Tyrosine phosphorylation of Syk is a critical step in human mast cell and basophil mediator release¹⁹. Cross-linking FcεRI on human basophils with IgE directed to NIP and NIP-BSA induces substantial tyrosine phosphorylation of Syk which was markedly reduced in cells pre-incubated with GE2 (Fig. 2). Thus, GE2 co-aggregation of FcεRI and FcγRII inhibits IgE-mediated Syk phosphorylation, which may contribute to the inhibition of histamine release.

Transgenic mice altered to express the human FcεRIα and with the murine FcεRIα chain knocked out demonstrate allergic reactivity mediated by passively administered human IgE antibody along with antigen^{20,21}. The human FcεRIα chain is coupled with the mouse β and γ chains to form a functional chimeric human/mouse FcεRI. This model takes advantage of the fact that murine mast cells also express FcγRIIb that can interact well with human IgG. This is in contrast to the lack of interaction between the murine FcεRI and human IgE. We used passive cutaneous anaphylaxis (PCA) to test the GE2 protein's ability to block IgE-driven FcεRI-mediated mast-cell release. We intradermally primed transgenic mice with 250 ng of chimeric human anti-NP (4-hydroxy-3-nitrophenylacetyl) IgE and simultaneously injected individual sites with saline, GE2 or IgE myeloma protein. Four hours later, mice were given a systemic challenge with 0.5 mg NP-BSA plus 1% Evans Blue intravenously. The intensity of the PCA at each site was assessed by the size of the skin bluing after 30 min. The size and color intensity of the reaction at the sites of GE2 injection were decreased compared with sites injected with an equivalent amount of human IgE myeloma (Fig. 3). Similar results were obtained using genetically engineered human IgE anti-dansyl antibody and dansyl-BSA as an antigen. A total of 30 mice were tested and the GE2 was 2–4 times more potent than purified control human IgE in its ability to block PCA.

Our results demonstrate that a single chimeric molecular human protein consisting of Fcγ1 plus Fcε directly inhibits *in vitro* histamine release from human basophils and also inhibits humanized FcεRI-mediated mast-cell degranulation in transgenic mice. GE2 was more potent than control human IgE in all assays. Even if the GE2 protein did not have a 15 amino-acid linker between the two segments of 'normal' human Fcγ1 and Fcε, it would likely be recognized as foreign and induce an antibody response. With the 15 amino-acid linker, it is highly likely that antibody against GE2 would be expressed and will be primarily directed at this linker region. In fact, such an antibody response to GE2 may well prove advantageous as antibodies against GE2 will lead to increased cross-linking of Fcγ and Fcε receptors and should enhance inhibition of basophil and mast-cell function as we have observed *in vitro* using an anti-IgG antibody. This truncated Fcγ–Fcε fusion protein, GE2, provides a novel approach for the immunotherapy of diseases mediated via the FcεR such as allergic asthma, allergic rhinitis, chronic urticaria, angioedema and anaphylaxis. Replacement of the Fcε sequence in our construct with specific allergen genes will result in chimeric bifunctional proteins. We predict that these proteins will have the ability to inhibit mast-cell and basophil reactivity in an antigen-specific manner rather than in the global fashion of GE2. Such novel antigen-specific reagents may be useful in allergen immunotherapy.

Methods

Construction and expression

To construct the human Fcγ–Fcε chimeric gene, the human IgE Fc region (CH2-CH3-CH4) was amplified from pAG vector (provided by S.L. Morrison), containing the whole ε genomic DNA. The 5' end primer was 5'-GCTCGAGGGTGGAGGCGGTTTCAGGCGGAG-GTGGCTCTGGCGGTGGCGGATCGTTCACCCCGCCACCGTGAAG-3', containing a flexible linker sequence and a *XhoI* site. The 3' end primer was 5'-GGCGGCCGCTCATTTACCGGGATTTACAGACAC-3', containing a *NotI* site. After amplification, PCR products were cloned into pCR2.1 vector (Invitrogen, Carlsbad, California) and sequenced. Then the *XhoI-NotI* fragment was inserted into *Sall-NotI* site of pAN expression

vector (from S.L. Morrison), containing the human genomic IgG $\gamma 1$ constant region from hinge through the end of CH3. The IgE Fc region was placed downstream of the IgG $\gamma 1$ constant region in frame with the CH3 and joined by a (Gly₄Ser)³ flexible linker. The expression vector containing the immunoglobulin Fc γ -Fc ϵ chimeric gene was transfected into SP2/0 cells. The Fc γ -Fc ϵ fusion protein GE2 was expressed in cell-culture supernatants and purified by using an anti-human IgE affinity column.

Western-blot analysis

The purified GE2 protein was run on 7.5% SDS-PAGE and then transferred into Immobilon transfer membranes (Millipore, Bedford, Massachusetts). For protein detection, blots were probed with either goat anti-human IgE (ϵ chain-specific) or goat anti-human IgG (γ chain-specific) conjugated to alkaline phosphatase (KPL, Gaithersburg, Maryland). Color development was performed with an alkaline phosphatase conjugated substrate kit (BIO-RAD, Hercules, California).

Binding analysis

Human FcR binding of the purified GE2 protein was tested by binding to Fc ϵ RI-transfected CHO3D10 cells and HMC-1 cells expressing Fc γ RII and then analyzed by flow cytometry (FACSCalibur) using the FITC-conjugated anti-human IgE and anti-human IgG1 reagents (Biosource, Camarillo, California).

Histamine release

Acid-stripped Percoll-enriched human blood basophils were sensitized with chimeric human anti-NIP IgE (10 μ g/ml) at 37 °C in a 5% CO₂ incubator and 1 h later, challenged with 50 ng of NIP-BSA (ref. 19). Histamine release was measured in the supernatants 30 min later. GE2 or control human myeloma IgE was added at various doses and times to test the effects on histamine release.

Measurement of Syk phosphorylation

NIP-sensitized basophils were incubated with or without GE2 (10 μ g/ml), washed and challenged for 2, 5 or 15 min with 50 ng/ml NIP-BSA. Treated or control NIP-IgE sensitized basophils were lysed in 400 μ l of 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Brij, 1 mM sodium orthovanadate and 1 μ g/ml each of antipain, leupeptin, aprotinin and phenylmethanesulfonyl fluoride (PMSF) and incubated for 10 min on ice. Immune complexes were generated by incubating clarified supernatants with anti-Syk pre-adsorbed to protein A-Sepharose beads (Pharmacia, Uppsala, Sweden). Proteins were separated by SDS-PAGE, transferred to nitrocellulose and phosphoproteins identified by anti-phosphotyrosine immunoblotting using enhanced chemiluminescence detection reagents (ECL, Amersham, Piscataway, New York). After probing with anti-phosphotyrosine antibody, blots were stripped for 30 min with 100 mM 2-mercaptoethanol, 2% SDS (w:v), and 0.5 M Tris-HCl buffer (pH 6.8) at 50 °C, and then reprobed with anti-Syk antibody (1 μ g/ml).

Passive cutaneous anaphylaxis

Transgenic mice expressing the human Fc ϵ RI α chain and with the murine Fc ϵ RI α chain knocked out²⁰ were primed intradermally with 250 ng of NP-specific recombinant human IgE (ref. 21) in 50 μ l saline. Individual sites were injected with saline, GE2 or IgE myeloma protein simultaneously. 4 h later mice were then given an intravenous challenge with 1.5 mg/ml of NP-BSA plus 1% Evans blue in 300 μ l saline solution. Cutaneous anaphylaxis was assessed visually by the blue dye leakage from blood vessels into the skin.

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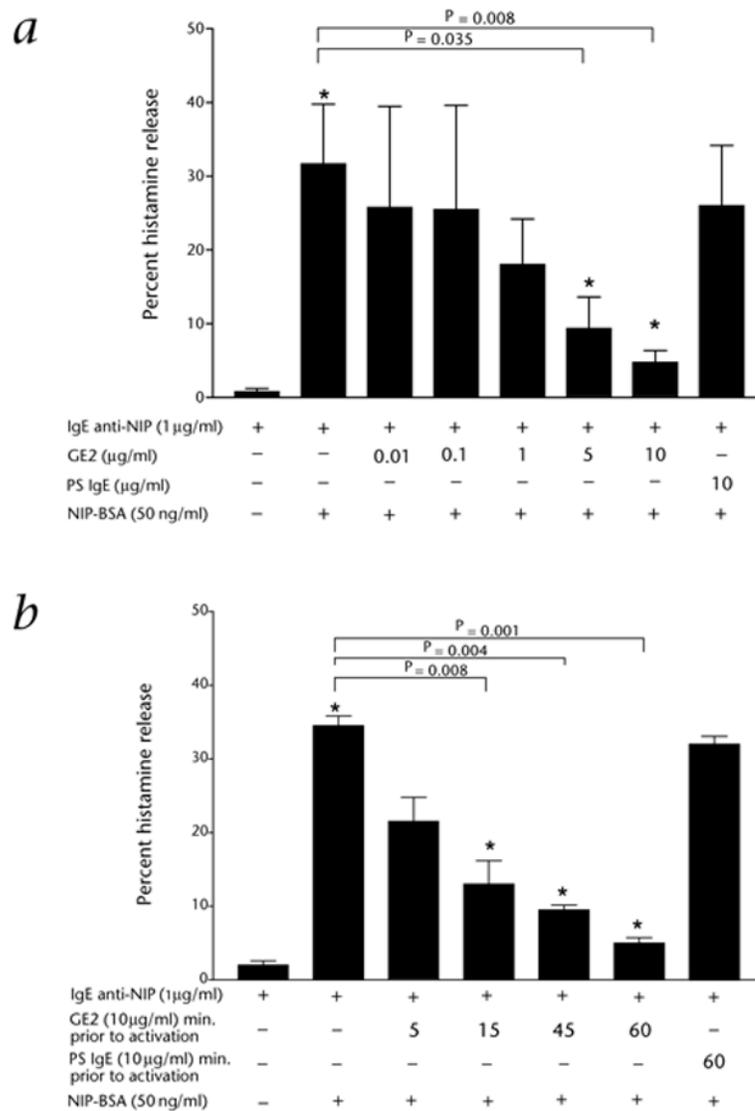
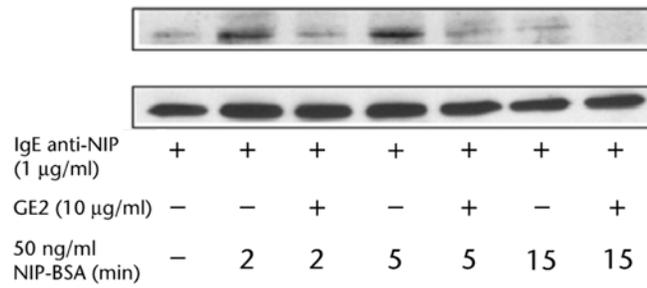


Fig 1. Dose- and time- dependent inhibition of basophil histamine release using GE2. **a**, Dose-dependence. Results are representative of 3 separate donors, each done in duplicate. **b**, Time-dependence. Results are representative of 2 separate donors, each done in duplicate. For both panels: *, significant differences in histamine release ($P < 0.05$), comparing the two indicated conditions. Total histamine in the donor basophils was $1.2 \mu\text{g}$ per 1×10^6 basophils.

**Fig 2.**

Co-aggregation of Fc γ RII and Fc ϵ R1 by GE2 inhibits Fc ϵ R1-mediated Syk phosphorylation. Immunoprecipitates were analyzed by western blotting with antibody against phosphotyrosine (top row), followed by anti-Syk antibody (bottom row). The top row represents phosphorylated Syk and the bottom row represents total Syk. Each lane shows Syk immunoprecipitated from 5×10^6 basophils. Results represent 2 separate experiments.

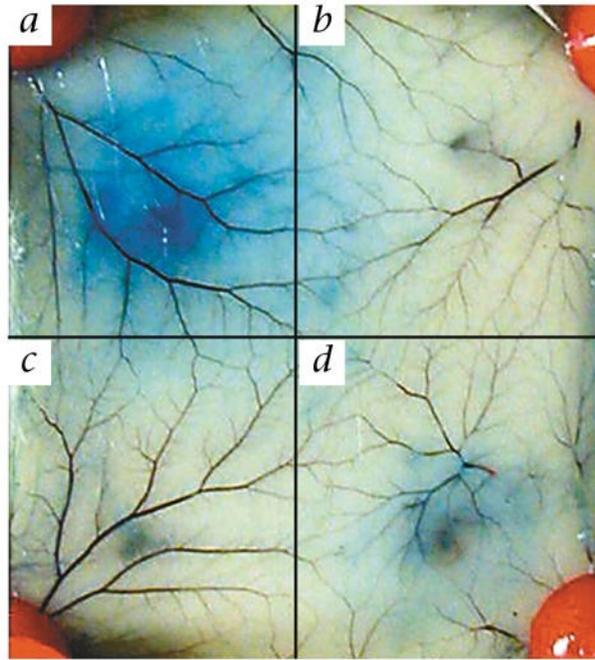


Fig 3.
In vivo immunoglobulin $Fc\gamma$ - $Fc\epsilon$ fusion protein inhibits IgE-mediated degranulation in transgenic mice. **a-d**, Mice were injected intradermally with the following: **a**, anti-NP IgE; **b**, saline; **c**, anti-NP IgE and GE2; **d**, anti-NP IgE and IgE myeloma.