A mouse Fcγ-Fcε protein that inhibits mast cells through activation of FcγRIIB, SH2 domain–containing inositol phosphatase 1, and SH2 domain–containing protein tyrosine phosphatases

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Background: A human Fcγ-Fcε fusion protein (GE2) designed to inhibit FcεRI signaling by coaggregating FcεRI with the inhibitory receptor FcγRIIB has been shown to inhibit mast cell activation and block cutaneous anaphylaxis. A critical issue remained as to whether the mechanism of GE2 inhibition is competition for IgE binding or inhibitory signaling through FcγRIIB.

Objective: Our aim was to define the *in vitro* and *in vivo* mechanism of action of a mouse homolog of GE2 (mGE) and to assess the potential of human GE2 (hGE2) for therapeutic administration.

Methods: The *in vitro* activity of mGE on mediator release and signaling pathways was characterized in IgE-sensitized bone marrow–derived mast cells (BMMCs). The *in vivo* activity of mGE was examined in mouse passive cutaneous and passive systemic anaphylaxis models, and the therapeutic activity of hGE2 was evaluated in *Ascaris suum*–sensitized cynomolgus monkeys.

Results: mGE inhibited release of histamine and cytokines by BMMCs from wild-type mice but not by BMMCs from FcγRIIB-deficient mice. In mice mGE blocked IgE-dependent anaphylaxis mediated by mast cells with sustained efficacy. In BMMCs mGE decreased spleen tyrosine kinase and extracellular signal-regulated kinases 1/2 phosphorylation and induced FcγRIIB phosphorylation and the subsequent recruitment of SH2 domain–containing inositol polyphosphate 5′ phosphatase (SHIP) 1 and SH2 domain–containing protein tyrosine phosphatase (SHP) 1/2 phosphatases. When administered therapeutically, hGE2 protected sensitized monkeys from local anaphylaxis for 3 weeks.

Conclusion: mGE-mediated inhibition of mast cell activation is associated with inhibitory signaling through FcγRIIB that results from activation of SHIP-1 and SHP-1/2 phosphatases. (J Allergy Clin Immunol 2008;121:441-7.)

Key words: *FcγRIIB, mast cell inhibition, FcεRI signaling*

Mast cells and basophils are key effectors in the initiation of IgE-associated type I hypersensitivity reactions. Their activation occurs when IgE bound to the high-affinity receptor FcεRI, a complex of α, β, and γ subunits, is aggregated by multivalent antigen. FcεRI aggregation initiates a signaling cascade that results in granule exocytosis and early release of preformed vasoactive and proinflammatory mediators, the rapid synthesis and release of lipid mediators, and the synthesis and delayed release of cytokines and chemokines.[1,2] One of the first events in FcεRI-mediated signaling is the activation of the tyrosine kinase Lyn and subsequent phosphorylation of immunoreceptor tyrosine-based activation motifs on the β and γ subunits of FcεRI. Recruitment and activation of the spleen tyrosine kinase (Syk) to the phosphorylated receptor subunits initiates downstream signaling events that include degranulation, mitogen-activated protein kinase activation, and phospholipase A2 activation.[3]

FcεRI signaling is negatively regulated by the inhibitory receptor for IgG, FcγRIIB. Coaggregation of FcεRI with FcγRIIB results in Lyn-mediated phosphorylation of the immunoreceptor tyrosine-based inhibitory motif (ITIM) on FcγRIIB, subsequent recruitment of cytoplasmic SH2 domain-containing lipid phosphatases, and inhibition of the signaling pathways leading to early and late mediator release.[1,3,4]

It has been demonstrated *in vivo* that high levels of IgG antibodies to an allergen can suppress IgE-mediated anaphylaxis, both by clearing and preventing allergen binding to IgE and by cross-linking allergen-IgE-FcεRI complexes with FcγRIIB.[1,4,5] This mechanism is thought to occur in human subjects exposed to allergens and forms the basis of allergen immunotherapy for patients.[6,7] Zhu et al[8] exploited this pathway and designed a fusion protein, designated GE2, comprising linked human IgG1-Fc and IgE-Fc regions that was hypothesized to inhibit mast cell activation by directly coaggregating FcεRI and FcγRIIB. Indeed, GE2 inhibited *in vitro* activation of human peripheral blood basophils[8] and cord blood–derived mast cells.[9] Because the human IgE-Fc does not bind rodent FcεRI,[10] *in vivo* efficacy for GE2 administered locally in the skin was demonstrated in a passive cutaneous anaphylaxis (PCA) model in transgenic mice expressing the human α chain of FcεRI and passively sensitized with human IgE[8] and in naturally sensitized rhesus macaques.[11]

It has been debated as to whether the inhibitory activity of GE2 results from competition for IgE binding to FcεRI rather than

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Abbreviations used
BMMC: Bone marrow–derived mast cells
DNP: Dinitrophenyl
ERK: Extracellular signal-regulated kinase
hGE2: Human GE2
ITIM: Immunoreceptor tyrosine-based inhibitory motif
mGE: Mouse homolog of human GE2
PCA: Passive cutaneous anaphylaxis
PSA: Passive systemic anaphylaxis
SHIP: SH2 domain–containing inositol polyphosphate 5′ phosphatase
SHP: SH2 domain–containing protein tyrosine phosphatase
Syk: Spleen tyrosine kinase
TLR: Toll-like receptor
TNP: Trinitrophenyl

coaggregation of FcεRI and FcγRIIB and generation of an inhibitory signal. A mouse homolog of GE2 would facilitate detailed *in vitro* mechanistic and *in vivo* efficacy studies to address the utility of GE2 for the treatment of allergic disease. In the present study we produced a mouse homolog of GE2 (mGE) and showed that it inhibits early and late mediator release by bone marrow–derived mast cells (BMMCs) from wild-type but not from FcγRIIB-deficient mice. *In vivo* mGE delivered systemically inhibited both PCA and passive systemic anaphylaxis (PSA). When human GE2 (hGE2) was administered therapeutically to *Ascaris suum*–sensitized cynomolgus monkeys, the animals were protected from local anaphylaxis for up to 3 weeks. FcεRI signaling in BMMCs was inhibited by mGE through FcγRIIB activation and recruitment of SH2 domain–containing inositol polyphosphate 5′ phosphatase (SHIP) 1 and SH2 domain–containing protein tyrosine phosphatases (SHP) 1/2. Together, these results support the feasibility of using an Fcγ-Fcε fusion protein in the treatment of IgE-mediated allergic diseases.

METHODS

Cells and antibodies

Bone marrow cells from BALB/c or C57BL/6 mice were cultured for 5 to 12 weeks in complete medium (RPMI 1640; ATCC, Manassas, Va) containing 10% FBS (HyClone, Logan, Utah), 10 μg/mL gentamicin (Sigma-Aldrich, St Louis, Mo), 55 μmol/L β-mercaptoethanol (Invitrogen, Carlsbad, Calif), and recombinant IL-3 (10 ng/mL; R&D Systems, Minneapolis, Minn). FcγRIIB$^{-/-}$ mice on a BALB/c background were purchased from Taconic Farms (Hudson, NY). Animal experiments were approved by the Biogen Idec (San Diego, Calif) and Charles River Laboratories (Worcester, Mass) Institutional Animal Care and Use Committees, and guidelines from the "Guide for the care and use of laboratory animals," Institute of Laboratory Animal Resources, National Research Council, National Academy Press (1996) were followed. Antibody reagents are described in the Methods section of the Online Repository at www.jacionline.org.

mGE construct

The mouse sequences H-$C_{\gamma 2a}$2-$C_{\gamma 2a}$3 and C_ϵ2-C_ϵ3-C_ϵ4 were amplified by means of PCR, assembled into the fusion protein H-$C_{\gamma 2a}$2-$C_{\gamma 2a}$3-$(Gly_4Ser)_3$-C_ϵ2-C_ϵ3-C_ϵ4, and expressed in CHO DG44 cells, as described in the Methods section of the Online Repository at www.jacionline.org. Secreted mGE was purified by using protein A chromatography and was a glycosylated disulfide–linked dimer of 158 kd. Each preparation was tested for endotoxin and purity and contained minimal aggregation (see Fig E1 in this article's Online Repository at www.jacionline.org).

hGE2 construct

The hGE2 molecule was assembled based on the GE2 construct of Zhu et al[8] in the pV90 vector (Biogen Idec) and was expressed in CHO cells. The reconstructed hGE2 did not contain epitope tags or nonnative sequences, with the exception of the Gly/Ser linker.

Cell activation

BMMCs (0.1×10^6 per sample) were sensitized with mouse IgE anti-trinitrophenyl (anti-TNP; 10 μg/mL) with or without mGE in complete medium for 2 hours at 37°C. Cells were washed and resuspended in HBSS containing calcium (Invitrogen) and TNP-BSA (0.2 μg/mL; Biosearch Technologies, Novato, Calif). After 1 hour at 37°C, EDTA was added to 0.04 mol/L. Histamine was quantified by means of ELISA (Research Diagnostics, Concord, Mass). Total histamine content of cells was measured after incubating BMMCs for 6 minutes at 100°C. When cytokine release was analyzed, 1×10^6 BMMCs per sample sensitized as above for 2 hours were incubated with TNP-BSA (1 μg/mL), LPS (1 μg/mL, Sigma-Aldrich), or both for 16 or 24 hours in complete medium at 37°C. TNF-α and IL-13 levels were quantified by means of ELISA. The results shown are representative of at least 3 independent experiments. In Figs 1 and 2 each bar represents the mean ± SEM.

Immunoprecipitation and immunoblot analysis

BMMCs ($10\text{-}40 \times 10^6$ per sample) were sensitized with IgE anti-TNP (10 μg/mL) for 2 hours and then stimulated with TNP-BSA (1 μg/mL). Cells were resuspended in lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride [PMSF], 1 mmol/L Na_3VO_4, and 0.1% SDS [Sigma-Aldrich]) and the 1X Halt Protease Inhibitor Cocktail Kit [Pierce, Rockford, Ill]) for 10 minutes on ice.

Passive anaphylaxis

For PCA, BALB/c mice were injected with mGE subcutaneously on the back flank 24 hours before challenge. Mouse IgE anti-dinitrophenyl (anti-DNP; 100 ng, Sigma-Aldrich) was injected intradermally into the right ear cartilage (pinna), and PBS was injected into the left pinna 4 hours before intravenous challenge with 100 μg of DNP-HSA in 1% Evans Blue dye (Sigma-Aldrich).[12] After 40 minutes, mice were euthanized, and pinnae were removed, incubated overnight in 2 mL of acetone with 0.5% sodium sulfate, and extravasated blue dye quantified at 620 nm. Background OD_{620} from each control pinna was subtracted. Photographs of Evans Blue dye extravasation in pinnae shown in Fig 3, *A*, are representative of 3 independent experiments. Fig 3, *B*, shows the mean ± SEM values for 3 mice.

For PSA, BALB/c mice (5 per group) were injected with mGE (5 mg/kg intraperitoneally) at various times up to 21 days before sensitization with IgE anti-DNP (20 μg intraperitoneally). Challenge with DNP-HSA (200 μg intravenously) was 24 hours after sensitization. After challenge, animals were monitored every 10 minutes for body temperature with a rectal thermometer and probe (Physitemp, Clifton, NJ).[13] In Fig 3, *C*, each point represents the mean ± SEM values for 5 mice. Results shown are representative of 4 independent experiments.

Skin testing in cynomolgus monkeys

A suum–sensitized male cynomolgus monkeys (Charles River Laboratories) were dosed subcutaneously with PBS or hGE2 at 10 mg/kg on 2 consecutive days. Two days later (day 1), animals were challenged intradermally at multiple sites with PBS (negative control), histamine phosphate (positive control), or *A suum* extract at $1{:}10^5$ or $1{:}10^6$ dilutions. Challenges were also