Original article

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.
Clinical implications: These studies support the feasibility of systemically delivering Fcγ-Fcε fusion protein in the treatment of IgE and FcεRI-mediated allergic diseases. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

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

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Disclosure of potential conflict of interest: E. Mertsching, H. Hess, K. Giza, E. Negrou, K. Hathaway, J. Chung, D. Perret, and M. R. Kehry are employed by Biogen Idec. L. Bafetti, S. Perper, and M. Shields were employed by Biogen Idec when this work was performed. A. Saxon has patent rights through his employment with the University of California.

Received for publication December 13, 2006; revised August 17, 2007; accepted for publication August 22, 2007.

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0091-6749/$32.00

doi:10.1016/j.jaci.2007.08.051

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

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 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

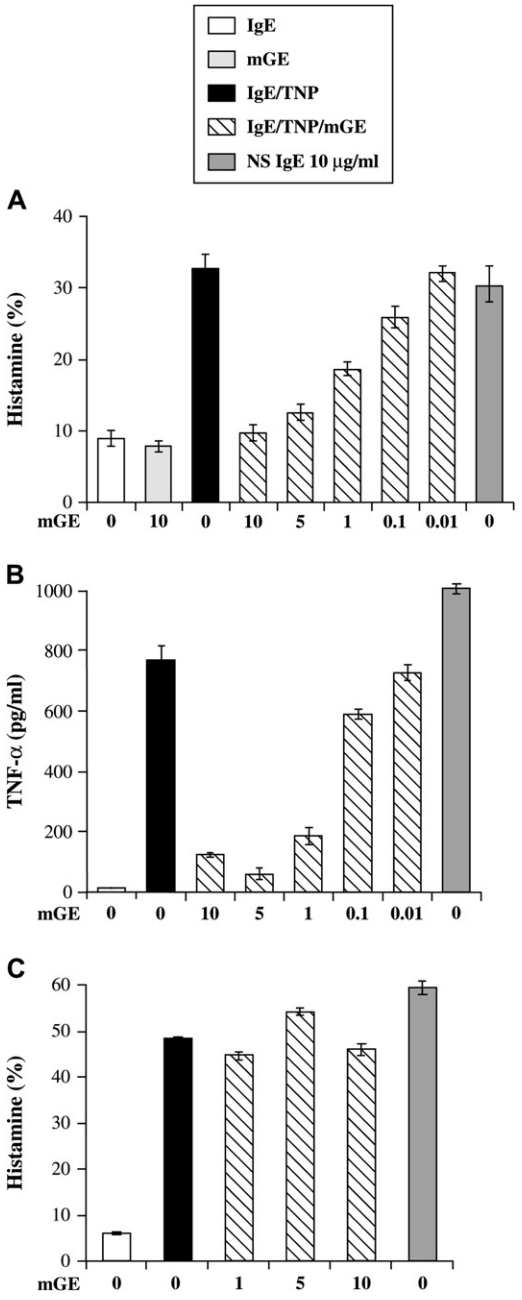

FIG 1. Inhibitory activity of mGE is dependent on expression of FcγRIIB. BMMCs were sensitized and challenged, and histamine **(A)** and TNF-α release **(B)** were quantified. Non–TNP-specific IgE *(NS)* or mGE was added with IgE anti-TNP, as indicated. **C**, BMMCs from FcγRIIB$^{-/-}$ mice were sensitized and challenged. mGE binding to wild-type and FcγRIIB$^{-/-}$ BMMCs was comparable (data not shown).

(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.

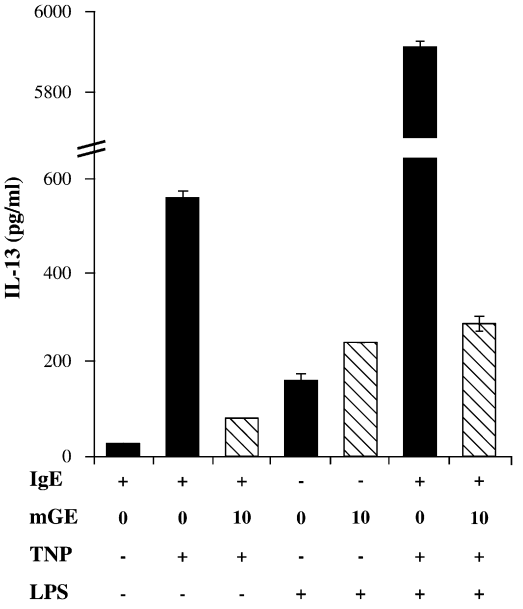

FIG 2. mGE inhibits synergistic activation of BMMCs through FcεRI and TLR4 engagement. BMMCs were incubated with IgE anti-TNP with or without mGE and stimulated with LPS, TNP-BSA, or both, as indicated. IL-13 release was measured after 16 hours. Each *bar* is the mean of replicate samples ± SEMs. Results are representative of 3 independent experiments.

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 performed before dosing with hGE2 (predose) and at days 7, 14, and 21. Ten minutes before challenge, a solution of 0.5% Evan's Blue dye was administered intravenously. Injection sites were measured 15 minutes after challenge and scored as 0 (no effect), 1 (mild), 2 (marked change), or 2.5 (worse reaction than histamine). Each group comprised 5 animals.

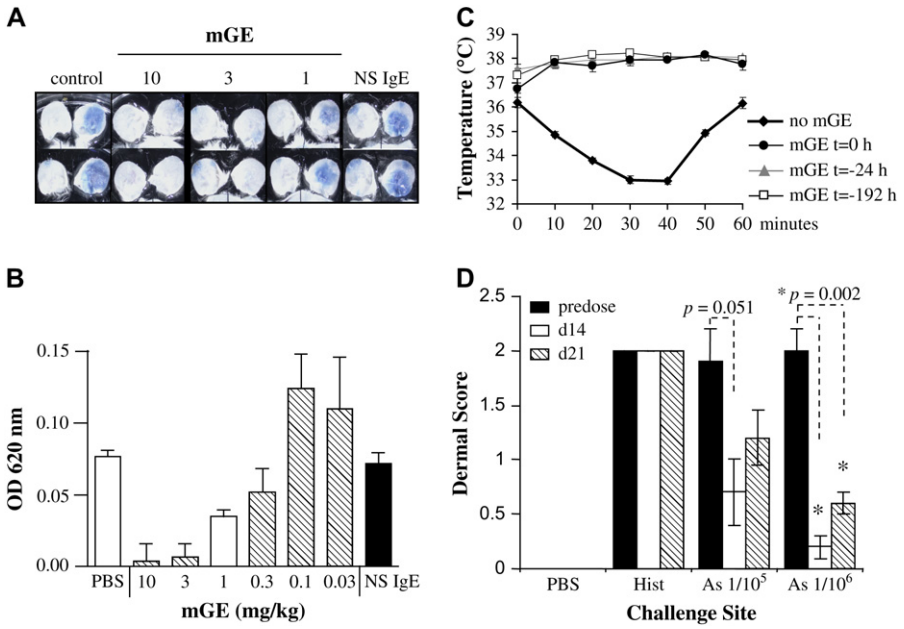

FIG 3. *In vivo* efficacy. **A** and **B**, PCA model. **A**, Mice were sensitized with IgE anti-DNP or PBS, and a non–DNP-specific IgE (*NS*; 10 mg/kg) or mGE was administered. **B**, Evans Blue dye was extracted and quantified. **C**, PSA model. **D**, Mean dermal scores (± SEM) in predosed and hGE2-dosed cynomolgus monkeys after challenge with PBS, histamine *(Hist)*, or *A suum* extracts.

Immunoprecipitation and immunoblot methods are described in the Methods section of this article's Online Repository at www.jacionline.org. The normalized ratios of the band intensity of phospho-FcγRIIB to total FcγRIIB or phospho-SHIP-1 to total FcγRIIB were calculated set to 1.0 for IgE only. The reduced amount of total FcγRIIB in mGE-treated BMMCs was not reproducible.

RESULTS

mGE inhibits IgE-dependent BMMC activation

We generated a mouse homolog of the human GE2 fusion protein, designated mGE, consisting of mouse IgG2a-Fc and IgE-Fc regions linked by a glycine/serine peptide. By means of flow cytometry, mGE bound to To test mGE activity *in vitro*, BMMCs exclusively through FcεRI, which is consistent with the low affinity of FcγRIIB for monomeric IgG (data not shown). To test mGE activity *in vitro*, BMMCs were sensitized with IgE anti-TNP (10 μg/mL) in the presence of various concentrations of mGE. After cross-linking IgE-bound FcεRI by means of the addition of TNP-BSA, histamine was quantified as a measure of mast cell degranulation. mGE abrogated histamine release of sensitized and antigen-challenged BMMCs (90% to 95% inhibition at 10 μg/mL) and exhibited a dose response (Fig 1, *A*). At a 10:1 ratio of IgE/mGE, mGE still reduced histamine release by more than 50%. mGE also similarly inhibited the release of the late mediators TNF-α (Fig 1, *B*) and IL-4 (data not shown). Histamine and cytokine release by BMMCs were not inhibited by nonspecific IgE (10 μg/mL; Fig 1, *A* and *B*), which suggests competition with IgE for FcεRI is likely not a major mechanism of action of mGE.

Under conditions in which BMMCs were simultaneously incubated with IgE and mGE (Fig 1), inhibition of degranulation was maximal. When mGE was added 15 minutes after IgE, only 50% inhibition of histamine release was found, and after 30 minutes, no effect was seen (see Fig E2 in this article's Online Repository at www.jacionline.org). These results confirm the slow off-rate of FcεRI-bound IgE and that mGE binding to FcεRI, as measured by inhibitory activity, appeared to be prevented in short time frames by saturating cell-surface receptors with IgE. Indeed, an incubation time of longer than 8 hours with IgE-saturated BMMCs was required for mGE to exhibit maximal inhibition of degranulation (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). During this time, the number of mGE molecules bound on the cell surface increased steadily, primarily because of expression of new FcεRI (see Fig E3, *B*, in the Online Repository at www.jacionline.org).

These results are consistent with the proposed mechanism for mGE inhibition as coaggregation of FcεRI with FcγRIIB; however, competition of mGE with specific IgE could not be excluded. To demonstrate a role for FcγRIIB in mGE inhibitory activity, BMMCs from FcγRIIB$^{-/-}$ mice were grown and sensitized with IgE anti-TNP in the presence and absence of mGE. In FcγRIIB-deficient BMMCs, mGE exhibited no inhibitory activity (Fig 1, *C*).

mGE blocks the synergistic effect of Toll-like receptor 4 and FcεRI cross-linking

It has been reported that the combination of Toll-like receptor (TLR) 4 engagement and FcεRI signaling on mast cells synergistically increases production of IL-9 and

IL-13.[14] To investigate whether mGE also inhibited this synergistic cytokine release, BMMCs were stimulated with a TLR4 ligand and FcεRI cross-linking in the presence and absence of mGE. mGE markedly reduced the synergistic increase in IL-13 (Fig 2) and TNF-α (data not shown) production without affecting the cytokines produced in response to TLR4 engagement alone.

In vivo activity

The *in vivo* activity of mGE was tested in 2 type I hypersensitivity models mediated by mast cells: PCA and PSA. For the PCA model, mice were injected subcutaneously with mGE, sensitized in the pinna with IgE anti-DNP, and challenged systemically with DNP-HSA. At doses of 10 and 3 mg/kg, mGE was efficacious in abrogating Evans Blue dye extravasation, and at a dose of 1 mg/kg, mGE exhibited partial efficacy (Fig 3, *A* and *B*). Animals dosed with a nonspecific IgE at 10 mg/kg did not show inhibition of the PCA reaction in the pinnae. Thus when administered at a site distal to the site of sensitization, mGE inhibited skin mast cell mediator release *in vivo*. Similar results were obtained when mGE and IgE were simultaneously injected in the back skin (L. Chan Allen; data not shown).

For the PSA model, mice were treated with PBS or mGE at various times before passive systemic sensitization with IgE anti-DNP (at t = 0). Challenge with DNP-HSA resulted in a transient decrease in body temperature over 30 minutes indicative of anaphylaxis, followed by recovery. mGE blocked the PSA response when administered between 1 and 8 days before sensitization (Fig 3, *C*). Similar inhibition of the PSA response was found when mGE was dosed 48 or 96 hours before sensitization and up to 12 days before sensitization (data not shown). Therefore mGE appeared to have a long *in vivo* residence time bound to FcεRI. Additionally, mGE dosed simultaneously with IgE also blocked the PSA response (Fig 3, *C*; t = 0). Thus mGE was shown to inhibit mast cell mediator release *in vivo*.

To study the therapeutic potential of the human homolog of mGE, hGE2 was administered to *A suum*–sensitized cynomolgus monkeys at days −3 and −2 before first challenge with *A suum* extract at $1{:}10^5$ and $1{:}10^6$ dilutions. Only a modest reduction in dermal scores was seen in animals treated with hGE2 and challenged at day 1. However, when the challenge was performed at days 7 (data not shown), 14, and 21 (Fig 3, *D*), hGE2 protected the monkeys from the skin anaphylaxis reaction induced by local administration of *A suum*. The values reached significance at the lowest concentration of *A suum*, and inhibition was maximal at day 14. These results indicated that hGE2 was efficacious when administered therapeutically.

Because mGE contains an IgG2a-Fc capable of binding Fcγ receptors, it was possible that systemic *in vivo* administration of mGE could either directly activate or deplete mast cells, depending on whether Fcγ receptor engagement on effector cells induced FcεRI cross-linking or antibody-mediated cellular cytotoxicity, respectively. Skin sections taken at various times after mGE dosing showed no histologic differences in mast cells, granule content, or mast cell numbers when compared with skin sections from PBS-treated mice (data not shown; see Fig E4 in this article's Online Repository at www.jacionline.org). These results were confirmed when serum histamine levels were measured 5 minutes and 1 hour after intravenous injection of mGE alone (data not shown).

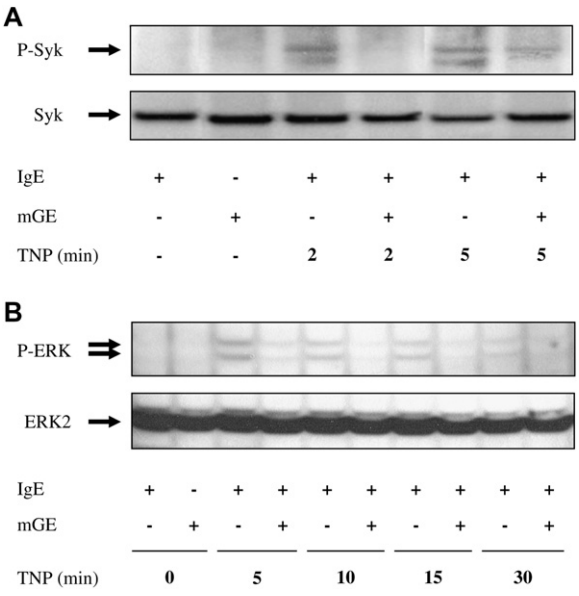

FIG 4. mGE inhibits Syk and ERK1/2 activation. **A**, Lysates of BMMCs sensitized and challenged as indicated were immunoprecipitated for Syk and immunoblotted with anti-phosphotyrosine *(P-Syk)* and anti-Syk antibodies. **B**, Immunoblots of lysates of BMMCs sensitized and challenged as indicated were probed with anti-phospho-ERK1/2 *(P-ERK)* and antibodies preferentially recognizing total ERK2. Results shown are representative of at least 3 independent experiments.

mGE decreases Syk and extracellular signal–regulated kinases 1/2 activation in BMMCs

The tyrosine kinase Syk plays a major role in the early signaling events after FcεRI cross-linking.[15,16] Syk phosphorylation was induced by TNP-BSA aggregation of IgE anti-TNP sensitized BMMCs (Fig 4, *A*). However, little or no Syk phosphorylation was found when BMMCs were also treated with mGE (Fig 4, *A*), demonstrating that mGE inhibited this early step in activation of the FcεRI signaling pathway.

The tyrosine kinase Lyn is constitutively associated with the FcεRI β chain, and the activation of Lyn is thought to be one of the first signaling events after FcεRI aggregation.[3] Lyn immunoprecipitated from TNP-BSA–activated BMMCs incubated with mGE showed no mGE-dependent changes in phosphorylation (data not shown).

Phosphorylation of downstream extracellular signal-regulated kinases (ERK) 1/2 was also examined in activated BMMCs treated with mGE. In the absence of mGE, ERK1/2 phosphorylation was increased maximally 5 minutes after FcεRI aggregation and decreased gradually

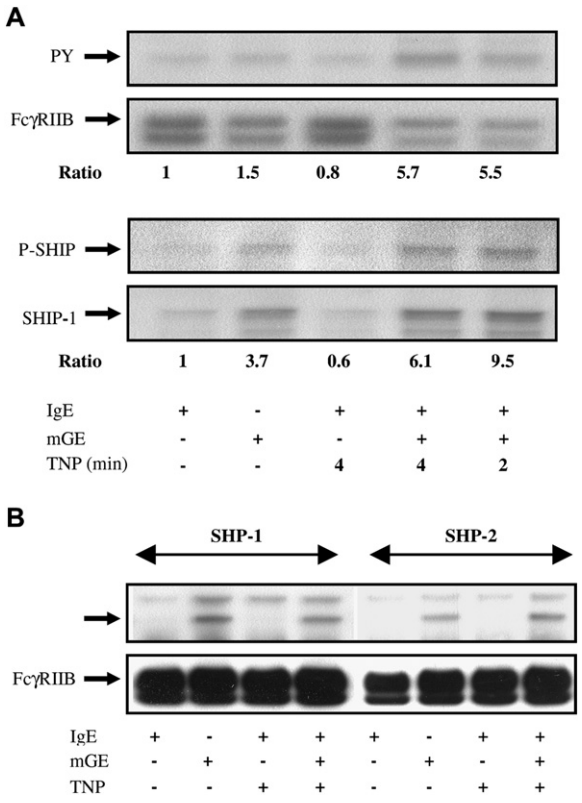

FIG 5. mGE mediates inhibitory signaling through FcγRIIB. **A**, FcγRIIB was immunoprecipitated from lysates of sensitized and challenged BMMCs. The blot was probed sequentially (from the top) with anti-phosphotyrosine *(PY)*, anti-FcγRIIB, anti-phospho-SHIP-1 *(P-SHIP)*, and anti-SHIP-1. **B**, Immunoblots were probed with anti-SHP-1 or anti-SHP-2 (*arrow* in *top panel*) or 2.4G2 (FcγRIIB identified by molecular weight). Results shown are representative of at least 3 independent experiments.

over 30 minutes (Fig 4, *B*). ERK1/2 phosphorylation was markedly decreased in activated BMMCs treated with mGE at all the time points examined up to 30 minutes (Fig 4, *B*).

mGE induces SHIP-1 and SHP-1/2 association with FcγRIIB

The lack of an inhibitory effect of mGE on BMMCs from FcγRIIB$^{-/-}$ mice suggested that mGE inhibition is mediated through FcγRIIB signaling. In mast cells coaggregation of FcεRI and FcγRIIB results in the phosphorylation of FcγRIIB and the recruitment of SH2-containing inositol 5' phosphatase-1 (SHIP-1) to the phosphorylated ITIM motif of FcγRIIB.[17] To further elucidate the mechanism of action of mGE, FcγRIIB and SHIP-1 phosphorylation were monitored in activated BMMCs treated with mGE. A minimal amount of phospho-FcγRIIB was detected in the absence of mGE, and SHIP-1 did not coprecipitate with FcγRIIB (Fig 5, *A*). In contrast, an increase in phosphorylated FcγRIIB levels was observed in challenged mGE-treated cells after 2 and 4 minutes, and the amount of SHIP-1 and phospho-SHIP-1 coprecipitating with FcγRIIB was increased (Fig 5, *A*). Interestingly, coprecipitation of SHIP-1 with FcγRIIB was also observed in cells incubated with mGE without IgE or TNP-BSA (Fig 5, *A*).

In mouse B cells and in transfected RBL-2H3 cells, Syk activity has been reported to be negatively regulated by the protein tyrosine phosphatase SHP-1.[18,19] SHP-1 and SHP-2 protein phosphatases have been found in BMMCs[20] but have not previously been shown to associate with phosphorylated FcγRIIB. BMMCs were incubated with mGE and activated with TNP-BSA for 4 minutes, and immunoprecipitates of lysates were run in duplicate lanes (Fig 5, *B*). SHP-1 and SHP-2 coprecipitated with FcγRIIB only in the presence of mGE. Consistent with our previous observations on FcγRIIB and SHIP-1 coprecipitation, mGE binding to BMMCs in the absence of FcεRI cross-linking also induced the association of FcγRIIB with SHP-1 and SHP-2 (Fig 5, *B*).

DISCUSSION

We generated a mouse homolog of the previously investigated human GE2 protein designed to inhibit mast cell activation by cross-linking the inhibitory receptor FcγRIIB with FcεRI. mGE inhibitory activity was characterized on IgE-sensitized antigen-activated BMMCs and was confirmed to be similar to inhibition of human mast cells by human GE2.[11] mGE was shown to inhibit BMMC activation at the level of mast cell release of both early- and late-phase mediators and required FcεRI binding. This is consistent with previously characterized inhibitory effects of FcγRIIB aggregation on FcεRI-dependent activation signals.[3] Also consistent with an FcγRIIB-mediated signaling mechanism, mGE did not decrease cytokine production induced by TLR4 signaling, a pathway that does not involve tyrosine kinase activation.[21]

Interestingly the marked synergy in IL-13 and TNF-α production resulting from TLR4 engagement and FcεRI cross-linking on BMMCs[14] was inhibited by mGE. This synergistic mechanism has been hypothesized to explain the exacerbation of clinical symptoms sometimes observed in asthmatic patients with bacterial infections. It is therefore possible that a GE2-like molecule might have additional therapeutic benefits in asthmatic individuals.

Critically, because mGE and human GE2 need to bind to FcεRI to exert inhibitory activity, the possibility that inhibition could solely result from competition with IgE for binding to FcεRI on mast cells had not been excluded in previous studies, and others have suggested this was the mechanism of action of GE2.[22] However, mGE did not show any inhibitory activity on BMMCs from FcγRIIB-deficient mice, even though it was able to bind surface FcεRI. mGE was also shown to stimulate FcγRIIB phosphorylation and association of inhibitory lipid and protein tyrosine phosphatases with FcγRIIB. Together, these results argue for inhibitory signaling through FcγRIIB as the major mechanism of action of mGE.

Previous *in vivo* studies with human GE2 in mice or rhesus macaques had only examined activity in locally

administered sites on the skin.[8,11] Our results demonstrate efficacy of mGE in inhibiting mast cell–mediated type I hypersensitivity reactions (PCA and PSA). A pharmacokinetic study of systemically administered mGE at 1, 3, and 10 mg/kg showed that mGE has good bioavailability through the subcutaneous route (approximately 85%), with a serum half-life of approximately 3 days (data not shown). Additionally, mGE alone did not deplete or activate mast cells in the skin or stomach (data not shown). To investigate the efficacy of an Fcγ-Fcε fusion protein when administered therapeutically, the human homolog of mGE, hGE2, was administered to *A suum*–sensitized cynomolgus monkeys. Results showed that even in the presence of pre-existing IgE specific for the parasite, the skin anaphylaxis reaction was profoundly reduced by hGE2. Importantly, 2 consecutive doses of hGE2 were sufficient to protect the animals for up to 3 weeks. These observations suggest that hGE2 might have potential as a therapeutic agent in the treatment of allergic diseases.

In activated BMMCs mGE reduced Syk and ERK1/2 phosphorylation and induced the phosphorylation of FcγRIIB and its association with the phosphatases SHIP-1, SHP-1, and SHP-2. These results are consistent with the decreased Syk phosphorylation and increased SHIP phosphorylation found in human cord blood mast cells treated with human GE2.[9] The decrease in Syk phosphorylation in BMMCs treated with mGE could be due to the activity of the SHP phosphatases. In B cells SHP-1 has been previously reported to associate with phosphorylated FcγRIIB[18]; however, the involvement of SHP-1/2 in FcγRIIB-mediated BMMC inhibition has not previously been demonstrated. BMMCs have been shown to express SHP-1 and SHP-2 that *in vitro* can bind highly phosphorylated ITIM-containing peptides derived from FcγRIIB, but association in mast cells could not be demonstrated.[9,23-25] In the rat RBL-2H3 cell line transfected with FcγRIIB, SHP-1 has been shown to associate with FcγRIIB only when FcγRIIB was hyperphosphorylated after pervanadate treatment. This level of phosphorylation was not attained when the FcγRIIB was cross-linked with antibodies.[25] It is possible that in comparison with an antibody to FcγR, mGE might transduce a stronger or more direct signal to activate and phosphorylate FcγRIIB, facilitating association with SHP proteins and decreasing phosphorylation of Syk. Consistent with this proposal, in a previous study on RBL-2H3 cells in which SHP association with FcγRIIB was not detected, Syk phosphorylation was unchanged.[24] The strong inhibition of Syk activation in BMMCs treated with mGE might indicate that SHP-1 and SHP-2 play an important role in the inhibitory mechanism. This more closely resembles the mechanism of FcγRIIB-mediated inhibitory signaling in B cells.[18]

Notably, mGE induced phosphorylation of FcγRIIB and inhibitory signaling, even though it did not coaggregate FcγRIIB with an antigen cross-linked FcεRI. In previous models used to study inhibitory signaling, FcγRIIB was coligated with FcεRI that was activated through the binding of IgE and multivalent antigen. mGE, however, was engineered to directly bind to the 2 receptors. A previous report has shown that cross-linking FcεRI and FcγRIIB separately with different antigens inhibited human basophil activation,[26] but the experimental design is challenging because of the low intrinsic affinity of FcγRIIB for IgG. It is possible that the linked Fc design of mGE can mediate translocation of both receptors to lipid rafts, providing activated Lyn and downstream signaling molecules.

In summary, we generated a mouse and a human Fcγ-Fcε fusion protein that inhibit mast cell activation *in vitro* and *in vivo*. hGE2 showed therapeutic activity up to 3 weeks after its administration to sensitized cynomolgus monkeys. mGE-mediated inhibition was associated with the activation of FcγRIIB, which led to recruitment of the phosphatases SHIP-1 and SHP-1/2 and resulted in downregulation of Syk. mGE provides an important tool to examine FcγRIIB-mediated mast cell regulation and study mast cell function in mouse models of allergic disease, including asthma and food allergy.

We thank Al Gill and Thomas Crowell for histology support; Cheryl Black, Gregg Hetu, and Candace Graff for pharmacokinetic analyses; and Christopher Kepley for advice on mast cell degranulation assays. We also thank Elise C. Martin at Charles River Laboratories for coordinating the cynomolgus monkey skin test study.

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