Modifications to an Fcgamma-Fcvarepsilon fusion protein alter its effectiveness in the inhibition of FcvarepsilonRI-mediated functions

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Allen LC, Kepley, CL, Saxon A, Zhang K. Modifications to an Fcgamma-Fcvarepsilon fusion protein alter its effectiveness in the inhibition of FcvarepsilonRI-mediated functions. *Journal of Allergy and Clinical Immunology* 2007; 120:462-8.

Made available courtesy of Elsevier: https://doi.org/10.1016/j.jaci.2007.04.019

Abstract:

Background: GE2, a human bifunctional Fcγ-Fcε fusion protein cross-links FcγRIIb and FcεRI on human mast cells and basophils and results in inhibition of FcεRI-mediated functions.
Objective: Three modified Fcγ-Fcε (GE) proteins were compared with GE2 for their effect on inhibition of FcεRI-mediated cellular responses.
Methods: GE2 was modified to potentially improve its therapeutic efficacy by increasing binding to FcγRIIb (GE S mutant) and decreasing binding to FcγRIII (GE H mutant) or reversing the Fcγ and Fcε domains and removing nonhuman linker sequences (E2G). These proteins were tested for their ability to bind a basophil-like cell line, block FcεRI-mediated degranulation in human basophils, and inhibit passive cutaneous anaphylaxis in human FcεRIα-transgenic mice.
Results: All 4 GE proteins bound cells that express FcεRI and FcγRIIb, although the original GE2 retained the strongest ability to bind to these cells. E2G was as effective as GE2 in its ability to inhibit anti-Fel d 1 IgE-mediated histamine release from human basophils and block passive cutaneous anaphylaxis reactions. The GE S and GE H mutants were less effective.
Conclusion: Optimization of GE2 as an inhibitor of FcεRI-mediated functions showed that effectiveness was maintained when potentially immunogenic linker sequences were removed and Ig domain positions were reversed, but specific residue changes within the IgG C_H2 domain aimed at enhancing GE2's inhibitory function by increasing FcγRII binding or additionally decreasing FcγRIII binding were not beneficial.
Clinical implications: GE2 and E2G molecules are effective inhibitors of FcεRI-mediated degranulation and are of interest as potential therapeutics for IgE-mediated allergic reactions.

Keywords: GE2 | IgE | IgG | FcεRI | FcγRIIb | allergy

Article:

Abbreviations used

HA: Hemagglutinin A
ITIM: Immunoreceptor tyrosine inhibitory motif
NP: 4-hydroxy-3-nitrophenylacetyl
PCA: Passive cutaneous anaphylaxis

Immediate hypersensitivity or allergic diseases, such as allergic asthma, allergic rhinitis, and most food allergies, are generally thought of as predominantly allergic antibody or IgE-mediated processes. The important role of IgE was recently highlighted by the inhibition of the early- and late-phase reactions in the lung and skin by treatment with anti-IgE.[1, 2] The presence of allergic antibody to common environmental allergen is common and becoming more prevalent,[3] and thus it is not surprising that diseases caused by IgE are also common and show increasing prevalence. IgE predominantly resides on mast cells and basophils bound to high-affinity IgE receptors, FcεRI. In initiating an allergic response, FcεRI-bound IgE binds multivalent allergens causing FcεRI cross-linking. This triggers a signaling cascade that results in both immediate mediator release and production of other biologically active molecules causing the classic symptoms of allergy.

We have previously shown that a human Ig fusion protein consisting of the hinge through C_H3 of the γ1 heavy chain plus C_H2 through C_H4 of the ε heavy chain (GE2 protein) can block the allergic response by co–cross-linking FcεRI and FcγRIIb inhibitory receptors on the cell surface.[4, 5] GE2 is a fusion of the Fcγ1 hinge–C_H2–C_H3 and the Fcε C_H2-C_H3-C_H4 that assembles as a 150-kd monomer containing 2 covalently linked Fcγ-Fcε chains. FcγRIIb is a negative regulatory molecule that contains an immunoreceptor tyrosine inhibitory motif (ITIM) and can inhibit FcεRI signaling on mast cells and basophils.[6, 7] GE2 blocks FcεRI-mediated functions of human basophils and mast cells and inhibits passive cutaneous anaphylaxis (PCA) in FcεRIα-transgenic mice and skin test reactivity in rhesus monkeys with dust mite allergy.[4, 8] GE2 also has the ability to inhibit Langerhans-like cell function through FcεRI-FcγRIIb cross-linking[9] and interfere in isotype switch and IgE production by B-cell function through FcεRII (CD23)–FcγRII cross-linking.[10]

In this article we compare the functional features of 4 proteins, the original GE2 and 3 GE2 modified proteins that will be collectively referred to as GE proteins, constructed in an effort to enhance the ability of GE2 to inhibit IgE-mediated processes (Fig 1). One of the modified proteins, the GE S mutant, wherein the serine at position 267 is replaced by an alanine, was constructed to enhance binding to FcγRIIb.[11] The second molecule, the GE H mutant, wherein the histadine at position 268 is replaced by an alanine, enhances binding to FcγRIIb, and decreases binding to FcγRIII.[11]A third protein, E2G, reversed the position of the Fcγ and Fcε domains, using the hinge region of Fcγ to function as the flexible linker between the 2 Fc regions and thereby removing potentially immunogenic linker sequences from the original construct.

We found that E2G was as effective as GE2, whereas the GE S and H mutants were surprisingly less effective than the original GE2 molecule. They form monomers with a small percentage of dimers in the preparations, and all except for the GE S mutant were able to bind a human basophil cell line, Ku812, better than native IgE. The GE2 and E2G proteins inhibited cellular degranulation better than the GE S and GE H mutants in basophils isolated from one individual. Both GE2 and E2G were more effective in blocking IgE-mediated PCA responses in human FcεRIα-transgenic mice than the GE S and GE H mutants. Overall, the effectiveness of these proteins to inhibit FcεRI-mediated degranulation and PCA followed a pattern as follows: E2G = GE2 > GE S mutant > GE H mutant.

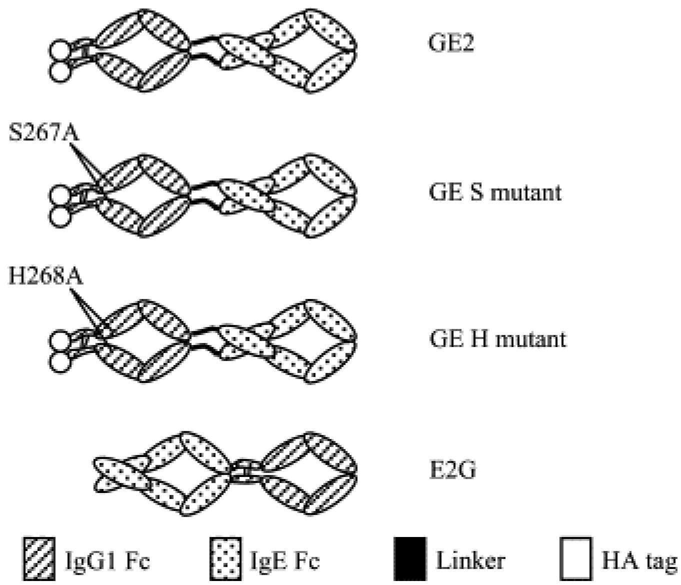

Fig 1. Schematic diagram of human GE proteins. The molecules are depicted with the N-terminus on the *left* and the C-terminus on the *right*. The Fcγ domains (γ hinge-Cγ2-Cγ3) are *striped*, the Fcε domains (Cε2-Cε3-Cε4) are *dotted*, the flexible linker is *black*, and the hemagglutinin tag is *white*. Amino acid substitutions are designated by their IgG1 EU index position.

Methods

Gene construction and expression

The GE2 construct, described previously,[4] consists of a hemagglutinin A (HA) epitope, 7 vector amino acids, the IgG1 hinge-C_H2-C_H3, 17 amino acids including a $(Gly_4Ser)_3$ linker, and IgE C_H2-C_H3-C_H4. We used nested PCR with primers 5′-GGCCAGATCTGA GCCCAAATCTTGT-3′, 5′-CCTCCCGCGGCTTTGTCTTGGC-3′, 5′-TTGACCTCAGGGTCTTCGTGTGCCACGTCCACCACCACGCAT-3′, and 5′-ATGCGTGGTGGTGGACGTGGCACACGAAGACCCTGAGGTCAA-3′ to introduce a S267A mutation within the IgG1 C_H2 domain of our Fcγ-Fcε gene and named it the GE S mutant. A similar nested PCR strategy with primers 5′-TTGACCTCAGGGTCTTCCG CGCTCACGTCCACCACCACGCAT-3′ and 5′-ATGCGTGGTGGTGGACGTGAGCGCGGAAGACCCTGAGGTCAA-3′ created the GE H mutant, containing H268A substitution. The *Bgl*II–*Sac*II fragment was ligated into the GE2 expression vector.[4] E2G reversed the Fcγ and Fcε sequences of GE2 by linking the 3′ end of IgE C_H2-C_H3-C_H4 with the 5′ end of the IgG1 hinge-C_H2-C_H3 by using a *Bgl*II site. The HA tag, vector sequences, and $(Gly_4Ser)_3$ linker present in GE2 were removed. The 5′ κ leader sequence was connected to the IgE C_H2 sequence by using overlap PCR and the primers 5′-AAGCTTGATATCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTG GGTTCCAGGTTCCACTGGTGAC-3′ and 5′-

TCCAGGTTCCACTGGTGACTTCACCCCGCCCACCGTGAAGATTTACAGTCGTCCTG CGACGGC-3′. The primer 5′-GGTACCAGATCTTTTACCGGGATTTACAGACACC-3′ was used to introduce a *Bgl*II site in place of the IgE stop codon. All the designed mutations were confirmed by sequencing. The product was cut with *Eco*RV-*Bgl*II and inserted into a plasmid containing a *Bgl*II site at the beginning of the IgG1 hinge and then placed in a final expression vector containing a CMV promoter (kindly provided by Dr S. L. Morrison).

Protein expression and purification

The linearized plasmid DNA (5 μg) was transfected by means of electroporation into 2 to 4 × 10^7 Ns0/1 myeloma cells. Expression was tested by means of ELISA and metabolic labeling, and then GE protein–producing cells were subcloned and grown in roller bottles. The cell supernatant was passed through a protein A-sepharose column (Sigma Aldrich, St Louis, Mo), and bound protein was eluted with citric acid, pH 4.5. One-milliliter protein fractions were immediately neutralized with 2 mol/L Tris, pH 8.0, and then dialyzed with PBS. For analytic purposes only, a small fraction of each GE protein was separated by means of gel filtrationon a fast protein liquid chromatography by using two 25-mL Superose 6 columns (Pharmacia, Uppsala, Sweden) with PBS and 0.02% NaN_3, pH 6.8, and a flow rate of 0.25 mL/min. The amount of monomer and aggregated material determined by means of an OD_{280} reading was quantitated by using Quanity One software (Bio Rad Laboratories, Inc., Hercules, Calif) and calculated as a percentage of the total protein eluted. The purified proteins were analyzed with SDS-PAGE under reducing and nonreducing conditions.

Flow cytometry

Binding to FcεRI was assessed by means of flow cytometry on the human basophil-like cell line Ku812 (kindly provided by Dr W. Vainchenker, Creteil, France), which expresses FcεRI and FcγRII. For each sample, 10^6cells were incubated with or without GE2 and IgE proteins at several concentrations at 4°C for 1 hour, followed by staining with fluorescein isothiocyanate–labeled goat anti-human ε chain (Sigma Aldrich) for 30 minutes at 4°C. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif), gating out dead cells and debris.

Histamine release

Human basophils were isolated from the buffy coat of human blood, as described previously.[4] The basophils were sensitized overnight with 10 μg/mL anti–4-hydroxy-3-nitrophenylacetyl (NP) IgE. The next day, 0.01 to 20 μg/mL of each of the mutants, 10 μg/mL IgE, or 10 μg/mL IgG was added to the basophils for 2 hours in the incubator at 37°C. Cells were washed and activated with optimal concentrations of NP-BSA for 30 minutes. Histamine release was measured as described previously.[7]

PCA

Mice expressing human FcεRIα but not murine FcεRIα[12] (kindly provided by J.-P. Kinet) were used to measure PCA, as described previously.[4] Several concentrations of GE proteins in 50 μL of human serum from a subject with cat allergy diluted 1:5 or 0.5 µg/mL purified anti-Fel d 1 human IgE were injected intradermally. Four hours later, the mouse was challenged intravenously with 200 µL of 1% Evan's blue dye in saline containing 10 to 15 µg of purified natural Fel d 1 (Indoor Biotechnology, Inc, Charlottesville, Va). After 30 minutes, the mouse was killed, and PCA was visualized by means of leakage of blue dye into the ventral surface of the skin through dilated blood vessels at the site of injection. PCA quantitation and end point titration was limited by the leaked dye appearance and distribution in the PCA spots, the locations of the skin injected, individual mice, and the number of sites per animal. Experiments were performed in triplicate.

Results

Characteristics of the purified GE proteins

The GE2, GE S mutant, and GE H mutant proteins, when separated by means of SDS-PAGE under nonreducing conditions, appeared as approximately 150-kd monomers composed of paired covalently bound GE heavy chains in contrast to normal H2L2 IgE at 190 kd and BSA at 66 kd (Fig 2, *A*). E2G was slightly smaller than the other GE proteins, with a molecular weight of about 145 kd, which is consistent with the removal of HA tag and linker sequences. A small fraction of the GE2- and E2G-purified material migrated at the approximate size of a GE protein dimer, or 300 kd (see below). Under reducing conditions with β-mercaptoethanoland separation on SDS-PAGE, the GE proteins migrated as a single band near 75 kd, as expected, whereas BSA appeared as a 66-kd band (Fig 2, *B*). When nondenatured protein samples were examined by using size exclusion chromatography, all 4 GE proteins contained a major peak, with the size of the expected GE protein monomers and a smaller fraction (2.8% E2G, 6.7% GE H mutant, 17.3% GE S mutant, and 17.6% GE2) that migrated at about the expected size for dimers (data not shown).

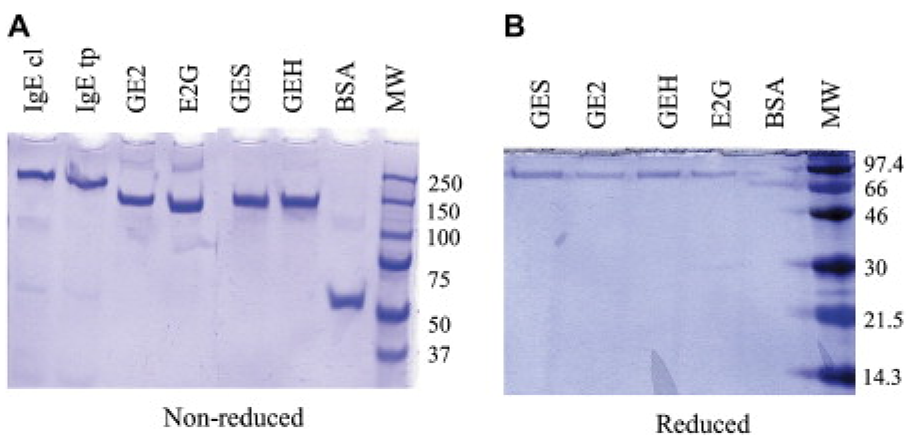

Fig 2. Purified GE proteins visualized on SDS-PAGE. **A**, Two micrograms of nonreduced samples of IgE isoforms, GE proteins, and BSA were run on a 5% phosphate gel. **B**, One microgram reduced samples of GE proteins and BSA were run on a 12% Tris-glycine gel. Molecular weight *(MW)* is indicated in kilodaltons on the *right*.

GE proteins showed differential ability to bind to FcεRI and FcγRIIb on Ku812 cells

Given the sequence differences and changes in domain positions of the GE2 proteins, we examined the ability of these proteins to bind to receptors on a human basophil-like cell line, Ku812. Ku812 expresses FcεRI and FcγRIIb, but not FcγRIIa and FcγRIII, on its surface.[5, 13, 14] The fusion proteins were predicted to bind cells better than IgE because they should bind to both FcεRI and FcγRIIb, whereas IgE would only bind to FcεRI. Using flow cytometry and a fluorescein isothiocyanate–conjugated goat anti-ε-chain antibody to detect GE proteins that bound Ku812 cells, we found that at low concentrations of protein, GE2, E2G, and GE H mutant proteins bound these cells at significantly higher levels than recombinant IgE (Fig 3). In comparison, GE2 displayed the highest level of binding to Ku812 cells, and the GE S mutant bound the least cells compared with the other GE proteins and IgE (Fig 3).

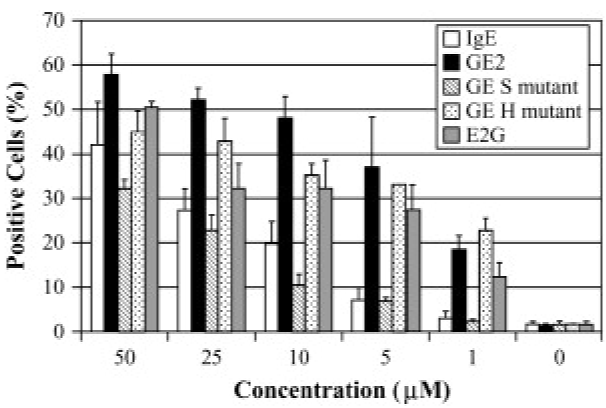

Fig 3. GE proteins and IgE bind a basophil-like cell line, Ku812. Cells were incubated with several concentrations of IgE or GE proteins, and protein bindingwas measured as the percentage of α-ε fluorescein isothiocyanate–positive cells detected by means of flow cytometry. *Error bars* represent the SD among 3 repetitions. The GE H mutant at 5 μmol/L had 1 measurement.

GE proteins inhibit FcεRI-mediated degranulation in human basophils

We have previously shown that GE2 blocks FcεRI-mediated histamine release in human basophils in a time- and dose-dependent manner.[4] We compared the ability of the GE mutant proteins to inhibit degranulation of human basophils isolated from different donors with an undetermined number of receptors. Cell samples, prepared in duplicate or triplicate, were sensitized with 10 μg/mL NP-IgE and activated with optimal concentrations of NP-BSA. All 4 GE proteins displayed an ability to decrease basophil degranulation; however, the extent of inhibition appeared to vary depending on the donor. In some donors GE2 and E2G were significantly more effective than the GE S and GE H mutants at 10μg/mL (Fig 4), whereas in other donors all 4 proteins displayed only minimal inhibition at 10 μg/mL, with no significant differences compared with the nonspecific IgE and IgG controls (Table I). The proteins also displayed a dose dependence that also varied depending on the donor cells used (Table II). In addition, when the GE proteins were tested on cells from the same donor over a 2-year time span at random intervals, the degree of inhibition varied, giving values of 10.3%, 25%, and 3.4% inhibition for GE2; 17.2%, 42.9%, and 37.6% for E2G; 5.7%, 17.5%, and 41.1% for the GE S mutant; and 24.1%, 40.9% and 33.6% for the GE H mutant (Table I, Table II, marked by asterisks). In all cases the IgE and IgG controls did not block degranulation, indicating histamine release was not inhibited by competition of FcεRI binding between NP-IgE and IgE nor was FcγRIIb inhibitory signaling triggered solely by monomeric IgG.

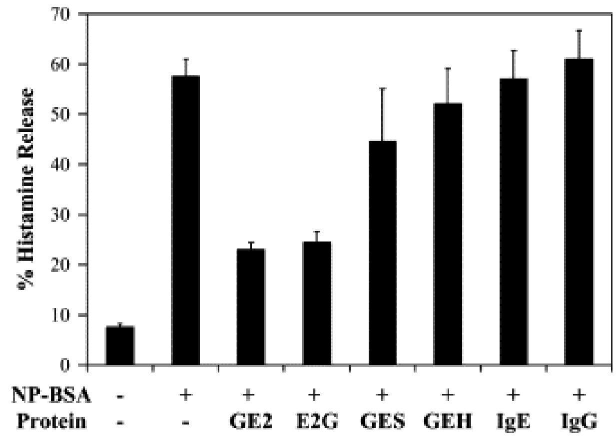

Fig 4. Effect of GE proteins on human basophil FcεRI-mediated degranulation. **A**, Basophils from a representative donor presensitized with 10 µg/mL anti-NP IgE, treated with 10 µg/mL protein, and activated with NP-BSA. Percentage of degranulation is taken from duplicate samples.

Table I. Effectiveness of GE proteins on degranulation from basophils isolated from different donors

Experiment	Spontaneous	ns IgE	GE2	E2G	GE S	GE H	ns IgG
1*	6.5 (2.1)	43.5 (21.9)	39.0 (21.2)	36.0 (21.2)	20.0 (7.1)	32.5 (16.3)	42.5 (20.5)
2	7.5 (0.7)	57.5 (3.5)	23.0 (1.4)†	24.5 (2.1)†	44.5 (10.6)	52.0 (7.1)	61.0 (5.7)
3	9.5 (2.1)	47.5 (7.8)	24.5 (4.9)†	28.0 (2.8)†	NA	NA	43.5 (2.1)
4	12.2 (9.9)	16.0 (11.4)	7.5 (0.1)†	8.7 (2.0)	16.9 (9.5)	12.3 (2.0)	47.3 (52.0)
5	4.2 (1.6)	43.3 (5.9)	29.0 (9.6)†	23.7 (4.2)†	41.0 (10.0)	39.7 (5.1)	37.7 (15.3)
6	9.0 (3.0)	29.3 (7.6)	18.0 (6.6)	19.7 (3.2)	23.0 (7.2)	19.7 (5.1)	30.7 (10.5)
7*	4.0 (1.0)	49.7 (6.1)	48.0 (3.0)	31.0 (10.0)†	41.0 (8.7)	33.0 (8.5)†	51.3 (9.6)
8	7.7 (5.7)	15.3 (4.9)	12.0 (3.6)	12.3 (1.5)	10.0 (4.6)	12.3 (4.5)	18.7 (2.1)
9	10.0 (3.6)	42.0 (13.0)	47.3 (9.0)	35.0 (7.0)	40.7 (11.9)	44.3 (10.0)	45.0 (7.2)

Percentage degranulation is reported in the presence of 10 µg/mL GE proteins, nonspecific IgE and nonspecific IgG, or spontaneous release showing SD (in parentheses).
ns, Nonspecific; *NA*, not applicable.
*Identifying the same donor.
†$P < .05$ compared with 10 µg/mL nonspecific IgE.

Table II. Dose effect of GE proteins on degranulation tested on basophils isolated from several donors

Protein	Spontaneous	ns IgE	0.01 µg/mL	0.1 µg/mL	1.0 µg/mL	10.0 µg/mL	20.0 µg/mL	ns IgG
GE2	2.5 (3.5)	39.0 (7.1)	41.5 (0.7)	42.5 (6.4)	33.0 (5.7)	35.0 (7.1)	NA	43.0 (2.8)
GE2*	7.5 (6.4)	28.0 (4.2)	33.5 (0.7)	30 (2.8)	26.0 (2.8)	21.0 (1.4)	NA	32.5 (6.4)
GE2	9.5 (2.1)	47.5 (7.8)	NA	48.0 (4.2)	37.5 (7.8)	24.5 (4.9)†	18.5 (2.1)†	43.5 (2.1)
E2G	2.5 (3.5)	39.0 (7.1)	41.0 (9.9)	38.0 (9.9)	40.0 (9.9)	37.5 (4.9)	NA	43.0 (2.8)
E2G*	7.5 (6.4)	28.0 (4.2)	27.0 (2.8)	23.0 (2.8)	21.0 (5.7)	16.0 (2.8)†	NA	32.5 (6.4)
E2G	9.5 (2.1)	47.5 (7.8)	NA	44.5 (3.5)	39.0 (11.3)	28.0 (2.8)†	12.5 (3.5)†	43.5 (2.1)
GE S	4.0 (1.0)	54.7 (10.2)	50.3 (2.1)†	48.7 (4.7)†	43.0 (10.1)	48.0 (3)†	NA	53.0 (11.8)
GE S	6.3 (1.5)	18.0 (5.3)	19.3 (7.2)	20.7 (6.8)	16.0 (6.2)	8.3 (6.5)	NA	23.3 (9.5)
GE S*	5.0 (3.6)	62.3 (15.2)	5.7 (16.1)	55.7 (10.5)	41.7 (16.9)	36.7 (12.7)	NA	59.7 (14.6)
GE S	10.7 (2.3)	41.3 (6.4)	28.7 (9.0)	24.7 (4.7)†	27.0 (5.3)	24.0 (7.2)†	NA	37.3 (16.3)
GE H	3.0 (2.8)	33.0 (5.7)	25.0 (4.2)	26.5 (3.5)	23.0 (2.8)	19.5 (2.1)†	NA	33.0 (7.1)

Percentage degranulation showing SD (in parentheses).
ns, Nonspecific; *NA*, not applicable.
*Identical donor.
†$P < .05$.

GE2 and E2G blocked FcεRI-mediated PCA in human FcεRIα-transgenic mice more effectively than the GE S and GE H mutant proteins

GE2 has been shown to inhibit FcεRI-dependent PCA in mice when coadministered with anti-NP IgE and challenged with NP-BSA, as well as in mice presensitized with human serum from a subject with cat allergy and challenged with the native Fel d 1 antigen.[4, 8] When we compared GE2 with the GE proteins, we found that E2G was equivalent to GE2 in blocking PCA at 5, 2.5, and 1 μg/mL (Fig 5, *A*). The GE S and GE H mutant proteins also completely blocked PCA reactivity at 5 μg/mL; however, both mutants displayed less inhibition of PCA reactivity than the original GE2 at lower concentrations (Fig 5, *B* and *C*). As a control, Fel d 1–specific IgE given without treatment of GE proteins resulted in a blue spot on challenge with Fel d 1, indicating strong positive PCA reactivity (Fig 5, panel 1). When nonspecific human myeloma IgE at 5 μg/mL was mixed with Fel d 1–specific serum IgE, it did not block the PCA response (Fig 5, *A*, panel 5). Each comparison experiment was performed on at least 3 mice and produced consistent results, although the sensitivity to allergen and treatment varied slightly between animals.

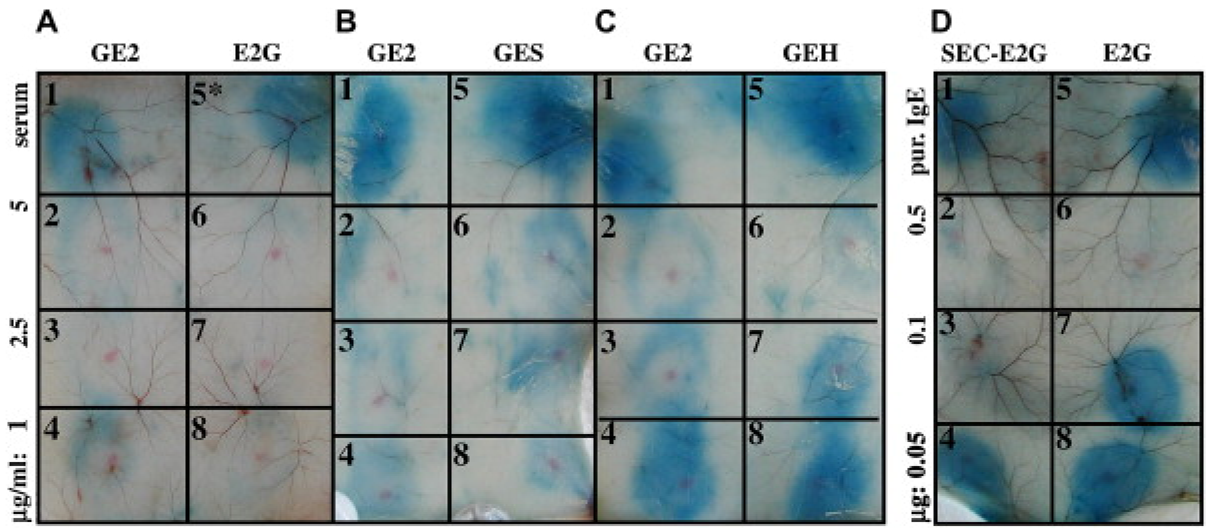

Fig 5. GE proteins block PCA: **A**, GE2 versus E2G; **B**, GE2 versus GE S mutant; **C**, GE2 versus GE H mutant; and **D**, size exclusion chromatography-purified E2G monomers versus E2G. Mice were injected with serum from a patient with cat allergy or purified IgE plus GE proteins and then challenged after 4 hours with Fel d 1 in Evans Blue dye. *Serum with 5 μg/mL myeloma IgE.

Purified monomeric E2G more effectively blocked FcεRI-mediated PCA than E2G preparations

Because the GE protein preparations contained a small fraction of aggregated protein, we were interested in determining how this affected the inhibitory properties of the molecules. The effectiveness of our E2G preparations were compared with size exclusion chromatography–purified E2G monomers (fractionated by Biogen Idec, Cambridge, Mass) by using PCA on FcεRIα-transgenic mice sensitized with purified anti-Fel d 1 IgE. Both E2G preparations completely blocked at higher doses, whereas only monomer E2G blocked at the lowest dose of protein (Fig 5, *D*).

Discussion

The success of GE2 in blocking allergic responses *in vitro* and in animal models gives promise for this molecule as a potential novel treatment of human allergic diseases.[4, 5] In seeking the best candidate for further drug development, we were interested in maximizing the benefits of co–cross-linking FcγRIIb receptors with FcεRI receptors on cells and minimizing the effects of potentially immunogenic sequences, such as a hemagglutinin tag and flexible linker.

When we looked at GE protein binding to Ku812 cells, we found that GE2, E2G, and GE H mutant bound cells better than IgE, whereas the GE S mutant had poorer binding than IgE. The lower binding of the GE mutants compared with that of GE2 was unexpected because previous studies by Shields et al[11] showed an increase in binding affinity (ratio of mutant/native intact Ig) of 1.84 for FcγRIIb (S267A), 1.44 for FcγRIIb, and 0.54 for FcγRIII (H268A). These small differences in binding of intact IgG molecules did not appear to correlate with an improved inhibitory effectiveness of the GE proteins. In the Ku812 cell model, the binding of the GE proteins measures the sum of the binding to both FcγRII and FcεRI. It is possible that the higher percentages of GE2-, E2G-, and GE H mutant–bound cells compared with lower binding of the GE S mutant could suggest that even though the Fcγ mutation increased binding under the Ig configuration (H2L2), the mutation could play an inhibitory role by directly blocking Fcγ access to FcγRII under the Fcγ-Fcε configuration. Likewise, this mutation might impede binding of the Fcε portion to FcεRI indirectly through the mutation, through an induced conformational change of the GE S mutant, or both. It was a concern that the lower binding of the GE S mutant, although only slightly less than that of IgE, might be partially due to the presence of aggregates in the preparation. Although an effort was made to isolate monomeric proteins, the aggregates could reform in solution after monomer purification. However, other factors must be involved in the lower binding seen for the GE S mutant because both GE2 and the GE S mutant contained similar concentrations of aggregated material (17.6% and 17.3%, respectively), and the overall binding was still greater for GE2 over the GE S mutant. This indicated that the difference in protein composition, a single amino acid residue in the FcγR binding region, likely changed the physical properties of the GE mutants, affecting binding affinity through a mechanism such as improper protein folding, decreased stability of the protein on the cell surface, or faster uptake and degradation in the Ku812 cell line.

FcεRI-mediated responses can be inhibited when FcεRI is coaggregated with FcγRIIb by original GE2 on basophils and cord blood–derived human mast cells.[6, 4, 7, 15] In some donors tested in this study, GE2 and E2G significantly inhibited degranulation compared with the GE S and GE H mutants that was consistent with the trend seen in the PCA studies. However, our basophil studies also showed that the GE proteins displayed differences in the level of inhibition between donors such that the proteins did not always show the same pattern of effectiveness. We also observed variability in GE protein effectiveness by using the same donor taken over time, suggesting that human basophils might have different phenotypic states that affect inhibition through the FcγRIIb receptor. Variation between and within subjects for *in vitro* basophil release is a well-recognized phenomenon.

It is likely that the number of receptors on the surface of the cells plays an important role in this process. Studies suggest that FcγRIIb expression is altered by a polymorphic promoter for human FcγRIIb,[16, 17] FcγRIIb expression on germinal center B cells in mice can be altered by older follicular dendritic cells,[18] and other studies show donor variability of FcγRII isoform expression in human monocytes,[19] platelets,[20] and natural killer cells.[21] Thus the expression of IgE and IgG receptors on human basophils and especially FcγRIIb and FcγRIIa expression on basophils from donors in whom no inhibition was observed are a likely factor in the different donor cell degranulation responses.

In addition to measuring histamine release from human basophils *in vitro*, we have measured the effects of GE2 on PCA in FcεRIα-transgenic mice. The mast cells in these transgenic mice bind the human Fcε of the GE proteins through their human FcεRIα chain while the murine FcγRIIb will bind the human Fcγ portion. E2G appeared to be equally as effective as GE2 in its ability to block PCA reactivity, whereas the GE S mutant and GE H mutant required higher doses of GE protein to completely block PCA reactivity. These results suggest that the mutations intended to increase binding to the FcγRIIb receptor might negatively affect ITIM signaling. High concentrations of nonspecific IgE were unable to block PCA reactivity, indicating that the GE proteins block FcεRI signaling specifically and not by competition with IgE for receptor.

In our GE protein preparations the small percentage of high-molecular-weight material appeared to represent denaturation of protein during the acidic elution from the protein A-sepharose column that did not properly refold on neutralization. Purified E2G monomer, which was able to block PCA reactivity at lower doses of protein (Fig 5), was more potent than the original E2G preparation. Although size exclusion chromatography–purified protein showed greater PCA activity, the differences did not appear to have a large affect on the comparison of the GE proteins. The GE2 and GE S mutant preparations contained the highest fraction of aggregate, suggesting that they would have the largest shift in effectiveness; however, the binding and inhibitory activity of the GE S mutant was significantly less than that of GE2. The GE H mutant and E2G preparations contained much less aggregate, yet did not perform dramatically better than GE2.

Overall, we have found that the GE2 and E2G constructs were the most effective inhibitors of IgE-mediated degranulation and showed the highest binding to a basophil-like cell line. Although the Fcγ and Fcε binding sites appeared to be functional in both 5′ and 3′ positions in the molecule, the E2G structure theoretically would have an advantage over GE2 in terms of the potential side effects derived from the potentially immunogenic linker sequences because the artificial synthetic linker sequence in GE2 was replaced by the natural IgG hinge region in E2G. Therefore E2G not only lacks the artificial linker sequence but also only has 1 fusion junction instead of 2 fusion junctions in GE2. For the GE S and GE H mutants, the lower Ku812 binding and decreased inhibition of histamine release compared with that of GE2 and E2G suggest that the serine residues at position 267 and the histidine residue at position 268 in the C_H2 domain of IgG1 are important in ITIM signaling. Amino acid substitutions, which have been shown to increase binding to the FcγRII receptors, decreased the effectiveness of the inhibitory properties of the GE molecules. It is possible that slightly lower affinity binding is critical for co–cross-linking of FcγRII and FcεRI receptors and necessary for ITIM signaling. Cell-surface movement of the receptors might be restricted, or receptor recycling could be affected as well.

In conclusion, given that GE2 and E2G are the most effective inhibitors of the 4 molecules, it would be beneficial for further drug development to consider both GE2 and E2G molecules as viable therapeutic options because the immunogenicity of the $(Gly_4Ser)_3$ flexible linker has not been determined.

We thank Tetsuya Terada, Michael J. Whitekus, and XuWei Yang for breeding and genotyping the transgenic mouse colony and for technical assistance and Biogen Idec for their discussions and financial support.

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