# Modifications to an Fcγ-Fcε fusion protein alter its effectiveness in the inhibition of FcεRI-mediated functions

**Lisa Chan Allen, PhD,[a] Christopher L. Kepley, PhD, MBA,[b] Andrew Saxon, MD,[a] and Ke Zhang, MD, PhD[a]** *Los Angeles, Calif, and Richmond, Va*

**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.**
**(J Allergy Clin Immunol 2007;120:462-8.)**

***Key words:*** *GE2, IgE, IgG, FcεRI, FcγRIIb, allergy*

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

From [a]the Hart and Louise Lyon Laboratory, Division of Clinical Immunology/Allergy, David Geffen School of Medicine of the University of California, Los Angeles, and [b]the Virginia Commonwealth University Health Systems, Richmond.

Supported by grants from the American Lung Association, Biogen Idec, DHSS USPHS AI-15251, and the Food Allergy and Anaphylaxis Network.
Disclosure of potential conflict of interest: L. C. Allen has patent licensing arrangements with UCLA and has received grant support from Biogen Idec. A. Saxon has patent licensing arrangements with the University of California on the GE2 platform and has received grant support from the National Institutes of Health (NIH). K. Zhang has patent licensing arrangements with the University of California on the GE2 platform and has received grant support from the NIH. C. L. Kepley has declare that he has no conflict of interest.
Received for publication September 7, 2006; revised February 19, 2007; accepted for publication April 9, 2007.
Available online June 4, 2007.
Reprint requests: Zhang Ke, MD, PhD, Hart and Louise Lyon Laboratory, Division of Clinical Immunology/Allergy, David Geffen School of Medicine of the University of California, Los Angeles, Los Angeles, CA 90095. E-mail: kzhang@mednet.ucla.edu.
0091-6749/$32.00

doi:10.1016/j.jaci.2007.04.019

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

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.

## 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′-GGCCAGATCTGAGCCCAAATCTTGT-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′-AAGCTTGATATCCACCATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCCAGGTTCCACTGGTGAC-3′ and 5′-TCCAGGTTCCACTGGTGACTTCACCCCGCCCACCGTGAAGATTTTACAGTCGTCCTGCGACGGC-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).

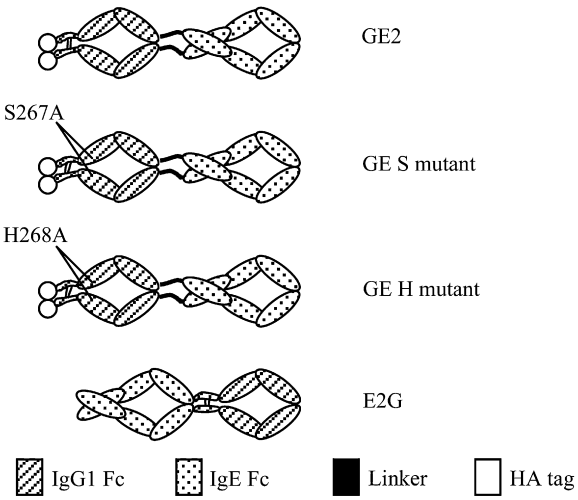


**FIG 1.** Schematic diagram of human GE proteins. The molecules are depicted with the N-terminus on the *left* and the C-terminus on the *right*. The Fcγ domains (γ hinge-Cγ2-Cγ3) are *striped,* the Fcε domains (Cε2-Cε3-Cε4) are *dotted,* the flexible linker is *black,* and the hemagglutinin tag is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

### Protein expression and purification

The linearized plasmid DNA (5 μg) was transfected by means of electroporation into 2 to $4 \times 10^7$ 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 filtration on 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, $10^6$ cells 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.