Inhibition of allergen-specific IgE reactivity by a human Ig Fcgamma-Fcepsilon bifunctional fusion protein

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Abstract:

Background: Coaggregating FcεRI with FcγRII receptors holds great potential for treatment of IgE-mediated disease by inhibiting FcεRI signaling. We have previously shown that an Fcγ-Fcε fusion protein, human IgG-IgE Fc fusion protein (GE2), could inhibit FcεRI-mediated mediator releases *in vitro* and *in vivo*.
Objective: We sought to test whether GE2 was capable of blocking mediator release from FcεRI cells sensitized with IgE *in vivo* or *in vitro* before exposure to GE2, a critical feature for GE2 to be clinically applicable.
Methods: GE2 was tested for its ability to inhibit Fel d 1–induced mediator release from human blood basophils from subjects with cat allergy, human lung-derived mast cells, human FcεRIα transgenic mice sensitized with human cat allergic serum, and rhesus monkeys naturally allergic to the dust mite *Dermatophagoides farinae*.
Results: Basophils from subjects with cat allergy and lung mast cells degranulate when challenged with Fel d 1 and anti-IgE, respectively. GE2 itself did not induce mediator release but strongly blocked this Fel d 1– and anti-IgE–driven mediator release. GE2 was able to block Fel d 1–driven passive cutaneous anaphylaxis at skin sites sensitized with human serum from subjects with cat allergy in human FcεRIα transgenic mice, but by itself, GE2 did not induce a passive cutaneous anaphylaxis reaction. Finally, GE2 markedly inhibited skin test reactivity to *D farinae* in monkeys naturally allergic to this allergen, with complete inhibition being observed at 125 ng.
Conclusion: GE2 is able to successfully compete for FcεRs and FcγRs on cells presensitized *in vitro* and *in vivo* and lead to inhibition of IgE-mediated reactivity through coaggregation of FcεRI with FcγRII.

Keywords: Allergen | IgE | FcεRI | anaphylaxis | fusion protein | immunotherapy | degranulation | mast cells | basophils | transgenic mouse

Article:

Abbreviations
GE2: Human IgG-IgE Fc fusion protein
NP: 4-Hydroxy-3-nitrophenylacetyl
PCA: Passive cutaneous anaphylaxis
PS: Purified human IgE myeloma protein

The importance of immediate hypersensitive diseases, including those affecting the airways, and severe food-induced reactions has become increasingly appreciated in the past decade. Traditional immunotherapy with increasing doses of injected allergen has proved effective, particularly in upper airway allergy. However, it is very labor intensive, is associated with significant adverse events, has shown variable efficacy in lower airway allergic disease (asthma), and has proved unsatisfactory for severe food allergy.[1] As a result, a variety of new immunologically based approaches are under development to treat these conditions. Such approaches include anti-IgE therapy,[2] antibodies directed to circulating cytokines believed to be key in the genesis of these allergic reactions (eg, anti-IL-4, anti-IL-5, and anti-IL-13),[3] antibodies directed to surface molecules (anti-CD23),[4]and new forms of immunotherapy, including peptide-based treatments[5]and immunotherapy with novel adjuvants (eg, CpG).[6]

We have developed and tested a novel Fcγ:Fcε human bifunctional Ig fusion protein, human IgG-IgE Fc fusion protein (GE2), designed to inhibit basophils, mast cells, and B cells by cocrosslinking FcεRI or FcγRII (CD23) with FcγRs (Fig 1).[7., 8., 9.] However, in those previous experiments, we showed that GE2 inhibits mast cell, basophil, and Langerhans-like cell function under conditions in which the cells *in vitro* or *in vivo* had been artificially sensitized with a humanized IgE mAb.[7., 8.] In the present study we extended the testing of GE2's function to the setting of naturally sensitized nonhuman primates, human basophils and mast cells, and humanized FcεRI cells in transgenic mice. We now report the results of this series of approaches, testing the ability of GE2 to block release from cells that have been sensitized *in vivo* before GE2 administration, an ability GE2 must possess if it is to be successful as a therapy in allergic subjects.

1. Methods

1.1. Allergy skin test reagents

Dermatophagoides farinae, Dermatophagoides pteronyssinus, and histamine were obtained from Allermed Laboratories. For screening skin testing, *D farinae* and *D pteronyssinus* were both used at 10,000 AU/mL, and histamine was used at 6 mg/mL. Multitest devices were obtained from Lincoln Diagnostics. A purified human IgE myeloma protein (PS) was used as an IgE control. PS IgE was purified from serum provided as a gift from Dr Ross McIntyre at Dartmouth Medical School.

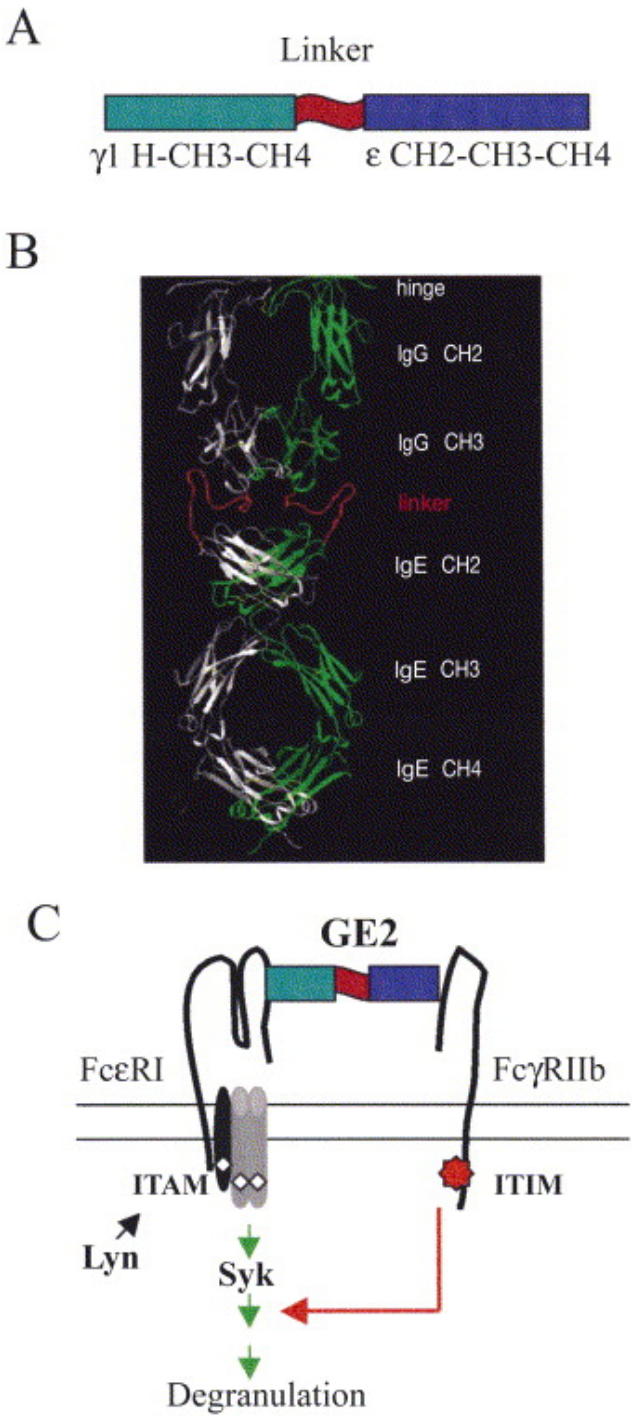

Fig 1. Diagram of GE2 fusion protein and the proposed mechanism by which it inhibits FcεRI-mediated functions. **A**, Schematic diagram of GE2 construct. **B**, Ribbon drawing for the 3-dimensional structure of the dimerized GE2. **C**, Proposed mechanism by which GE2 inhibits FcεRI-mediated degranulation.[7] The tetrameric αβγ2 FcεRI receptor normally is activated by crosslinking of the IgE-primed FcεRI with multivalent antigen, resulting in the activation of Lyn, which in turn phosphorylates ITAMs within the FcεRI and creates docking sites for Syk kinase family members that result in signal propagation, which culminates in mast cell activation and degranulation. GE2, by binding FcεRI and FcγRIIb as shown, cocrosslinks FcγRIIb with FcεRI, resulting in the blocking of IgE-dependent degranulation. This inhibition results from the ITIM activation in FcγRIIb, which leads to the interruption of Syk signaling.[7] *ITAM*, Immunoreceptor tyrosine-based activation motif; *ITIM*, immunoreceptor tyrosine-based inhibition motif.

1.2. Basophil purification

Basophils were obtained from donors who had positive skin test responses for cat dander (Fel d 1). Donors were taking no medications at the time of donation. Highly purified (>95%) basophils were obtained as described previously.[10]

1.3. Measurement of basophil degranulation

Purified basophils were washed, and GE2 was added (0-10 μg/mL) for 2 hours at 37°C in a 5% CO_2 incubator. The cells were centrifuged, and the supernatants were removed and stored at −70°C to later determine any prerelease of mediators. Cells were washed and activated in Dulbecco modified Eagle medium with 1% BSA, 1 mmol/L $MgSO_4$, and 1 mmol/L $CaCl_2$ with or without optimal concentrations of Fel d 1 (100 ng/mL, Indoor Biotechnologies). As a control, PS IgE was substituted for GE2. After 30 minutes, the supernatant was removed for histamine analysis, as described previously.[11., 12.]

1.4. Lung tissue mast cell release

Fresh human lung tissue chunks of approximately the same size (0.15 g) were washed 4 times with RPMI plus 2% FCS/Pen-strep. Tissue was incubated with GE2, PS IgE, or nonspecific human IgG for 2 hours in the same media at 37°C. Cells were washed 1 time and challenged for 45 minutes with anti-human IgE (3 μg/mL) in Tyrodes buffer. The spontaneous release group received only medium and no anti-IgE. Tissue was centrifuged at 4°C, and the supernatant was removed and stored at −70°C. Fresh medium was added, freeze-thawed 4 times, and centrifuged at 5000*g* to remove tissue debris for the purpose of determining the total amount of β-hexosamine in the tissue. The supernatants were used to calculate the total levels of β-hexosamine remaining in the tissue after FcεRI challenge.

1.5. Dust mite allergy testing of rhesus monkeys

Monkeys were sedated with ketamine and then tested on the abdominal skin at 4 sites with a MultiTest device. Tests consisted of *D farinae* and *D pteronyssinus* at 10,000 AU/mL, histamine (6 mg/mL), and saline. Monkeys were scored positive if they showed a wheal equal to or larger than 1 cm to *D pteronyssinus* or *D farinae* and to histamine. Reactivity in the animals on this epicutaneous testing was clearly distinct from that observed in human subjects. The histamine and antigen reactions were marked by areas of wheals that were white, with a lack of any surrounding erythema. Of 44 animals screened, 5 had negative histamine reactions, and 1 animal showed areas of confluent wheals and was judged to be of indeterminate status. Thus of the 38 animals evaluable, 12 were considered to have positive results to *D farinae* (a frequency of 32% positivity). None of the monkeys showed reactivity to *D pteronyssinus*. Seven of the monkeys with positive results then underwent graded intradermal dose testing on the abdomen with 0.05 mL of *D farinae* ranging from 5 to 500 AU/mL. A dose of *D farinae* causing midlevel reactivity was thereby established for each animal (Table I). All procedures involving monkeys were performed at the Sabana Seca Station of the Caribbean Primate Research Center, University of Puerto Rico.

Table I. Summary of inhibition of skin test results

Animal no.	Treatment								
	GE2, 250 ng	PS, 250 ng	GE2, 125 ng	PS, 125 ng	GE2, 62.5 ng	PS, 62.5 ng	*D farinae*(dose)*	Histamine	Saline
	Diameter of bluing reaction (mm)								
R80	0	10	0	16	3	14	12 (50)	14	0
M221	0	6	0	10	0	13	11 (100)	12	0
M23A	0	8	2	9	3	12	10 (50)	12	0
LO6	0	10	0	12	7	13	9 (100)	11	0
I070	0	8	0	14	16	18	17 (25)	22	0
Mean (SD), mm	0.0† (0)	8.4 (1.3)	0.4†(0.9)	12.2 (2.8)	5.8‡(5.6)	14.0 (2.1)	10.4 (3.1)	14.2 (4.0)	0

∗Intradermal challenge dose individualized for animal.
†$P < .0001$ compared with their corresponding PS IgE.
‡$P < .01$ compared with their corresponding PS IgE.

1.6. Inhibition of *D farinae* skin reactivity by GE2 in rhesus monkeys

One month after the initial allergy testing, 5 monkeys previously determined to be sensitive to dust mite allergen were administered intradermal injections to the abdomen of 0.05 mL of increasing concentrations of GE2, or control myeloma human IgE protein PS saline was administered at control sites. Five hours later, the animals were administered 5 mL of 0.5% Evans Blue dye intravenously, and the abdominal skin test sites were challenged with saline (negative control), histamine (positive control), or the dose of *D farinae* selected from the earlier dose-response testing. Sites were photographed 30 minutes later, and the largest diameter of bluing was measured. Differences were examined by using the Student *t* test.

1.7. Passive cutaneous anaphylaxis in human FcεRIα chain transgenic mice

The human FcεRIα chain transgenic mice (kindly provided by Dr Jean-Pierre Kinet, Harvard Medical School, Boston, Mass)[13., 14.] were intradermally injected with 50 μL of 1:5 diluted serum from a patient with cat allergy (no. 9632, Plasmalab) to sensitize the skin mast cell sites. Four hours later, these same sites were injected with GE2 or control materials. A further 4 hours later, the mice were challenged intravenously with 10 μg of purified native Fel d 1 antigen plus 1% Evan blue in a volume of 200 μL. Mice were killed 30 minutes after the intravenous challenge.

1.8. Statistical analysis

Data were analyzed by using *t* tests with the Welch correction to calculate the statistical significance between different treatments versus control. *P* values of .05 or less were considered significant. Statistics were performed with GraphPad Prism.

1.9. Protection of research subjects

The testing of rhesus Monkeys was approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico and the UCLA Animal Research Committee. The murine procedures (passive cutaneous anaphylaxis [PCA]) were approved by the UCLA Animal Research Committee. All the studies involved in human mast cells and basophils were approved by the Human Studies Committees at Virginia Commonwealth University Health Systems.

2. Results

2.1. GE2 inhibits allergen-induced degranulation from basophils and lung mast cells sensitized *in vivo*

We have previously shown that GE2 inhibited IgE-mediated reactivity in FcεRI-bearing cells passively sensitized with 4-hydroxy-3-nitrophenylacetyl (NP)–IgE.[7., 8.] Here we tested whether GE2 could inhibit degranulation of basophils naturally sensitized to Fel d 1 *in vivo* by using cells obtained from donors with cat allergy. Basophil histamine release was inhibited by GE2 in a dose-dependent fashion (n = 3, Fig 2), with up to 78% inhibition at 10 μg/mL GE2 (P = .03) when cells were challenged with Fel d 1 (100 ng/mL). This inhibition of degranulation did not result simply from competition between GE2 and Fel d 1–specific IgE because cells treated with nonspecific myeloma IgE (PS) responded to antigen as robustly as cells incubated in medium only. These results demonstrate that GE2 can inhibit mediator release from basophils naturally sensitized *in vivo* with Fel d 1–specific IgE. Importantly, GE2 alone, at all concentration tested, failed to trigger release, indicating that GE2 itself would not cause degranulation (Fig 2).

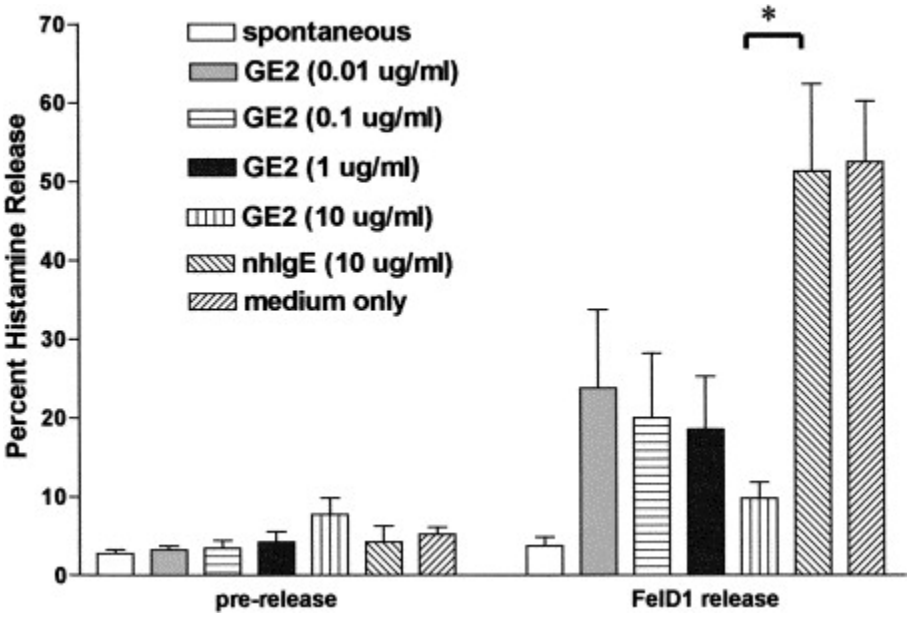

Fig 2. GE2 inhibits FcεRI-mediated degranulation from basophils obtained from donors with cat allergy. Basophils were incubated with or without GE2 or nonspecific control IgE (PS) in Iscove medium at 37°C. The cells were incubated with or without 100 ng/mL Fel d 1 for 30 minutes, and mediator release was measured in the supernatants. Results are represented as the SEM of 3 experiments from 3 separate donors (± SEM). The *asterisk* indicates a statistically significant P value of .03.

We also examined the ability of GE2 to inhibit anti-IgE–driven mast cell release in human lung tissue that had bound IgE *in vivo*. Anti-IgE–driven mediator release from lung fragments was inhibited in a dose-dependent fashion, with almost complete inhibition of the induced release occurring with *ex vivo* treatment of the lung fragments at 50 μg/mL GE2 (P = .04, Fig 3). Furthermore the FcεRI dependence of this inhibition was shown because GE2-treated mast cells still could be triggered by calcium ionophore, which is known to function through an IgE-independent pathway (data not shown).

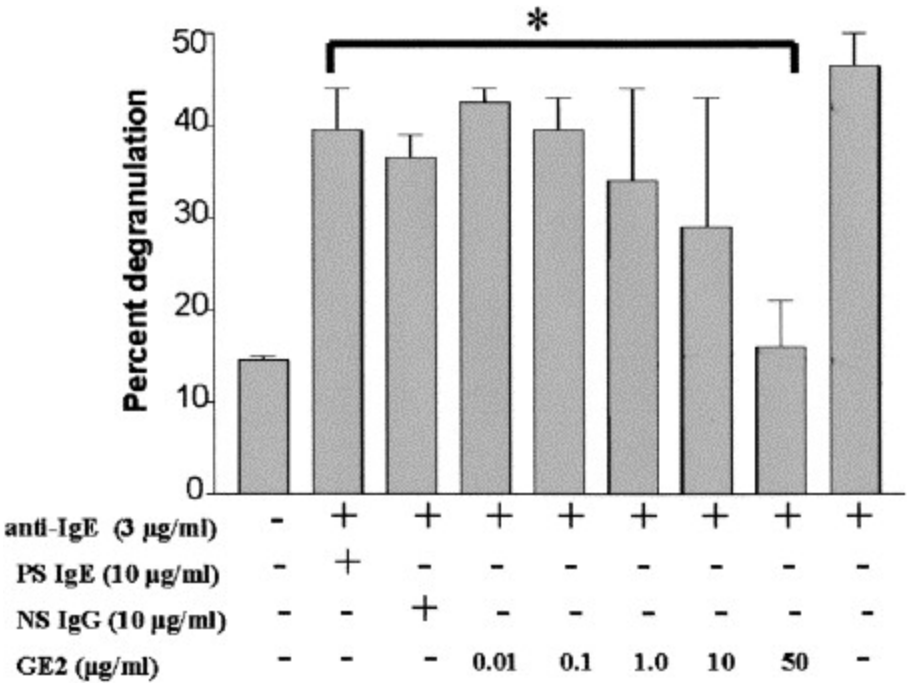

Fig 3. GE2 inhibits FcεRI-mediated degranulation from tissue mast cells. Mast cell–containing lung chunks were incubated with or without GE2 or nonspecific IgE (PS) or IgG in Iscove medium at 37°C. The tissue chunks were incubated with or without 1 µg/mL anti-IgE for 45 minutes, and mediator release was measured in the supernatants. Fresh medium was added back to the tissue, which was freeze-thawed 3 times and centrifuged to obtain the total amount of histamine. Results are represented as the SEM of 2 experiments (± SEM). The *asterisk* indicates a statistically significant *P* value of .04.

2.2. GE2 blocks PCA reactivity in human FcεRIα chain transgenic mice presensitized with serum from a patient with cat allergy

Although we have previously shown that GE2 is able to compete with coadministered monoclonal human IgE to block PCA reactivity in transgenic mice,[7] we had not shown whether cells presensitized *in vivo* in transgenic mice could be inhibited by the subsequent administration of GE2. To test this, transgenic mice were locally sensitized at individual skin sites with serum from a subject highly allergic to cat. Four hours later, GE2 or control material was administered to these sites, and after a further 4 hours, the mice were then challenged intravenously with Fel d 1.

As shown in Fig 4, serum from a patient with cat allergy (no. 9632) was able to produce strong PCA reactivity in human FcεRIα transgenic mice on Fel d 1 challenge (Fig 4, *A*). Treatment of the serum from a patient with cat allergy at 56°C for 30 minutes, conditions known to abolish IgE binding to FcεRI but not IgG binding to its corresponding receptors, abolished PCA reactivity (Fig 4, *G*), showing that Fel d 1–specific IgE, but not specific IgG, was responsible for the PCA reactivity observed. These results indicated that mast cell degranulation was triggered by Fel d 1–specific IgE and showed that the human FcεRIα transgenic mice could be used as an *in vivo*model of human allergic reactivity. PCA reactivity was inhibited in a dose-dependent manner by GE2 protein injected 4 hours later, with complete inhibition occurring at 2 µg (Fig 4, *B-D*). Reactivity was not blocked with administration of equal amounts of human IgE or human IgG with this protocol (data not shown). Purified myeloma IgE (PS IgE), NP-specific

IgE, and nonallergic human serum (Fig 4, *E, F*, and *H*) were not able to function as sensitizing reagents in place of the serum from a patient with cat allergy, showing that the PCA reaction was mediated by anti-Fel d 1–specific IgE. Additionally, when an intravenous challenge of Fel d 1 was given immediately after local administration of GE2, there was no local reaction (data not shown), demonstrating that GE2 itself is not causing release at the injection sites.

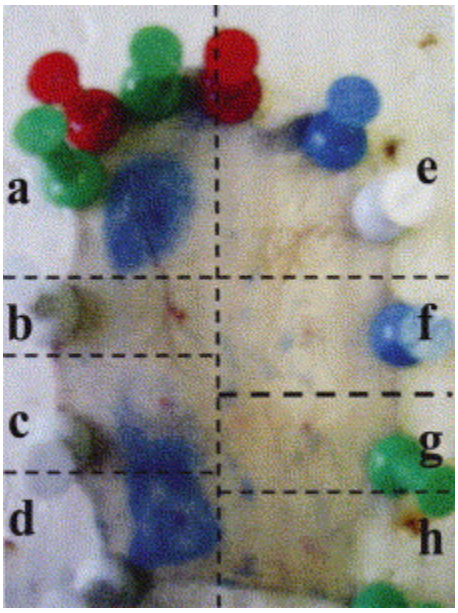

Fig 4. GE2 inhibits Fel d 1–specific IgE-mediated mast cell degranulation *in vivo*. The skin on the back of FcεRIα transgenic mice was injected with serum (9632) from a patient with cat allergy (1:5 dilution; *a*); serum with 2 μg of GE2 administered 4 hours later *(b)*; serum with 0.2 μg of GE2 *(c)*; serum with 0.02 μg of GE2 *(d)*; myeloma IgE (PS, 250 ng; *e*); NP-specific IgE (250 ng; *f*); heat-inactivated serum 9632 (56°C for 30 minutes; *g*); or nonallergic serum *(h)*. After another 4 hours, the mice were challenged intravenously with 10 μg of purified Fel d 1 plus 1% Evans blue dyein 200 μL and killed 30 minutes after challenge. The results are representative of 3 experiments with similar results.

2.3. GE2 inhibits naturally occurring skin test reactivity to dust mite in rhesus monkeys

Rhesus monkeys have been reported to express skin test reactivity and serum IgE directed toward dust mites.[15] We therefore undertook to determine whether GE2 could inhibit skin test reactivity in rhesus monkeys. *D farinae*–reactive monkeys were first screened and identified. *D farinae*–reactive monkeys then underwent graded inhibition testing with GE2 (62.5-250 ng) versus purified human IgE myeloma protein (PS) as a control. This control was necessary because some inhibition from IgE alone (or the IgE portion of GE2) might be expected. Five hours after the test proteins or saline were injected, the sites were then challenged with *D farinae* at a dose individualized for each animal (Table I). GE2 protein effected complete inhibition at 250 ng and showed complete inhibition in 4 of 5 animals at 125 ng (Fig 5 and Table I). At 62.5 ng, GE2 still exhibited clear inhibition of reactivity compared with saline or control (Fig 5). In contrast, PS IgE did not show significant inhibitory effects on skin test reactions in any of the doses tested (Fig 5). These results clearly indicate that GE2 protein is able to inhibit naturally occurring dust mite allergen–induced allergic skin reactivity in nonhuman primates in a dose-dependent fashion.

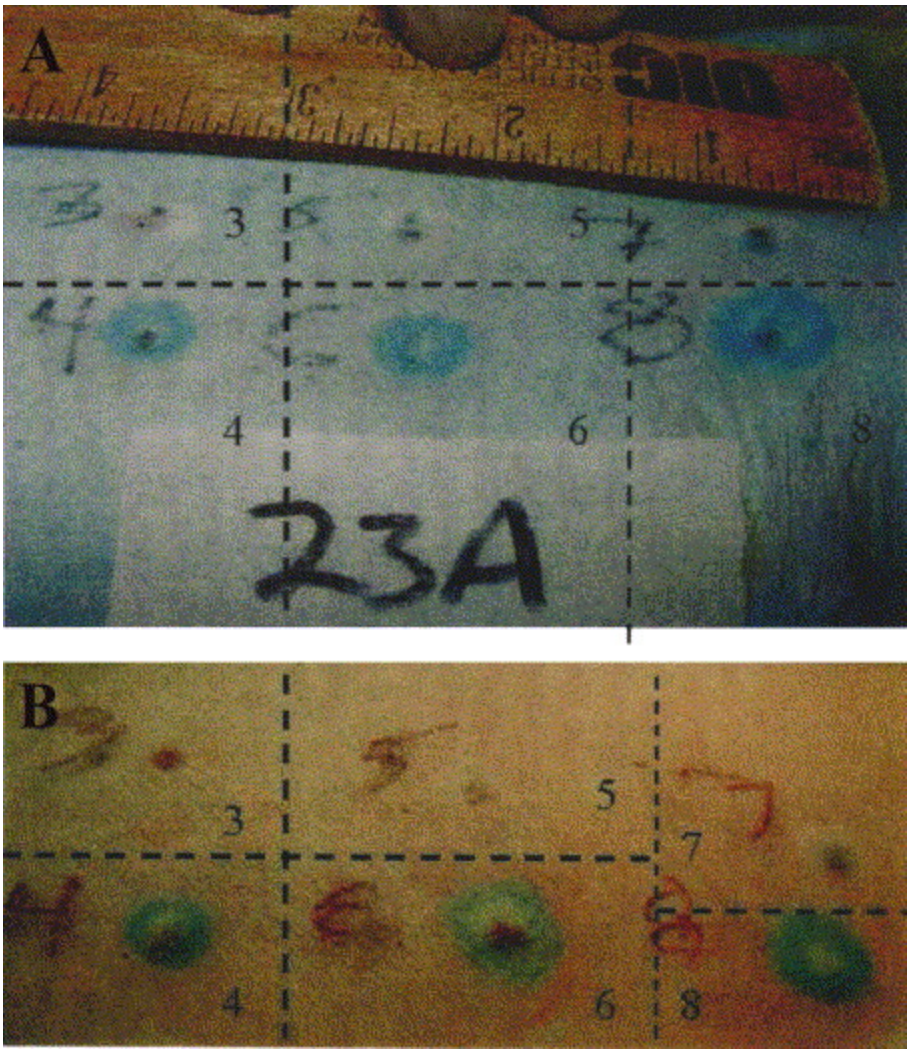

Fig 5. GE2 inhibits dust mite–induced mast cell degranulation *in vivo* in rhesus monkeys. Sites 3, 5, and 7 were injected with 250, 125, and 62.5 ng of GE2, whereas sites 4, 6, and 8 were injected with 250, 125, and 62.5 ng of control IgE (PS IgE), respectively. Five hours later, 5 mL of 0.5% Evans blue dye was injected intravenously, and the GE2 and PS IgE-injected spots were challenged with a predetermined dose of *D farinae* allergen. The skin reaction was recorded 30 minutes after challenge. **A**, Skin test results from monkey M23A. **B**, Skin test results from monkey R80. The results are representatives of the 5 monkeys, the results of which are shown in Table I.

3. Discussion

Mast cells and basophils are the primary cell types responsible for initiating the cascade of IgE-mediated allergic inflammation that results in various signs and symptoms of allergic diseases. On allergen-driven crosslinking, IgE bound to its high affinity receptor, FcεRI, expressed on mast cells, basophils, or both, triggers degranulation and cytokine production. Recent studies have revealed that mast cells also might play a key role in autoimmune disease.[16] Thus mast cells also appear to be important in inflammatory arthritis,[17] experimental allergic encephalomyelitis,[18] multiple sclerosis,[19., 20., 21.] and certain type of autoimmune skin disease, such as bullous pemphigoid.[22] Activation and subsequent degranulation of mast cells through FcεRI crosslinking (in both human subjects and mice), FcγRIII (in mice), or both are thought to be the critical steps for these processes.[16] Therefore inhibition of mast cell activation,

degranulation, and cytokine production provides for a potential therapeutic target for the prevention, treatment, or both of allergic disorders, as well as autoimmune diseases.

The previously constructed GE2 was shown to inhibit FcεRI-mediated functions of basophils, mast cells, and Langerhans-like dendritic cells *in vitro* and *in vivo*.[7., 8.] This activity was shown to be related to the ability of GE2 to coaggregate FcεRs and FcγRs (Fig 1). However, in those experiments the purified humanized IgE mAb (anti-NIP or anti-dansyl IgE) was used in the experiments to trigger IgE-dependent degranulation or mediator release. Whether GE2 would be able to block (1) human naturally produced allergen-specific IgE-derived reactivity and (2) reactivity in which the target cells had been presensitized by allergen-specific IgE *in vivo* remained to be tested. These questions were necessary to test the potential clinical relevance of GE2 for the inhibition of FcεRI-mediated reactivity signaling.

In this report we approached the ability of GE2 to block already sensitized cells by using a variety of experimental systems. The mediator release of the Fel d 1–specific IgE-presensitized basophils from subjects highly allergic to cats and the lung mast cells loaded with IgE *in vivo* was significantly inhibited by GE2. GE2 also exhibited strong inhibitory effects on sera from sensitized patients with cat allergy, naturally occurring cat allergen Fel d 1–induced allergic degranulation of mast cells in FcεRIα transgenic mice, and on naturally occurring dust mite allergen *D farinae*–mediated allergic skin reactions in rhesus monkeys. These results, obtained from the more clinically relevant settings in contrast to the use of artificial human IgE that was used in the previous experiments,[7., 8.] further validate the potential of GE2 as a therapeutic approach to treat IgE-mediated allergic diseases. Given that GE2 is able to inhibit degranulation of FcεRI-bearing cells triggered by NP-, Dansyl-, Fel d 1–, and dust mite-specific IgE in various experimental systems (and this report),[7., 8.] it is evident that GE2 functions to broadly inhibit degranulation through a nonallergen-specific fashion. Such a feature provides the basis for GE2 as a general inhibitor for IgE-mediated, but not limited to allergen-specific, allergic reactions.

The allergic reaction to dust mite allergen *D farinae* in monkeys provided us a more clinically relevant naturally occurring experimental system to test GE2's efficacy to inhibit an allergen-specific allergic response. Allergic skin reactions were strongly inhibited by GE2 but not by equal amounts of nonspecific IgE, indicating that the inhibitory effects of GE2 were due to the specific property of GE2 and not simply an Fcε effect. Clearly, mere competition for occupancy of FcεRI by Fcε was not sufficient to inhibit the allergen-induced skin reaction. This supports our molecular studies showing that GE2 exerts its strong inhibitory effects through negative signaling of FcεRI-FcγRIIb coaggregation.[7] This *in vivo* result is consistent with our previous *in vitro* observation that GE2 was able to prevent the phenotypic change of degranulation of mast cells (Kepley at al, unpublished observation).

Taken together, the data demonstrate that GE2 functions in a nonallergen-specific manner to inhibit IgE-dependent allergic reactions *in vitro* and *in vivo*, including artificially and naturally sensitized basophils and mast cells, including in nonhuman primates and transgenic mice expressing humanized FcεRI cells. In addition, other studies have shown that GE2 inhibits B-cell IgE production and human Langerhans cell proallergic activity.[8., 9.] It should be noted that all the tests conducted thus for were either experiments *in vitro* or local tests *in vivo*. The effects of GE2 on inhibiting systemic allergic responses, such as asthma, should be tested when an appropriate

animal model is available. Overall, these studies provide strong support for the next step of initiating human clinical trails with the GE2 therapeutic platform.

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