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# Systemic administration of an Fc $\gamma$ –Fc $\epsilon$ -fusion protein in house dust mite sensitive nonhuman primates<sup>☆</sup>

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## KEYWORDS

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**Abstract** Crosslinking Fc $\epsilon$ RI and Fc $\gamma$ RIIB receptors inhibits mast cell and basophil activation, decreasing mediator release. In this study, a fusion protein incorporating human Fc $\gamma$  and Fc $\epsilon$  domains, hGE2, was shown to inhibit degranulation of human mast cells and basophils, and to exhibit efficacy in a nonhuman primate model of allergic asthma. hGE2 increased the provocative concentration of dust mite aeroallergen that induced an early phase asthmatic response. The treatment effect lasted up to 4 weeks and was associated with reduction in the number of circulating basophils and decreased expression of Fc $\epsilon$ RI on repopulating basophils. Repeat hGE2 dosing induced production of serum antibodies against human Fc $\gamma$  and Fc $\epsilon$  domains and acute anaphylaxis-like reactions. Immune serum induced histamine release from human IgE or hGE2-treated cord blood-derived mast cells and basophils *in vitro*. These results indicate that repeat administration with hGE2 induced an antibody response to the human molecule that resulted in activation rather than inhibition of allergic responses.

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**Abbreviations:** ANOVA, Analysis of variance; AU, Allergy units; b-IgE, Biotinylated chimeric mouse/human IgE; ns-IgE, Non-specific human IgE; CBDMC, Cord blood-derived mast cells; CBDB, Cord blood-derived basophils; cDNA, Complementary deoxyribonucleic acid;  $C_{dyn}$ , Dynamic compliance;  $C_{max}$ , Maximum serum concentrations; Dp, *Dermatophagoides pteronyssinus*; Df, *Dermatophagoides farinae*; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunoassay; Fc $\epsilon$ , Fragment crystallizable of immunoglobulin subclass E (C $\epsilon$ 2–C $\epsilon$ 3–C $\epsilon$ 4 domains); Fc $\epsilon$ RI, Receptor subclass I for the Fc region of immunoglobulin subclass E; Fc $\gamma$ , Fragment crystallizable of immunoglobulin subclass G (C $\gamma$ 2–C $\gamma$ 3 domains); Fc $\gamma$ RIIB, Receptor subclass IIB for the Fc region of immunoglobulin subclass G; Fisher's LSD, Fisher's test for least significant difference; HBSS, Hank's balanced salt solution, HDM, House dust mite; HRP, horseradish peroxidase; IgE, Immunoglobulin subclass E; IgG, Immunoglobulin subclass G; IL-4, Interleukin 4; IL-13, Interleukin 13; ITIM, Immunoreceptor tyrosine-based inhibitory motif;  $K_D$ , Dissociation constant; PBS, Phosphate-buffered saline; PE, Phycoerythrin;  $R_L$ , Lung resistance; RR, Respiratory rate;  $T_{max}$ , Time to reach  $C_{max}$ ; TNP, Trinitrophenyl; TV, Tidal volume;  $t_{1/2}$ , half-life.

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## Introduction

Allergic asthma manifests as immune reactivity to inhaled antigens that progresses to chronic inflammation, remodeling of the airway wall, and reoccurring airway obstruction. In sensitized individuals, allergen exposure induces mediator release and responses from diverse cells types, including mast cells, basophils, eosinophils, epithelial cells, fibroblasts, and smooth muscle cells [1]. While the pathogenesis of allergic asthma is not completely understood, IgE binding to its cognate receptor, FcεRI, is involved, and down-regulation of FcεRI activation and signaling is an effective therapeutic strategy.

The IgE Fc has a high affinity for FcεRI ( $K_D \sim 10^{-10}$  M), a hetero-tetrameric receptor ( $\alpha\beta\gamma_2$ ) expressed on mast cells and basophils [2,3]. IgE has a short *in vivo* half-life in the circulation, but the half-life of IgE bound to FcεRI on mast cells and basophils is thought to be at least 2 weeks [4]. Thus, a transient increase in circulating IgE can lead to prolonged priming of these cells. Antigen-dependent aggregation of FcεRI via bound IgE results in activation and recruitment of protein tyrosine kinases, including Lyn, Syk, and Fyn, to phosphorylated immunoreceptor tyrosine-based activation motifs (ITAM) in the cytoplasmic domains of the receptor  $\beta$  and  $\gamma$  chains [2,3]. In contrast to the excitatory signaling subsequent to IgE-dependent cross-linking of FcεRI, aggregation of FcεRI with inhibitory Fcγ receptors on the same cell can lead to inhibitory signaling. In mast cells, the inhibition is mediated primarily by FcγRIIB, that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic domain [5,6]. Co-aggregation of FcεRI with FcγRIIB results in the phosphorylation of the ITIM on FcγRIIB and subsequent recruitment of inhibitory phosphatases, followed by suppression of the FcεRI signaling cascade and inhibition of degranulation [5,6]. In allergic patients antigen desensitization treatment is thought to induce IgG responses to allergens that, in part, result in IgG complexes that indirectly co-engage FcεRI and FcγRIIB [7,8].

In 2002, Saxon and colleagues designed a chimeric protein, GE2, to directly cross-link FcεRI and FcγRIIB and thereby down-regulate basophil and mast cell activation [9]. GE2 was comprised of the Fc region of human IgG1 (hinge-Cγ2–Cγ3) linked to the Fc region of human IgE (Cε2–Cε3–Cε4) [9]. Consistent with its design, GE2 inhibited basophil and mast cell degranulation, passive cutaneous anaphylaxis, skin wheal and flare reactivity, and B cell switching to IgE production [9–11].

The present study evaluated the use of an Fcγ–Fcε fusion protein as a therapeutic in allergic asthma. A fusion protein, hGE2, was generated that was similar in structure to GE2, but lacked an epitope tag and several non-native amino acids that were present in the original molecule. Activity of hGE2 was confirmed *in vitro* and in localized skin reactions, and was subsequently administered systemically to cynomolgus macaques with well-documented sensitivity to aerosolized house dust mite (HDM) allergen [12]. Treatment with hGE2 inhibited the early phase reaction to aerosolized allergen for more than 4 weeks in a dose-dependent manner and reduced the number of circulating basophils. Repeat dosing was associated with a progressive immune response against both the Fcε and Fcγ portions of the molecule and acute anaphylactic-like reactions.

## Methods

### Animals

The animal model has been described in detail previously [12,13]. At the time of the study, the animals ranged from 4 to 8 years of age, weighed 2.5 to 7 kg, and exhibited a history of house dust mite (HDM) antigen sensitivity for more than 4 years. The animals were naive to human antibodies. Some of the animals had been treated previously with recombinant human lactoferrin. Animal husbandry was conducted according to the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press. Protocols were approved by the Institutional Animal Care and Use Committee of East Carolina University.

### hGE2

The bifunctional hGE2 molecule was assembled based on the human GE2 construct of Zhu et al. [9] in the pV90 vector (Biogen Idec). hGE2 did not contain epitope tags or non-native sequences, with the exception of the Gly/Ser linker. The C-terminal Lys residue from the human  $\gamma 1$  chain was deleted to guard against internal proteolytic cleavage. The 566 amino acid fusion protein contained a heavy chain leader sequence followed by residues 226–477 of human  $\gamma 1$  tethered to residues 248–610 of human  $\epsilon$  by a 15 amino acid Gly/Ser linker. The final construct was expressed in Chinese Hamster Ovary cells. Protein was purified from culture supernatants by affinity chromatography on Protein A Sepharose, hydrophobic interaction chromatography, and gel filtration on Superdex 200 (GE Healthcare) to remove aggregates. Pooled fractions were >99% monomer and contained less than 0.1 EU/mg of protein. The amino acid sequence, disulfide bond pattern, and glycan composition of hGE2 were confirmed by mass spectrometry. The lots of hGE2 in PBS used for this study ranged in concentration from 7 to 18.3 mg/ml. hGE2 was delivered subcutaneously as either a single dose, or two doses administered 24 h apart.

### Generation of cord blood-derived mast cells and basophils

Human basophils were differentiated from CD34<sup>+</sup> cord blood progenitors (AllCells, LLC, Emeryville, CA) by 4- to 5-weeks of culture in complete medium [RPMI 1640 (ATCC, Manassas, VA), 10% FBS (HyClone, Logan, UT), 10  $\mu$ g/ml gentamicin (Sigma-Aldrich, Saint Louis, MO), 55  $\mu$ M  $\beta$ -mercaptoethanol (Invitrogen, Inc., Carlsbad, CA)] containing recombinant human IL-3 (1 ng/ml) and recombinant human TGF- $\beta 1$  (10 ng/ml; both from R&D Systems, Minneapolis, MN). Mast cells were differentiated from CD34<sup>+</sup> cord blood cells for 9- to 12-weeks of culture in complete medium supplemented with recombinant human IL-6 and stem cell factor (50 and 100 ng/ml, respectively; R&D Systems). Basophils were >98% pure. Mast cells were enriched using anti-c-kit coupled magnetic beads (Miltenyi Biotec, Auburn, CA).

### *In vitro* histamine release assays

Cells ( $1 \times 10^5$  in 100  $\mu$ l) were sensitized at 37 °C with biotinylated chimeric mouse/human IgE (10  $\mu$ g/ml; JW8/5/

13; ECACC) for 2 days (basophils) or 3 to 4 days (mast cells) in the presence or absence of hGE2 or competitor IgE (ns-IgE). Incubation with IgE or hGE2 resulted in similar levels of Fc $\epsilon$ RI up-regulation (data not shown). After sensitization, cells were washed twice, resuspended in Hank's Balanced Salt Solution (HBSS) containing calcium (Invitrogen, Inc.) and challenged with either streptavidin (0.02  $\mu$ g/ml; Fisher Scientific, Pittsburgh, PA) or goat anti-human IgE ( $\epsilon$ -chain specific; 1  $\mu$ g/ml, Sigma-Aldrich) for 1 h at 37 °C. Histamine in the supernatants was analyzed by ELISA (Beckman Coulter, Brea, CA). Experiments were repeated at least three times with different preparations of basophils and mast cells. The percentage of histamine released was calculated as follows: (histamine released) / (total histamine released after the cells were heated at 100 °C for 6 min)  $\times$  100. In comparing different experiments and treatments the values for percentage histamine release obtained from IgE-sensitized and challenged cells were normalized to 100%.

### Pharmacokinetic analysis

Naive male cynomolgus macaques received a single subcutaneous dose (1, 10, or 30 mg/kg) of hGE2 in PBS. Serum samples were collected and levels of hGE2 quantified by ELISA as follows. Plates (96-well MaxiSorp, Nunc) were coated with mouse anti-human IgE (G7-18, 5  $\mu$ g/ml; BD Biosciences), serum samples applied in serial dilutions, and hGE2 detected with mouse anti-human IgG1 (hinge) -HRP conjugate (4E3, 1:10,000; SouthernBiotech). A standard curve was used to quantify hGE2 levels. The lower limit of quantification in the assay was 0.26  $\mu$ g/ml in undiluted serum. Pre-dose levels of circulating hGE2 were below the lower limit of quantification (<263 ng/ml). Data were analyzed using a noncompartmental analysis extravascular input model (WinNonlin Model 200, Pharsight). Individual concentrations and sampling times were used for calculation of maximum serum concentrations ( $C_{max}$ ), time to reach  $C_{max}$  ( $T_{max}$ ), clearance, volume of distribution, and circulating half-life ( $t_{1/2}$ ).

### Pulmonary function testing

Pulmonary function testing was performed as described in detail previously [13]. Animals were anesthetized with 2.0 mg/kg Telazol® and maintained on propofol (10 to 15 mg/kg/hr). Respiratory rate (RR), tidal volume (TV), dynamic compliance ( $C_{dyn}$ ) and lung resistance ( $R_L$ ) were measured by standard computer analysis of tracheal airflow and esophageal pressure. Saline was delivered via a Devilbiss ultrasonic nebulizer for 4 min (2 ml/min delivered), and pulmonary function was monitored for two min to establish baseline parameters. Animals were exposed to aerosolized HDM for 4 min, and pulmonary function monitored for 15 min. Arterial oxygen saturation was monitored by pulse oximetry (SurgiVet Model V3304, Harvard Apparatus, Holliston, MA) throughout the protocol, and supplemental O<sub>2</sub> was delivered if readings fell below 70%.

### Flow cytometry analyses of peripheral blood basophils

Venous blood (2 ml) was collected, and red blood cells lysed (1 $\times$  RBC Lysis Buffer; eBioscience, San Diego, CA). Basophils

were identified by flow cytometry as IgE<sup>+</sup> and CD123<sup>++</sup> or as IgE<sup>+</sup> and Fc $\epsilon$ RI<sup>+</sup>. We found that anti-human CD203c antibodies (Beckman Coulter, Fullerton, CA) that strongly stain human basophils did not bind to cynomolgus macaque basophils. White blood cells (1 $\times$ 10<sup>6</sup>/sample in PBS, 2 mM EDTA, 2% FBS) were stained with FITC-anti-human IgE (Vector Laboratories, Burlingame, CA), phycoerythrin (PE)-anti-human CD123 (IL-3 receptor  $\alpha$  chain; BD Biosciences, Franklin Lakes, NJ) and with biotin-anti-human IgG1 hinge (SouthernBiotech, Birmingham, AL) to detect hGE2 bound on basophils. Alternatively, blood cells were stained with FITC-anti-human IgE, PE-anti-human Fc $\epsilon$ RI $\alpha$  (eBioscience) and biotin-anti-human IgG1 hinge. In both stainings, biotin was detected using streptavidin allophycocyanin (APC, BD Biosciences). Cells were analyzed on a FACSCalibur (BD Biosciences) in the presence of a viability marker. Fc $\epsilon$ RI<sup>+</sup> cells strongly stained with anti-IgE and anti-CD123 and were confirmed to be HLA-DR<sup>-</sup>, CD3<sup>-</sup>, CD19<sup>-</sup>, CD14<sup>-</sup> (data not shown). The number of basophils per sample was calculated by multiplying the percentage of basophils among total live cells determined by flow cytometry with the number of live cells counted in a hemocytometer.

### Determination of anti-hGE2 and anti-HDM titers

Serum antibodies binding hGE2 or HDM antigen were measured by ELISA. For anti-hGE2 determination, microtiter plates were coated overnight with hGE2, (0.25  $\mu$ g/ml, 4 °C), washed, and blocked with SuperBlock (Pierce). Serially diluted serum samples were added to the wells, and incubated for 90 min at room temperature. After washing, bound antibodies were detected with biotinylated hGE2 and streptavidin-HRP (BD Biosciences). Mouse anti-human IgE (Maine Biotechnology, Portland, ME) and goat anti-human IgG (Fc-specific; SouthernBiotech) were used as reference standards for antibodies to IgE Fc and IgG1 Fc, respectively. Resulting titers to IgG1 Fc were therefore not comparable with titers to IgE Fc.

HDM-specific IgE was measured by coating plates with dust mite allergen (10  $\mu$ g/ml/well, overnight at 4 °C in 0.01 M sodium bicarbonate buffer, pH 9.6). The plates were washed and blocked with 10% newborn calf serum. Serum samples were diluted 1:125 and incubated in the prepared plates overnight at 4 °C. IgE was detected with biotinylated goat anti-human IgE (Vector Lab, Burlingame, CA), streptavidin-conjugated horseradish peroxidase (BD Pharmingen, San Diego, CA) and 3, 3', 5, 5' tetramethylbenzidine (BD Pharmingen). Positive and negative control sera were obtained from known HDM-sensitive and naive animals.

### Statistics

The response to aerosolized allergen for each individual animal was expressed as a percent change from the saline control period. The drug effect was evaluated by comparing the responses to allergen observed in the vehicle and drug treatment arms of the protocol. Responses of individual animals were averaged. Statistical significance was determined using Student's two-tailed *t*-test to compare two groups, and ANOVA with Fisher's LSD post-hoc test to evaluate 3 or more groups (level of significance: *P*<0.05). All values are reported as means  $\pm$  standard error.

## Results

### Validation of hGE2 activity *in vitro*

Human CBDB and CBDMC primed with biotinylated IgE released histamine when stimulated to degranulate with streptavidin (Fig. 1). Streptavidin, hGE2, or biotinylated IgE alone did not induce appreciable release of histamine. Addition of 1 and 10  $\mu\text{g/ml}$  hGE2 to the wells reduced IgE/streptavidin-induced histamine release in a dose-dependent manner, with maximum inhibition (75–80%) observed with 10  $\mu\text{g/ml}$  (data on dose dependence not shown). Non-biotinylated IgE (ns-IgE) at a concentration of 10  $\mu\text{g/ml}$  did not inhibit histamine release induced by biotinylated IgE and streptavidin, indicating that hGE2 was not inhibiting histamine release by competing with sensitizing IgE. These results were similar to those previously reported for GE2 [9,11].

### Pharmacokinetic profile

A single subcutaneous dose of hGE2 (1, 10, or 30 mg/kg) was delivered to non-sensitized animals. Following administration, dose-dependent elevation of hGE2 in serum was observed with a  $C_{\text{max}}$  between 24 and 72 h (mean  $T_{\text{max}} = 37 \pm 15$  h,  $n=9$ ; Fig. 2).  $T_{\text{max}}$  was independent of dose. From 1 to 10 mg/kg, the  $C_{\text{max}}$  increased 20-fold, which was more than proportional to the increase in dose. From 10 to 30 mg/kg, the  $C_{\text{max}}$  increased 2.6-fold, which was slightly less than the 3-fold increase in dose. The serum half-life ( $t_{1/2}$ ) appeared greater in the 1 mg/kg dose group ( $t_{1/2} = 127$  h,  $n=2$ ) than in the other groups ( $t_{1/2} = 56 \pm 9$  h,  $n=6$ ).

### Pulmonary allergic sensitivity

GE2 and hGE2 were previously shown to inhibit cutaneous allergic reactions in *Dermatophagoides farinae*-sensitive

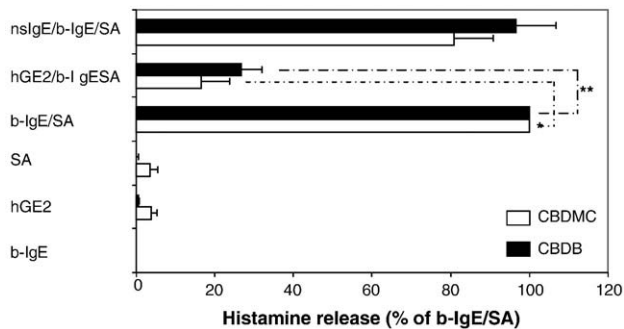


**Figure 1** Inhibition of histamine release from CBDB and CBDMC. Cells were incubated with biotinylated IgE (b-IgE) in the presence or absence of hGE2 (10  $\mu\text{g/ml}$ ) or non-specific IgE (ns-IgE, 10  $\mu\text{g/ml}$ ). Up-regulation of  $\text{Fc}\epsilon\text{RI}$  was similar after sensitization of the cells with IgE or hGE2 alone (data not shown). Degranulation was stimulated with streptavidin (SA). Maximum histamine release ranged from 53 to 86% of total histamine content for CBDMC and from 43–78% for CBDB. The percentage of histamine release was calculated after setting the max release of IgE/SA at 100%. Data were obtained in 4 independent experiments using cells from 3 different donors for basophils and 4 different donors for mast cells. \* $P=0.001$ , \*\* $P<0.001$ .

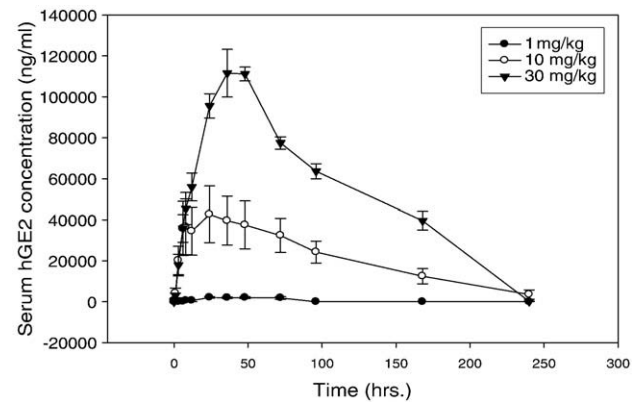


**Figure 2** Pharmacokinetic profile for single dose administration of hGE2 in non-sensitized cynomolgus macaques. Concentrations of hGE2 were quantified in serum from naive cynomolgus macaques following a single subcutaneous injection of hGE2 at 1, 10, or 30 mg/kg doses. Values represent means  $\pm$  SE, 3 animals in each dose group.

rhesus macaques (*Macaca mullata*) [11] and *Ascaris suum*-sensitive cynomolgus macaques (*Macaca fascicularis*) [20]. We repeated this protocol with hGE2 in HDM-sensitive, hGE2-naive cynomolgus macaques. The results replicated what was reported previously (data not shown). The ability of hGE2 to inhibit responses to aerosolized HDM was then tested in HDM-sensitive animals, some of which had been used for skin testing of hGE2, and others that were naive to the protein. Based on the pharmacokinetic modeling, animals were treated with two 5 mg/kg doses of hGE2 administered subcutaneously 24 h apart. The provocative dose of HDM that induced a 100% increase in RR or  $R_L$ , 50% decrease in  $C_{\text{dyn}}$ , or  $\text{SaO}_2$  less than 70% was determined for each animal 2 weeks prior to treatment with hGE2. Four days after the first hGE2 treatment, the HDM challenge was repeated. Increasing doses of aerosolized HDM were delivered until a respiratory

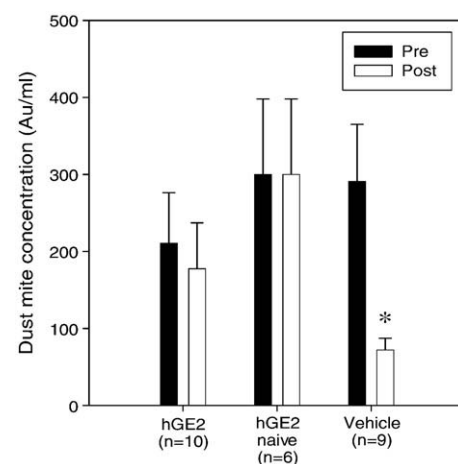


**Figure 3** Effect of  $2 \times 5$  mg/kg doses of hGE2 on sensitivity to aerosolized HDM. The provocative concentration of HDM was determined 2 weeks before (black bars) and 4 days after treatment (white bars) with hGE2 or vehicle. hGE2 (naive): subset that had no exposure to hGE2 prior to this dosing. \*Different from all groups ( $P \leq 0.05$ ).

response was observed (i.e., 100% increase in RR or  $R_L$ , 50% decrease in  $C_{dyn}$ , or  $SpO_2 < 70\%$ ) or the previously determined provocative dose of HDM was achieved. A significant decrease in the provocative dose of HDM was observed in the vehicle control group during the second challenge, but not in the hGE2 treatment group (Fig. 3). A subset of the hGE2-treated animals had prior exposure to hGE2 from skin testing (Table 1). When these animals were excluded from the analysis, the remaining animals (hGE2 (naive) group in Fig. 3) still exhibited a significant difference from the vehicle control group, indicating that inhibitory effect was independent of prior exposure to hGE2.

To verify the reduced sensitivity to HDM, serum collected at the time of allergen challenge 4 days after treatment with vehicle or hGE2 was assayed for HDM-specific IgE. HDM-specific IgE concentration was 35% ( $\pm 11\%$ ,  $n=7$ ) less following hGE2 treatment compared to vehicle treatment.

In a subsequent experiment, animals were treated with vehicle or a single subcutaneous dose of 1 or 10 mg/kg hGE2. The sensitivities to aerosolized HDM were compared before and up to 8 weeks after the treatment (Fig. 4). Unlike the protocol presented above, the maximum allergen concentration delivered during the second challenge was not limited to the provocative dose of HDM determined before treatment, thereby providing information on the degree of desensitization induced by hGE2. At both 4 days and 4 weeks after treatment with 10 mg/kg hGE2, 4 of 4 animals exhibited reduced sensitivity to aerosolized HDM, with a mean increase in the HDM provocative dose of  $2323 \pm 127$  AU/ml. Following treatment with hGE2, the animals were exposed to the highest concentration of aerosolized HDM (2500 AU/ml for 4 min) without achieving the minimum changes in RR, TV,  $C_{dyn}$ ,  $R_L$ , or  $SpO_2$  used to define a positive provocation. Thus, a single dose of 10 mg/kg hGE2 abolished the asthmatic response. It should be further noted that this level of allergen challenge would have been lethal in untreated animals. After treatment with 1 mg/kg of hGE2, 1 of 5 animals exhibited decreased allergic sensitivity at both 4 days and 4 weeks after treatment. Another animal exhibited decreased sensitivity at

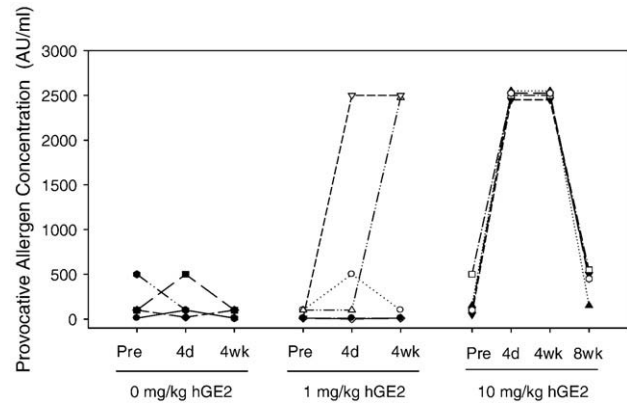


**Figure 4** Sensitivity to aerosolized HDM allergen before and after treatment with a single dose of hGE2. Animals were treated with 0, 1, or 10 mg/kg of hGE2 administered subcutaneously. The provocative concentration of aerosolized HDM allergen was determined for up to 8 weeks following the treatment. Individual animals are represented as different symbols.

4 weeks, but not 4 days. No consistent change was observed in the vehicle control group (Fig. 4). In summary, treating twice with 5 mg/kg doses (Fig. 3) or once with a 10 mg/kg dose (Fig. 4) decreased allergic sensitivity relative to the control animals, but the single 10 mg/kg dose appeared more efficacious (i.e., the allergic response was blunted even at the highest permissible levels of aeroallergen).

All of the animals that exhibited decreased allergic sensitivity 4 days after treatment with a single dose of hGE2 had been exposed to the drug in prior experiments (Table 1). The one animal that exhibited decreased sensitivity 4 weeks after treatment, but not 4 days after treatment, had no prior exposure to hGE2. This observation raised the question of whether the initial exposure to hGE2 affected the outcomes to subsequent drug exposure, perhaps through an immune response to the fusion protein.

**Table 1** hGE2 exposure record

| Animal ID | Intradermal dosing |              | Subcutaneous dosing |              |                 |              |                  |              |
|-----------|--------------------|--------------|---------------------|--------------|-----------------|--------------|------------------|--------------|
|           | Date               | Dose (ng/kg) | Date                | Dose (mg/kg) | Date            | Dose (mg/kg) | Date             | Dose (mg/kg) |
| 3512      | 11/9/05            | 101          |                     |              |                 |              |                  |              |
| 3513      | 11/10/05           | 203          | <b>12/16/05</b>     | <b>5</b>     |                 |              |                  |              |
| 3514      |                    |              | 3/24/06             | 5            | 3/25/06         | 5            | 8/14/2006        | 1            |
| 3517      | 11/9/05            | 156          | 3/24/06             | 5            | 3/25/06         | 5            | <b>8/14/2006</b> | <b>10</b>    |
| 3521      |                    |              |                     |              |                 |              | 07/24/06         | 1            |
| 3523      | 11/8/05            | 122          |                     |              |                 |              |                  |              |
| 3524      |                    |              | 12/15/05            | 5            | 12/16/05        | 5            | 07/24/06         | 10           |
| 3532      |                    |              | 5/11/06             | 5            | 5/12/06         | 5            |                  |              |
| 3534      | 11/10/05           | 292          | 5/18/06             | 5            | 5/19/06         | 5            | 8/7/2006         | 1            |
| 3541      |                    |              | 12/16/05            | 5            | <b>12/17/05</b> | <b>5</b>     | <b>8/7/2006</b>  | <b>10</b>    |
| AT8G      |                    |              | 12/15/05            | 5            | 12/16/05        | 5            | 8/7/2006         | 1            |
| AX5E      |                    |              | 5/11/06             | 5            | 5/12/06         | 5            |                  |              |
| CR25      | 11/8/05            | 102          | 3/23/06             | 5            | 3/24/06         | 5            | <b>8/14/2006</b> | <b>10</b>    |
| CR8H      |                    |              |                     |              |                 |              | 8/14/2006        | 1            |

Bold indicates the treatment was followed by an adverse reaction (See Table 2).

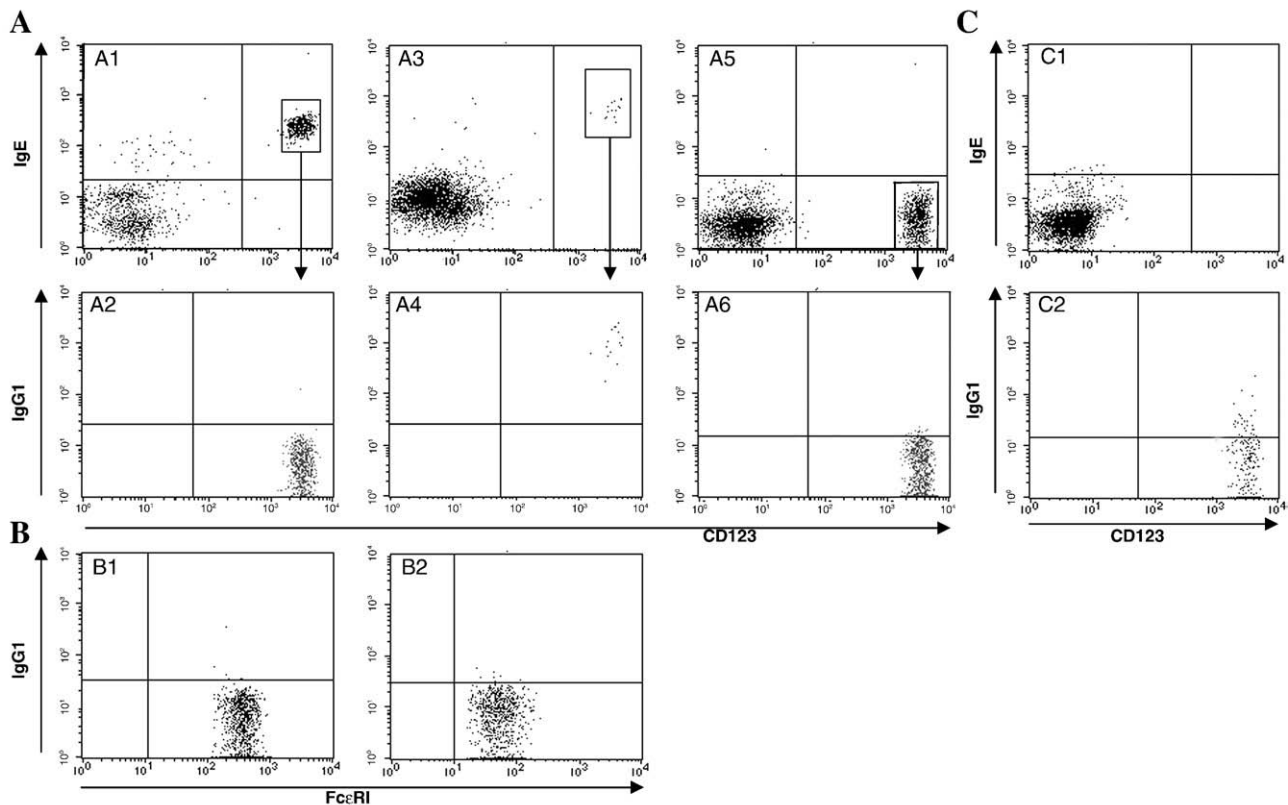


**Figure 5** FACS analysis of blood basophils following hGE2 treatment. Basophils were isolated from one responder animal in the 10 mg/kg (A) and 1 mg/kg (C1) dose groups; and a non-responder in the 1 mg/kg group (C2); before (A1, A2), 24 h after (A3, A4, C1, C2) and 2 weeks after (A5, A6) dosing. (B) IgE<sup>+</sup> basophils from A, stained for IgG1 hinge and FcεRI prior (left) and 2 weeks after hGE2 dosing (right).

### Binding of hGE2 to blood basophils and reduction of basophil numbers

Blood cells were collected before and after treatment with hGE2, stained for expression of CD123 (IL-3 receptor  $\alpha$  chain) and surface IgE, and double-positive cells were analyzed with an antibody to the hinge region of human IgG1 as an indicator of hGE2 binding. Representative histograms from responders in the 10 and 1 mg/kg groups (Figs. 5A, B and C1) and a non-responder in the 1 mg/kg group (Fig. 5C2) are shown. IgE<sup>+</sup> CD123<sup>++</sup> cells/basophils were readily detectable 2 weeks before treatment (Fig. 5, panel A1). The number of basophils was markedly reduced 24 h after treatment with hGE2 (Fig. 5A, panel A3; Fig. 5C1; Fig. 6), and any remaining cells co-stained for IgG1 hinge, indicating bound hGE2 (Fig. 5, panel A4). In the majority of animals, the number of basophils was restored to pretreatment levels 2 weeks after treatment (Fig. 5A, panel A5; Fig. 6), but the cells exhibited little or no IgE bound to the cell surface (Fig. 5A, panel A5) and no bound hGE2 (Fig. 5A, panel A6). FcεRI expression on basophils was decreased 5-fold 2 weeks after treatment (Fig. 5B). In contrast, 24 h after hGE2 dosing a non-responder animal in the 1 mg/kg group exhibited relatively high numbers of basophils with very low levels of hGE2 bound to the surface (Fig. 5C2). Animals that exhibited allergic desensitization following hGE2 treatment had a profound reduction in blood basophils (Fig. 6). Basophil numbers recovered over 2 weeks, but their ability to bind IgE remained low due to low expression of FcεRI receptors, a characteristic of immature basophils.

### Immune response to hGE2

Repeat dosing with hGE2 one to five months after an initial treatment was associated with an increase in the incidence of adverse events observed 1 to 4 h after the follow-up treatment (see Table 1 for detailed exposure history, Table 2 for adverse events). Diphenhydramine (2.5 mg/kg) and intravenous fluids

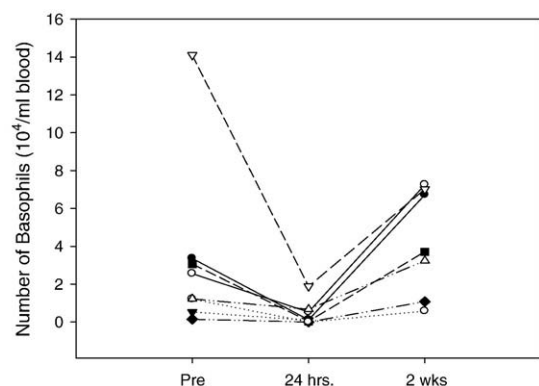


**Figure 6** Number of basophils in peripheral blood before and after treatment with a single dose of hGE2. Animals were treated with 1 or 10 mg/kg hGE2 (white and black symbols, respectively) administered subcutaneously. Blood samples were collected 2 weeks before, and 24 h and 2 weeks after treatment. The numbers of CD123<sup>+</sup>/IgE<sup>+</sup> cells were determined by flow cytometry. Symbols represent different animals.

**Table 2** Anti-hGE2 titers following systemic treatment

| Subject | Initial systemic exposure |                           |                           | Follow-up systemic exposure |                           |                                     | Epitope identification       |                              |
|---------|---------------------------|---------------------------|---------------------------|-----------------------------|---------------------------|-------------------------------------|------------------------------|------------------------------|
|         | hGE2 dose                 | Anti-hGE<br>( $\mu$ g/ml) | Acute reaction            | hGE2 dose                   | Anti-hGE<br>( $\mu$ g/ml) | Acute reaction                      | Anti-IgG Fc<br>( $\mu$ g/ml) | Anti-IgE Fc<br>( $\mu$ g/ml) |
| 3521    | 1 $\times$ 1 mg/kg        | 17                        |                           |                             |                           |                                     | 36                           | 4                            |
| CR8H    | 1 $\times$ 1 mg/kg        | 118                       |                           |                             |                           |                                     |                              |                              |
|         |                           |                           | Lethargy, slow/weak pulse |                             |                           |                                     |                              |                              |
| 3513    | 1 $\times$ 5 mg/kg        | 5                         |                           |                             |                           |                                     |                              |                              |
| AX5E    | 2 $\times$ 5 mg/kg        | 3                         |                           |                             |                           |                                     |                              |                              |
| 3532    | 2 $\times$ 5 mg/kg        | 58                        |                           |                             |                           |                                     |                              |                              |
| AT8G    | 2 $\times$ 5 mg/kg        | 2                         |                           |                             |                           |                                     |                              |                              |
| 3514    | 2 $\times$ 5 mg/kg        | ND                        |                           | 1 $\times$ 1 mg/kg          | 92                        |                                     |                              |                              |
| 3524    | 2 $\times$ 5 mg/kg        | 1                         |                           | 1 $\times$ 10 mg/kg         | 1146                      |                                     | 83                           | 149                          |
| 3541    | 2 $\times$ 5 mg/kg        | 7                         | Rash                      | 1 $\times$ 10 mg/kg         | 563                       | Hives, lethargy<br>Facial swelling, | 40                           | 259                          |
|         |                           |                           |                           |                             |                           | lethargy                            | 488                          | 769                          |
| CR25    | 2 $\times$ 5 mg/kg        | ND                        |                           | 1 $\times$ 10 mg/kg         | 2227                      | Itching, emesis                     | 53                           | 86                           |
| 3517    | 2 $\times$ 5 mg/kg        | ND                        |                           | 1 $\times$ 10 mg/kg         | 375                       |                                     |                              |                              |

Anti-hGE2 titers in serum were determined 14 to 35 days after initial and follow-up systemic treatments with hGE2. ND: not done due to insufficient amount of serum. Animals 3513, 3517, CR25 were used for intradermal skin testing, and received intradermal injections of hGE2 3 months before the first systemic treatment. Detailed exposure history is presented in Table 1. Prior to initial treatment with hGE2 animals had not previously been exposed to human therapeutic antibodies.

reversed the symptoms. Blood samples were therefore drawn and serum analyzed for anti-hGE2 antibodies. Prior to hGE2 exposure no anti-hGE2 reactivity was detected. With repeat dosing, anti-hGE2 titers increased, with reactivity directed against both the human IgG and IgE Fc portions of the hGE2 molecule (Table 2).

The ability of the immune sera to directly stimulate histamine release was subsequently investigated *in vitro* using human CBDMC. CBDMC were sensitized with human IgE and challenged to release histamine by addition of polyclonal goat anti-IgE antibody or cynomolgus macaque serum with anti-hGE2 titers (Fig. 7A). Minimal histamine released was observed upon exposure of the cells to IgE, hGE2, anti-IgE, or macaque serum alone. Sensitizing the cells with human IgE and cross-linking the cell surface-bound IgE with polyclonal anti-IgE antibody induced strong release of histamine. Serum from animals with high anti-hGE2 titers induced an equivalent release of histamine from human mast cells primed with human IgE *in vitro*. Additionally, serum from animals with high anti-hGE2 titers induced histamine release from CBDMC treated *in vitro* with hGE2. The amount of histamine released from hGE2-treated CBDMC was approximately 50% of the maximal histamine released from IgE-treated CBDMC (Fig. 7A). Serum from normal cynomolgus macaques not dosed with hGE2 did not exhibit histamine-releasing activity on CBDMC sensitized with human IgE (Fig. 7A). Plotting the histamine release against the anti-GE2 titers for this limited dataset revealed a positive correlation (Fig. 7B). Similar results were obtained using human CBDB (data not shown).

## Discussion

The goal of this study was to determine the feasibility of inhibiting allergic responses to inhaled allergens using a fusion protein containing Fc $\gamma$  and Fc $\epsilon$  domains that was designed to cross-link Fc $\epsilon$ RI and Fc $\gamma$ RIIB on basophils and

mast cells. Previous work demonstrated that GE2 inhibited histamine release from mast cells and basophils *in vitro*, and reduced wheal formation in intradermal allergen testing of allergic mice and rhesus macaques [11]. The fusion protein generated for this study, hGE2, exhibited functionality similar to original GE2 *in vitro* and *in vivo*. In *Ascaris suum*-sensitized monkeys, systemically-delivered hGE2 protected the animals from the skin reaction induced by allergen challenge for up to 3 weeks [15]. In the current study, systemic treatment with hGE2 is demonstrated to reduce sensitivity of allergic cynomolgus macaques to aerosolized HDM relative to sensitivity of vehicle treated animals. This potentially beneficial therapeutic effect of hGE2 was accompanied by production of macaque antibodies against the human Fc $\gamma$  and Fc $\epsilon$  regions of the molecule. Immune serum containing antibodies against the human Fc $\gamma$  and Fc $\epsilon$  regions stimulated histamine secretion from human mast cells *in vitro*.

GE2 was designed to cross-link Fc $\epsilon$ RI and Fc $\gamma$ RIIB, down-regulate mast cell and basophil activation, and inhibit IgE-stimulated degranulation [11,14]. The current study confirms and extends the previous findings by demonstrating that hGE2 induced long-term loss of HDM sensitivity. In the model used for the current study, the acute response to aerosolized HDM includes decreased  $C_{dyn}$  indicative of altered peripheral lung function, increased  $R_L$  indicative of bronchoconstriction, and histamine-dependent alterations in breathing patterns secondary to activation of lung sensory receptors and respiration reflexes [13]. Changes in RR, TV,  $C_{dyn}$ ,  $R_L$ , and  $SpO_2$  were utilized to define the provocative dose of allergen. Consequently, the significant increase in provocative concentration of allergen observed following treatment with hGE2 reflected all the parameters and not a shift to an alternative pattern of responsiveness (e.g., increase in airway resistance accompanied by a decrease in RR and increase in TV). Based on previous experience with this model, the large doses of allergen tolerated by the sensitized

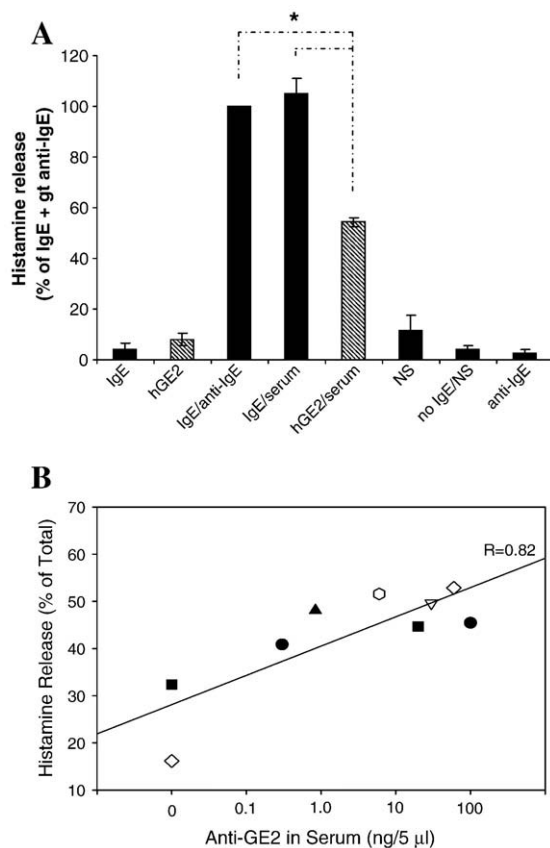


**Figure 7** Histamine release from human CBDMC. (A) CBDMC were primed with human IgE or hGE2 and challenged with anti-human IgE (gt anti-IgE), serum from 5 to 7 hGE2-treated animals (macq serum) or non-treated animals (norm macq serum). Values are mean  $\pm$  SE,  $n=3$  experiments. (B) Histamine release vs. anti-hGE2 titers in serum from 9 HDM-sensitive animals. \* $P<0.001$ .

animals after treatment with hGE2 would have rapidly resulted in lethal Hb desaturation and anaphylaxis, in the absence of any therapeutic intervention. Thus, systemic delivery of hGE2 was very effective in reducing allergic sensitivity. This effect of hGE2 was attributed to its direct inhibitory effect on basophils and mast cells and not to an immune response against the molecule since efficacy was observed in hGE2-naïve animals 4 days after initial exposure, which is too short a time period to mount a significant immune response.

The effect of hGE2 on allergic sensitivity was prolonged compared to its clearance from the blood. Subcutaneous delivery of 10 mg/kg hGE2 was associated with a half-life in blood of 56 h, yet the sensitivity to allergen was reduced for at least 4 weeks. The discrepancy was attributed to a prolonged effect on the target cells, basophils and mast cells. After repeat dosing, hGE2 caused a transient reduction in the number of circulating basophils. The mechanism of this reduction in basophil number is unknown. It is possible that these cells were depleted after binding hGE2 either through antibody-dependent cytotoxicity mediated through the human IgG Fc or through negative signaling events followed by cell death. Another possibility is circulating basophils re-located into tissues. Basophils that returned to the circulation had an approximate 5-fold decrease in their expression

of Fc $\epsilon$ RI. The lack of receptor-bound IgE on these cells indicated they were newly-formed immature basophils. No data were generated on lung mast cell numbers due to the difficulty in enumerating this subpopulation in non-terminal protocols, but the alteration in lung sensitivity to aeroallergen was consistent with a reduction in the number and or activation state of these cells.

In addition to confirming the potential therapeutic effects of an Fc $\gamma$  and Fc $\epsilon$  fusion protein, the results revealed a potentially adverse side effect: production of antibodies against the human Fc $\epsilon$  and Fc $\gamma$  domains that were capable of inducing histamine secretion from hGE2 primed basophils and mast cells. Two of the three animals that exhibited clinical reactivity following systemic administration had previously been exposed to small doses of the fusion molecule through local administration during skin testing. This prior exposure may account for particularly high titers and reactivity seen on the second systemic administration in that it was essentially the third dose in those two animals (CR25 and 3517). It is likely that the immune response to hGE2 in this study was a cross-species effect, as retrospective sequence analysis of cynomolgus  $\epsilon$  chain cDNA and comparison with human  $\epsilon$  chain cDNA revealed much greater differences in predicted amino acid sequences (85.5% amino acid sequence identity; E. Garber, personal communication; data not shown) than between cynomolgus and human  $\gamma$ 1 chain sequences (91.8% amino acid sequence identity). This hypothesis could be tested by constructing a GE2 molecule based on cynomolgus macaque Fc $\epsilon$  and Fc $\gamma$  domains. However, even in the same species recombinant proteins can be immunogenic due to variations in folding or glycosylation, aggregation, or allelic sequence differences. It should be noted immunogenicity of proteins in nonhuman primates may not be predictive of immunogenicity in humans [16,17]. In cases where human IgGs are immunogenic in NHP, the effects are primarily limited to immunogenicity of the antibody idiotype, rather than constant region and the immune response serves to neutralize or clear them more rapidly and is not necessarily detrimental. In the case of hGE2, the molecule is unique as compared to other therapeutic antibodies in that the Fc $\gamma$ -Fc $\epsilon$  fusion protein is designed to directly bind activating receptors on histamine-secreting cells, which increases the chance that an immune response against the fusion protein could lead to receptor cross-linking and anaphylactic reactions. The ability of hGE2 to prime human mast cells and basophils *in vitro* for degranulation by sera containing anti-human IgE and anti-human IgG antibodies emphasizes this point. *In vitro*, hGE2 primed mast cells or basophils degranulated to a lesser extent than IgE primed cells, indicating potential inhibitory activity of hGE2 (Fig. 7A), however the *in vivo* adverse reactions were significant (Table 2). The reactions were consistent with histamine-mediated processes, and in a couple of cases rapidly progressed to a clinical state resembling anaphylaxis. No attempt was made to further characterize the reactions, but rather the animals were treated with fluids and anti-histamine to stabilize their condition.

The failure of previous studies on GE2 to uncover the potential adverse reactions may reflect differences in protocols. The current study involved repetitive systemic treatments with hGE2, whereas the previous study involved a single local injection during skin testing. Unpublished

observations in mice indicate that mouse GE2 (mGE2) exhibits minimal immunogenicity. We injected mice intraperitoneally with 4 doses of 150 mg mGE2 in Freund's adjuvant or the mGE2 linker sequence conjugated to KLH. One of three mice injected with mGE2 developed a low titer to mGE2. After 4 immunizations with the linker sequence, three out of three mice showed a good titer to peptide but no reactivity to mGE2 (S. Miklasz, unpublished observations). Six subcutaneous doses of mGE2 delivered over 9 days were devoid of adverse clinical events (S. Perper and H. Hess, unpublished observations).

*In vitro* priming of mast cells by exposure to GE2 alone in the absence of IgE was not investigated previously. Instead, Zhang and colleagues treated cells with GE2 and IgE simultaneously and cross-linked receptor-bound IgE with antigen [11] or treated *in vivo* IgE-sensitized cells with GE2 followed by antigen [13]. In the present study, naive cells were incubated with hGE2 and then directly stimulated with antibodies to IgE or IgG. Rather than increasing the inhibitory properties of hGE2 by engaging additional Fc $\gamma$ RIIB receptors, these polyclonal serum antibodies recognizing hGE2 led to histamine release. The ability of the sera to induce release from IgE-treated cells *in vitro* would be readily accounted for by the development of anti-human Fc $\epsilon$ . It is possible that antibodies in the serum of treated cynomolgus monkeys directed to the human Fc $\gamma$  region of hGE2 could potentially block interaction of the Fc $\gamma$  region with Fc $\gamma$ RIIB; but inhibition would likely be only partial or indirect, since residues in the Fc $\gamma$ 1 region that differ in sequence between human and cynomolgus macaque do not overlap with amino acid residues mapped as binding Fc $\gamma$ RIIB (S. Demarest and E. Garber, personal communication and ref. [18]). Thus, the exact mechanism behind the stimulatory *in vivo* effect of the macaque anti-hGE2 antibodies remains to be determined.

Taken together, the results of this study provide evidence that systemic delivery of an Fc $\gamma$  and Fc $\epsilon$  fusion protein can decrease sensitivity to inhaled allergens for prolonged periods. Decreased sensitivity appeared closely related to a decrease in sensitized basophils and possibly mast cell numbers. However, potential immunogenicity coupled with the specific targeting of activating receptors by GE2 on histamine-secreting cells makes the likelihood of anaphylactic reactions in allergic subjects an ongoing concern as development of antibodies against biological pharmaceutical agents is common even within species, and anaphylactic reactivity has been a reported outcome [19,20].

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