Published in final edited form as:

J Allergy Clin Immunol. 2013 January ; 131(1): 213–21.e1-5. doi:10.1016/j.jaci.2012.10.018.

Blockade of peanut allergy with a novel Ara h 2–Fcγ fusion protein in mice

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Abstract

Background—Conventional immunotherapy for peanut allergy using crude peanut extracts is not recommended because of the unacceptably high risk of anaphylaxis. Allergen-specific immunotherapy is not currently undertaken for peanut allergy.

Objectives—The objective of this study was to develop a novel peanut-human fusion protein to block peanut-induced anaphylaxis.

Methods—We genetically designed and expressed a novel plant-human fusion protein composed of the major peanut allergen Ara h 2 and human IgG Fcγ1. We tested the Ara h 2–Fcγ fusion protein (AHG2)'s function in purified human basophils. Transgenic mice expressing human FcεRIα and a murine peanut allergy model were used.

Results—AHG2 inhibited histamine release induced by whole peanut extract (WPE) from basophils of patients with peanut allergy, whereas the fusion protein itself did not induce mediator release. AHG2 inhibited the WPE-induced, peanut-specific, IgE-mediated passive cutaneous anaphylaxis in hFcεRIα transgenic mice. AHG2 also significantly inhibited acute anaphylactic reactivity, including the prototypical decrease in body temperature in WPE-sensitized mice challenged with crude peanut extract. Histologic evaluation of the airways showed that AHG2 decreased peanut-induced inflammation, whereas the fusion protein itself did not induce airway inflammation in peanut-sensitized mice. AHG2 did not exert an inhibitory effect in mice lacking FcγRII.

Conclusion—AHG2 inhibited peanut-specific IgE-mediated allergic reactions in vitro and in vivo. Linking specific peanut allergen to Fcγ can provide a new approach for the allergen immunotherapy of peanut allergy.

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Disclosure of potential conflict of interest: D. Zhu has received grants and travel support from the National Institutes of Health (NIH); the Food Allergy and Anaphylaxis Network; the American Academy of Allergy, Asthma & Immunology (AAAAI); the Northwestern Memorial Foundation; and the Food Allergy Initiative. L. C. Grammer has received grants and travel support from the NIH, the Food Allergy and Anaphylaxis Network, and S&C Electric Co; has consultant arrangements with Astellas Pharmaceuticals; is employed by Northwestern University and the Northwestern Medical Faculty Foundation; has received payment for lectures from the AAAAI; and has received royalties from Lippincott and Up To Date. R. P. Schleimer has received grants from the NIH and has consultant arrangements with Intersect ENT, Glaxo Smith Kline, and Allakos. The rest of the authors declare that they have no relevant conflicts of interest.

Keywords

Food allergy; peanut; fusion protein; immunotherapy; anaphylaxis; mast cells; basophils

Food allergy is a health problem of national importance that is increasing in prevalence worldwide. Peanut is one of the most allergenic foods.¹ Approximately 1.5 million persons in the United States have peanut allergy, and 50 to 100 of these die each year from the accidental ingestion of foods containing peanuts or peanut extracts.² Affected subjects can experience severe symptoms ranging from urticaria to anaphylaxis. In addition, peanut sensitivity usually appears at an early age and often persists throughout life. Because of the severity of the allergic reaction and the wider use of peanuts as protein extenders in processed foods, the risk to the peanut-sensitive subject is increasing.³ Unlike allergic diseases to inhaled allergens, no effective treatment exists for peanut allergy other than strict avoidance, rapid intervention with epinephrine when reactions do occur, or both.⁴ However, the peanut-hypersensitive population is still at constant risk of accidentally ingesting peanuts caused by misleading or absent product labeling, contamination from hidden allergens, and the extensive use of peanut products in processed foods.⁵ Even kissing has been shown to transfer enough peanut antigens to induce a reaction in sensitive subjects.⁶ The ever-present risk of a serious or even fatal reaction takes an enormous emotional toll on the families of children with peanut allergy. Standard subcutaneous immunotherapy (SCIT) that is effective for inhalant allergy has consistently been proved too dangerous in the setting of severe food allergies.

Because food allergy is often an IgE-mediated disease, there have been a variety of attempts to develop safe and effective immunotherapy for food allergy. Currently reported immunotherapy includes antigen-specific and nonspecific approaches. Antigen-specific approaches include oral, sublingual, and epicutaneous immunotherapy. A recent study found that peanut oral immunotherapy can induce desensitization.⁷ Another study by Kim et al⁸ also demonstrated that peanut-specific sublingual immunotherapy can induce significant desensitization after 12 month of therapy. In addition, using dietary isoflavones can protect mice against peanut sensitization by suppressing dendritic cell maturation.⁹ Nonspecific approaches include anti-IgE antibodies and a Chinese herbal formulation.¹⁰ Although the new approaches show promising results,¹¹ no approved immunotherapy is available for the treatment of peanut allergy.¹²

The aim of our study is to develop a safe immunotherapy using a novel peanut-human fusion protein consisting of the major peanut allergen Ara h 2¹³ and human IgG Fc γ 1 (Fig 1, A). We hypothesize that this protein will inhibit whole peanut extract (WPE)-induced allergic reactions by indirectly cross-linking inhibitory Fc γ RIIb with peanut-specific IgE bound to Fc ϵ RI (Fig 1, B). This concept is based on the fact that peanut allergy is an IgE-mediated hypersensitivity reaction,¹⁴ and during responses, cross-linking of the high-affinity IgE receptors (Fc ϵ RI) through IgE bound to multivalent peanut antigen results in the activation of degranulation of mast cells and basophils.^{15, 16} Previous studies have demonstrated that aggregating the inhibitory receptor Fc γ RIIb with Fc ϵ RI leads to the inhibition of degranulation.^{17–19} We found that using an Fc γ -allergen construct to indirectly crosslink Fc γ RIIb and Fc ϵ RI through the antigen-specific IgE also inhibits degranulation. Subsequent studies have proved this concept in experiments related to cat allergy.^{20, 21} Despite being highly effective in these studies, conventional immunotherapy for cat allergy is already available and relatively safe, but this is not the case for peanut allergy. In this study we determined the efficacy of a peanut allergen-Fc γ fusion protein as an inhibitor of WPE-induced degranulation *in vitro* and *in vivo*, as well as in a murine model of peanut allergy. Overcoming the acute anaphylaxis induced by peanut allergen should enable subsequent

studies to determine whether this chimeric molecule can induce tolerance by using standard immunotherapeutic approaches.

METHODS

Mice and reagents

Six-week-old female C57BL/6 and *Fcgr2b^{tm1Ttk}* mice were purchased from the Jackson Laboratory (Bar Harbor, Me). They were maintained on peanut-free chow under specific pathogen-free conditions. Standard guidelines for the care and use of animals were followed. WPE was purchased from Greer Laboratories (Lenoir, NC). The purified peanut allergen Ara h 2 and anti-Ara h 2 antibody were purchased from Indoor Biotechnologies (Charlottesville, Va). The cDNA for the major peanut allergen Ara h 2 was kindly provided by Dr Steve Stanley (University of Arkansas School of Medicine, Little Rock, Ark).

Construction and expression of Ara h 2–Fcγ fusion protein

The cDNA of Ara h 2 was inserted into the expression vector pSecTag-IgG₁, which contains genomic DNA of a human immunoglobulin IgG_{1h} Fc fragment. The expression vector containing the chimeric construct of Ara h 2–Fcγ was transfected into CHO cells with Lipofectamine (Invitrogen, Carlsbad, Calif). After selection with zeocin, the Ara h 2–Fcγ fusion protein (AHG2) was expressed in cell-culture supernatants and purified with a Protein A affinity column. The purified protein was characterized by means of Western blotting and ELISA. AHG2 was run on 4.5% to 15% SDS-PAGE and then transferred into Immobilon transfer membrane (Millipore, Temecula, Calif). For protein detection, the blot was incubated with serum from a patient with peanut allergy (no. 15815, 17019, or 18885) overnight. After washing, the blot was probed with a horseradish peroxidase–conjugated goat anti-human IgE Fc–specific antibody (KPL, Gaithersburg, Md) and detected with ECL (GE Healthcare, Piscataway, NJ). To confirm that AHG2 binds to human FcγRII, we incubated AHG2 with HMC-1, a human mast cell–like line that expresses FcγRII but not FcεRI. After washing, we stained the cells with anti-human IgG₁ Fc–specific antibody conjugated with fluorescein isothiocyanate and performed flow cytometry.

Purification of human basophils

Human basophils were purified by means of Ficoll gradient centrifugation (GE Healthcare), followed by negative selection with magnetic beads (Miltenyi Biotec, Auburn, Calif). Basophil purities were greater than 90%, as determined by means of both acid toluidine blue staining and fluorescence-activated cell sorting analysis (BD LSRII, BD FACSDiva; BD, San Jose, Calif) with CD203c and CD123 antibodies.²² We obtained informed consent from all human subjects, as approved by the Institutional Review Board at Northwestern University.

Passive cutaneous anaphylaxis in human FcεRIα chain transgenic mice

The human FcεRIα chain transgenic mice were kindly provided by Dr Jean-Pierre Kinet.²³ The mice were intradermally injected with 50 μL of serum from a patient with peanut allergy (51.3 kU/L; no. 15815; PlasmaLab, Seattle, Wash) to sensitize the skin mast cells. The different doses of AHG2 were added to the patient's serum before passive sensitization. Four hours later, the mice were challenged intravenously with 100 μg of WPE plus 1% Evans blue in a volume of 200 μL. The mice were killed 30 minutes after the intravenous challenge.

Murine peanut allergy model

Mice were sensitized as described by Sun et al,²⁴ although with slight modifications. The mice were orally sensitized with 500 µg of WPE along with 10 µg of cholera toxin (List Biological Laboratories, Campbell, Calif) once a week for 4 weeks. Sensitized mice were intravenously challenged with 100 µg of WPE 2 weeks after the last sensitization. Before the challenge, sensitized mice were subcutaneously treated with different doses of AHG2. The airway tissues were collected 2 days after the challenge.

Assessment of hypersensitivity reactions

Anaphylactic symptoms were evaluated 30 minutes after the challenge by using a defined scoring system (Table I). Scoring of symptoms was performed in a blinded manner by 3 independent investigators.

Measurement of core body temperatures

After the challenge, body temperatures were measured every 10 minutes with a rectally inserted thermal probe (Physitemp Instruments, Clifton, NJ).

Statistics

All data are reported as medians and ranges, unless otherwise stated. Differences between groups were analyzed by using nonparametric statistics. A *P* value of less than .05 was considered significant.

RESULTS

AHG2 inhibited WPE-induced histamine release and degranulation

We have constructed and expressed a fusion protein consisting of the major peanut allergen Ara h 2 plus the hinge, CH2, and CH3 regions of human IgG₁. Western blotting confirmed that AHG2 had the predicted size of 44 kDa (Fig 2, A). The amino acid sequence of AHG2 was confirmed by using mass spectrometry. Further probing found that AHG2 was recognized by both monoclonal anti-Ara h 2 and anti-human IgG Fc-specific antibodies (Fig 2, A). In addition, the ELISA or Western blot result showed that the peanut allergen Ara h 2 component of AHG2 was recognized by peanut-specific IgE (Fig 2, B, and see Fig E1 in this article's Online Repository at www.jacionline.org). Flow cytometry showed that AHG2 binds to FcγRIIb expressed on HMC-1 cells (Fig 2, C). These results demonstrate that the fusion protein AHG2 was expressed properly, having both peanut allergen and Fcγ antigenic or functional moieties intact.

To confirm that AHG2 itself does not cause an allergic reaction, we tested AHG2 on human basophils and humanized transgenic mice. Primary human basophils were purified from the white blood cell filters by using a Basophil Isolation Kit (Miltenyi Biotec), according to the manufacturer's methodology. The purity of basophils was determined to be greater than 90% by means of both acid toluidine blue staining and fluorescence-activated cell sorting analysis with CD203c and CD123 antibodies. We sensitized the purified basophils with serum from a patient with peanut allergy (no. 15815, 18885, or 17019; PlasmaLab; serum IgE levels are shown in Fig E2 in this article's Online Repository at www.jacionline.org). After 24 hours, the sensitized cells were washed and treated with different doses of AHG2 for 1 hour. The supernatants were then collected to determine histamine levels. The results show that AHG2 did not induce any histamine release from human basophils, whereas WPE did (data not shown).

Before testing AHG2 *in vivo*, we first set up a peanut-specific IgE-mediated passive cutaneous anaphylaxis (PCA) model in transgenic mice that expressed human IgE receptors.²³ The mice were separately intradermally sensitized with 50 μ L of serum from a patient with peanut allergy (no. 15815), normal human serum, anti-4-hydroxy-3-nitrophenylacetyl (NP) IgE, and heat-inactivated serum from a patient with peanut allergy (no. 15815). After 4 hours, mice were then intravenously challenged with 100 μ L of WPE plus 1% Evans blue. Thirty minutes later, we measured the size of the area of bluing at each site to determine the intensity of the PCA. We found that WPE induced degranulation solely in peanut-specific IgE-sensitized mice (Fig 3, A).

Human Fc ϵ RI α transgenic mice were intradermally sensitized with 50 μ L of various concentrations of serum from a patient with peanut allergy (no. 15815, 17019, and 18885) to test the fusion protein *in vivo*. Four hours later, mice were then intravenously injected with 50 μ g of purified Ara h 2 or 100 μ g of AHG2 (which carried an equivalent amount of Ara h 2) with 1% Evans blue. We found that the peanut allergen Ara h 2 induced degranulation (shown as vascular leak indicated by blue skin), whereas AHG2 did not (Fig 3, B and C). The transgenic mouse sensitized with serum from a patient with peanut allergy plus different doses of AHG2 was intravenously challenged with peanut allergen Ara h 2 four hours later. We found that 1 μ g of AHG2 completely blocked Ara h 2-induced vascular leak (Fig 3, D). In addition, we demonstrated that AHG2 did not block the NP-BSA-induced, NP-specific, IgE-mediated PCA reaction in transgenic mice (data not shown). We therefore concluded that AHG2-induced inhibition is peanut specific.

Although our results show that AHG2 completely blocked Ara h 2-induced degranulation, it must block the antigen-induced allergic reaction to WPE to be therapeutically effective. To test this, we sensitized purified human basophils with serum from a patient with peanut allergy. After 24 hours, we washed the cells and treated the sensitized cells with different doses of the AHG2 fusion protein. Two hours later, we challenged the cells with WPE. After 30 minutes, we immediately collected the supernatants and quantitated the histamine concentration using ELISA. The results showed that the AHG2 fusion protein partially inhibited WPE-induced, peanut-specific, IgE-mediated histamine release in human basophils in a dose-dependent manner (Fig 4, A). Similar results were found when AHG2 was tested in RBL-2H3 cells sensitized with sera from 2 different patients with peanut allergy (see Fig E3 in this article's Online Repository at www.jacionline.org). Furthermore, we found that AHG2 significantly inhibited WPE-induced degranulation in human Fc ϵ RI α transgenic mice (Fig 4, B). The transgenic mice were intradermally sensitized with 50 μ L of serum from a patient with peanut allergy (no. 15815) alone and in combination with different doses of AHG2. Four hours later, the mice underwent an intravenous challenge with WPE (0.5 mg) plus 1% Evans blue. The result showed that AHG2 dose-dependently inhibited degranulation induced by WPE in peanut-specific IgE-sensitized transgenic mice (Fig 4, B). We obtained consistent results from testing AHG2 in groups of 5 mice (Fig 4, C). Similar results were produced when we sensitized the same mice with sera from 2 different patients with peanut allergy (no. 17019 and no. 18885, see Fig E4 in this article's Online Repository at www.jacionline.org).

AHG2 inhibited peanut-induced acute systemic anaphylaxis in mice

Using a murine peanut allergy model,²⁴ we next induced acute severe systemic anaphylaxis by intravenously challenging the peanut-sensitized mice. Female C57BL/6 mice were orally sensitized with 500 μ g of WPE plus 10 μ g of cholera toxin in 100 μ L of saline once a week for 4 weeks. Two weeks after the last sensitization, the mice were intravenously challenged with 100 μ g of WPE. The clinical symptoms were evaluated by 3 experienced investigators using a scoring system (Table I). Core body temperatures of WPE-challenged mice were also measured every 10 minutes. We found that peanut allergen caused a severe acute

anaphylactic reaction in WPE-sensitized mice (Fig 5, *A*), whereas AHG2 fusion protein itself did not cause allergic symptoms (data not shown). When the sensitized mice were treated with AHG2 protein, their symptom scores were significantly improved (Fig 5, *A*). AHG2 modestly inhibited the decrease in body temperature in sensitized mice on WPE challenge (Fig 5, *B*). The histamine levels between the 2 groups did not show a statistically significant difference (Fig 5, *C*). When peanut-challenged mice were treated with different doses of AHG2 and challenged again with WPE, AHG2 also showed inhibition of WPE-induced anaphylaxis (see Fig E5 in this article's Online Repository at www.jacionline.org). To determine whether the AHG2-induced inhibition is mediated by the inhibitory receptor Fc γ RII, we tested AHG2 in Fc γ RII-deficient mice.²⁵ Using the same procedures as before, we orally sensitized the Fc γ RII knockout mice. When sensitized mice were challenged with WPE, we found that the anaphylactic reaction exhibited by the mice was enhanced. AHG2 did not block either WPE-induced anaphylaxis or the decrease in body temperature in Fc γ RII-deficient mice (Fig 6).

AHG2 blocked peanut-induced inflammation in airways

Peanut-induced acute anaphylaxis is often associated with airway inflammation.²⁶ To observe WPE-induced airway inflammation, we collected bronchoalveolar lavage fluid and lung tissues from WPE-sensitized mice after a WPE challenge. Histologic evaluation showed that there were marked inflammatory infiltrates in the airways of WPE-challenged mice. Differential cell counts in bronchoalveolar lavage fluid showed that AHG2 fusion protein significantly inhibited antigen-induced neutrophil and eosinophil infiltration (Fig 7, *A*), although the lymphocyte count did not show a significant difference. The AHG2 protein decreased inflammatory infiltration induced by WPE in lung tissue as well (Fig 7, *B*).

DISCUSSION

SCIT is an effective therapy for allergies.²⁷ SCIT can modify different aspects of the immune system by inducing a shift of T_H2 cells to T_H1 cells, epitope-specific T-cell anergy, allergen-specific regulatory T cells, and allergen-specific IgGs. However, SCIT requires numerous administrations of allergen and can cause severe adverse events that range from local allergic reactions to fatal anaphylaxis.²⁸ This study is the first report on the peanut allergen–Fc γ fusion protein's ability to block severe peanut-induced anaphylaxis. We demonstrated that this fusion protein induced inhibition in a murine model of peanut allergy. The fusion protein itself does not cause an allergic reaction in sensitized subjects. This approach of using the major allergen in conjunction with Fc γ might provide a novel immunotherapy for food allergy.

In the systemic model we found that AHG2 did not completely block the WPE-induced anaphylactic reaction, unlike in previous skin tests. It is possible that AHG2 is inactive in the systemic model because of the interference of mast cell-independent or IgE-independent mechanisms. Previous studies demonstrated that there are 2 pathways involved in systemic anaphylaxis in this model: the classic IgE/Fc ϵ RI/mast cell-mediated pathway and the alternate IgG/Fc γ RIII/macrophage-mediated pathway.²⁹ When the amount of antigen is small, the IgE-dependent pathway is favored. However, larger amounts of antigen readily induce the alternative pathway. Therefore we can increase AHG2-induced inhibition by further decreasing the challenge allergen amount.

Unlike cat allergy, which is caused primarily by a single allergen, Fel d 1, there are as many as 11 proteins from peanuts that are identified as allergens. More than 90% of peanut-hypersensitive subjects react to 3 main allergens: Ara h 1, Ara h 2, and Ara h 3.³⁰ Here we found that a fusion protein composed of the major peanut allergen Ara h 2 and Fc γ inhibited WPE-induced anaphylaxis and airway inflammation. Future studies will be required to

determine whether the WPE-induced reaction might be further reduced or even completely inhibited if we were to use 3 fusion proteins in conjunction: Ara h 1–Fcγ, Ara h 2–Fcγ, and Ara h 3–Fcγ.

In conclusion, we have constructed a peanut allergen–human IgG Fcγ fusion protein. Our results demonstrate that this fusion protein inhibited WPE-induced, peanut-specific, IgE-mediated histamine release in human basophils, as well as allergic responses in transgenic mice. Through the inhibitory receptor FcγRII, AHG2 partially inhibited the WPE-induced acute anaphylactic reaction and airway inflammation in the murine peanut allergy model. AHG2 itself did not induce anaphylaxis and inflammation in WPE-sensitized mice, suggesting that all properties of the allergen were mitigated by the presence of the FcγRIIb binding moiety. This approach of using the allergen in conjunction with Fcγ might provide the first step toward a therapy that will induce an allergen specific immunotherapy response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Supported by National Institutes of Health grant R21 AI088808; the Food Allergy and Anaphylaxis Network; the American Academy of Allergy, Asthma & Immunology; the Northwestern Memorial Foundation; and the Food Allergy Initiative.

Abbreviations used

AHG2	Ara h 2–Fcγ fusion protein
NP	4-Hydroxy-3-nitrophenylacetyl
PCA	Passive cutaneous anaphylaxis
SCIT	Subcutaneous immunotherapy
WPE	Whole peanut extract

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Clinical implications: Peanut allergen-Fc γ fusion protein can be used for allergen immunotherapy of peanut allergy.

FIG. 1.

Diagram of AHG2-induced inhibition. **A**, Computerized 3-dimensional structure of dimerized AHG2. **B**, Proposed mechanism by which AHG2 inhibits FcεRI-mediated degranulation. *ITAM*, Immunoreceptor tyrosine-based activation motif; *ITIM*, immunoreceptor tyrosine-based inhibitory motif; *MAPK*, mitogen-activated protein kinase; *PI3K*, phosphoinositide 3-kinase; *PLC*, phospholipase C; *SHIP*, Src homology domain 2-containing inositol phosphatase; *Syk*, spleen tyrosine kinase.

FIG. 2.

Characterization of AHG2 fusion protein. **A**, AHG2 was confirmed by using Western blotting and probed with antibodies specific for human IgG Fc and Ara h 2. **B**, AHG2 was recognized based on peanut-specific IgE. **C**, AHG2 bound to Fc γ RIIb expressed on HMC-1 cells. *FITC*, Fluorescein isothiocyanate; *SSC*, side scatter.

FIG. 3.

AHG2 blocked Ara h 2-induced vascular leak in transgenic mice. **A**, WPE induced peanut-specific IgE-mediated-PCA in transgenic mice. *NS*, Nonallergic serum; *PS*, serum from a patient with peanut allergy. **B**, Ara h 2 induced PCA mediated by different concentrations of peanut-specific IgE from serum from a patient with peanut allergy. *PS*, Serum from a patient with peanut allergy; *1:1PS*, 1:1 dilution of serum; *1:5PS*, 1:5 dilution of serum; *1:10PS*, 1:10 dilution of serum. **C**, AHG2 did not induce peanut-specific IgE-mediated PCA in transgenic mice. **D**, AHG2 inhibited Ara h 2-induced, peanut-specific IgE-mediated PCA in a dose-dependent manner. *PS*, Serum from a patient with peanut allergy; *PS-0.1*, serum

with 0.1µg of AHG2; *PS-1*, serum with 1 µg of AHG2; *PS-10*, serum with 10 µg of AHG2. Each experiment was repeated with 3 transgenic mice.

FIG. 4.

AHG2 inhibited WPE-induced allergic reactions *in vitro* and *in vivo*. **A**, AHG2 inhibited WPE-induced histamine release in human basophils. Results are representative of 3 separate experiments, each done in duplicate. **B**, AHG2 inhibited the peanut-specific IgE-mediated PCA reaction in a dose-dependent manner. **C**, The average blue density of 5 mice for each of the above tests. *PS*, Serum from a patient with peanut allergy; *PS-0.01*, serum with 0.01 μ g of AHG2; *PS-0.1*, serum with 0.1 μ g of AHG2; *PS-1*, serum with 1 μ g of AHG2.

FIG. 5.

AHG2 inhibited systemic anaphylaxis caused by the peanut extract in mice. Four groups of mice were sensitized with the peanut antigen. Group 1 ($n = 5$) was a control, group 2 ($n = 9$) was challenged with WPE, group 3 ($n = 9$) was treated with 1 mg/kg AHG2 (*AHG2-L*) and then challenged with WPE, and group 4 ($n = 9$) was treated with 10 mg/kg AHG2 (*AHG2-H*) and then challenged with WPE. Each mouse was evaluated for their symptoms score (**A**), body temperature (**B**), and histamine level in blood (**C**). Data are representative of 2 separate experiments. $*P < .05$.

FIG. 6.

AHG2 lost its inhibitory effect in Fc γ RIIb-deficient mice. Three groups of Fc γ RIIb knockout mice were sensitized and challenged with WPE. Group 1 (n = 6) was a control challenged with saline, group 2 (n = 6) was not treated with AHG2, and group 3 (n = 7) was treated with 10 mg/kg AHG2 before WPE challenge. Each mouse was evaluated for their symptoms score (**A**) and body temperature (**B**). Data are representative of 2 separate experiments.

FIG. 7. AHG2 inhibited WPE-induced inflammation in the airways of WPE-sensitized mice. **A**, Differential cell counts in bronchoalveolar lavage fluid. The bronchoalveolar lavage fluid was collected from each mouse of the above groups. The results are representative of the average of each group. * $P < .05$. **B**, Histologic airway changes. Representative histologic sections of lung tissues from the mice in Fig 5 were stained with hematoxylin and eosin. Bar = 100 μ m.

TABLE I

Anaphylactic symptom score table

Score	Symptoms
0	No clinical symptoms
1	Repetitive mouth/ear scratching and ear canal digging with hind legs
2	Decreased activity, self-isolation, puffiness around eyes and/or mouth
3	Periods of motionlessness for >1 min, lying prone on stomach
4	No responses to whisker stimuli; reduced or no response to prodding
5	End point: tremor, convulsion, death