

Anti-IgE mAb Suppresses Systemic Anaphylaxis through the Inhibitory IgG Receptor Fc γ RIIB in Mice

– Interaction between Anti-IgE and Fc γ RIIB –

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ABSTRACT

Background: Anti-IgE mAb which binds circulating but not receptor-bound IgE has been shown to be effective in treatment for asthma and other allergic diseases. However, the mechanisms by which anti-IgE mAb influences the pathophysiological responses are remained to be illustrated. This study was undertaken to examine the therapeutic efficacy of non-anaphylactogenic anti-mouse IgE mAb using murine models of IgE-induced systemic fatal anaphylaxis. **Methods:** Active systemic anaphylaxis was induced by either penicillin V (Pen V) or OVA and passive systemic anaphylaxis was induced by either anaphylactogenic anti-mouse IgE or a mixture of anti-chicken gamma globulin (CGG) IgG1 mAb and CGG. The binding of the Fc portion of anti-IgE to CHO-stable cell line expressing mouse Fc γ RIIB was examined using flow cytometry. Fc fragments of anti-IgE mAb were prepared using papain digestion. The expression of phosphatases in lungs were assessed by Western blotting and immunohistochemistry. **Results:** Anti-IgE mAb prevented IgE- and IgG-induced active and passive systemic fatal reactions. In both types of anaphylaxis, anti-IgE mAb suppressed antigen-specific IgE responses, but not those of IgG. Anti-IgE mAb neither prevented anaphylaxis nor suppressed the IgE response in Fc γ RIIB-deficient mice. The Fc portion of anti-IgE mAb was bound to murine Fc γ RIIB gene-transfected CHO cells and inhibited systemic anaphylaxis. Anti-IgE mAb blocked the anaphylaxis-induced downregulation of Fc γ RIIB-associated phosphatases such as src homology 2 domain-containing inositol 5-phosphatase (SHIP) and phosphatase and tensin homologue deleted on chromosome ten (PTEN). **Conclusion:** Anti-IgE mAb prevented anaphylaxis by delivering nonspecific inhibitory signals through the inhibitory IgG receptor, Fc γ RIIB, rather than targeting IgE. (*Immune Network* 2007;7(3):141-148)

Key Words: anti-IgE mAb, anaphylaxis, Fc γ RIIB

Introduction

Anaphylaxis is the most urgent and potentially serious manifestation of immediate hypersensitivity, but effective therapies for this condition are not currently available. IgE plays a central role in the pathogenesis of immediate hypersensitivity with anaphylaxis (1).

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Suppression of IgE production has long been considered the ideal treatment against allergic diseases. Many investigators have attempted to suppress IgE responses with a variety of approaches, including the use of immunosuppressive drugs (2), modified allergens (3-5), and cytokine inhibitors (6-9). Efforts to create a specific inhibitor that would block the binding of IgE to its high affinity receptor (Fc γ R1) without the capacity to induce degranulation have resulted in the development of an anti-human IgE mAb (omalizumab) that binds circulating but not receptor-bound IgE (10). This mAb has been shown to be effective in reducing serum-free IgE levels (11,12) and preventing passive

sensitization of human tissue and blocking allergen-induced IgE synthesis in vitro as well as in vivo (13-15). Anti-IgE is currently being tested in clinical trials as a potential treatment for asthma and allergic rhinitis (16,17). One recent clinical study has shown that anti-IgE significantly and substantially increases the threshold of sensitivity to peanuts on oral food challenge in patients with a history of immediate hypersensitivity to peanuts, in which IgE plays a major role (18).

However, in addition to IgE, IgG is also implicated in fatal anaphylaxis in humans (19) and animals (6, 20-22). Therefore, it is necessary to determine the extent of therapeutic efficacy of anti-IgE in anaphylaxis involving both IgE and IgG Abs.

In this study, the therapeutic efficacy of non-anaphylactogenic rat anti-mouse IgE, which recognizes an epitope within the Fc γ R1-binding region of murine IgE, was investigated in two types of active fatal anaphylaxis caused by either penicillin V (Pen V) in which IgE Ab plays a major role (9,23) or OVA in which either IgE or IgG Ab is responsible for the fatal reaction (20-22). We found that anti-IgE mAb prevented both types of active fatal anaphylaxis by providing nonspecific inhibitory signals via the inhibitory IgG receptor, Fc γ RIIB.

Materials and Methods

Animals. Specific pathogen-free male C57BL/6 mice were obtained from the Korean Institute of Chemistry Technology (Daejeon, Republic of Korea). Fc γ RIIB knock-out mice (Fc γ RIIB^{-/-}) derived from breeder pairs of B6;129S4-Fcgr2b^{tm1Rav}/J stock and the original wild-type strain (B6129SF2/J) were obtained from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in a laminar flow cabinet and maintained on standard laboratory chow ad libitum. Mice were 7-8 weeks old at the start of each experiment. Wistar rats were purchased from SLC, Inc. (Shizuoka, Japan). All experimental animals used in this study were cared for under a protocol approved by the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School.

Reagents. Pen V, OVA (grade V), and BSA (fraction V) were purchased from Sigma Chemical Co. (St. Louis, MO). CGG was purchased from Accurate and Chemical Scientific (Westbury, NY). Bordetella per-

tussis was obtained from the National Institute of Health (Seoul, Republic of Korea).

Abs. The non-anaphylactogenic mAbs (R35-92, rat IgG1) were purchased from BD Biosciences (Palo Alto, CA). The control mAb, J4-1 (9), which secretes rat IgG1 with specificity for the hapten, nitrophenol (NP), was kindly provided by Dr. F. D. Finkelman, Uniformed Services University of Health Science (Bethesda, MD). The anaphylactogenic anti-mouse IgE mAbs (LO-ME-2, rat IgG2a) were obtained from Zymed Laboratories Inc. (San Francisco, CA). Mouse IgG1 mAbs against CGG (20) were employed to induce passive systemic anaphylaxis. J4-1 and anti-CGG were prepared as ascites in pristane-primed nude mice. Preparations were precipitated in 45% ammonium sulfate and dialyzed against PBS (pH 7.2); protein was quantitated prior to use.

Induction of active and passive systemic anaphylaxis. Pen V- and OVA-induced active systemic anaphylaxis was induced as described previously (9,23). IgE- and IgG-induced passive anaphylaxis were induced by i.v. injection of LO-ME-2 (20 μ g) and i.v. injection of a mixture of anti-CGG mAb (2.0 mg) and CGG (500 μ g), respectively. After the challenge injections were given, the mice were observed for 1 h, and signs of shock and the number of deaths were recorded as described previously (9,23).

Measurement of antigen-specific serum levels of IgE and IgG. Blood samples were collected from orbital venous plexus under anesthesia 3 h before the challenge, and antigen-specific serum IgE and IgG levels were determined using a passive cutaneous anaphylaxis (PCA) reaction and ELISA as described previously (9,23).

Determination of plasma histamine. Post-challenge blood (10 min) was taken from the heart, which was cut open, and blood was then mixed with a 0.1 vol of 3.8% ice-chilled citrate solution and centrifuged immediately using an Eppendorf microfuge. The plasma was stored at -20°C until use. Plasma histamine was measured as described previously (23).

RNA isolation and reverse transcription (RT). Total mouse RNA was isolated from the spleen by using TRIZOL reagent (Invitrogen, Carlsbad, CA). cDNAs were synthesized by reverse transcription from 1 μ g of total RNA isolated from a mouse spleen source using a SuperScriptTM II Reverse Transcriptase (Invitrogen). PCR was performed with the Rotor-Gene 3000 System

(Corbett Research, Mortlake, Australia) programmed at 95°C for 5 min., followed by 30 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 3 min, using the Amplitaq Gold DNA polymerase (Perkin Elmer Applied Biosystems, Norwalk, CT). The primers used were as follows: Fc γ RIIb, 5'-AAC TGC AGG AGA GCA ACT GGA CTG TC-3' (sense) and 5'-CCC AAG CTT CTG GAA CCT GCT TTT TCT TG-3' (antisense).

Cloning and DNA sequence analysis. The full-length Fc γ RIIb cDNAs obtained from RT-PCR amplifications were digested with appropriate restriction enzymes, separated by electrophoresis in 1% agarose gel containing ethidium bromide, purified, and concentrated using a QIAquick PCR purification column (Qiagen, Chatsworth, CA). After PCR amplification, the PCR product was inserted into the pcDNA3.1 expression vector (Invitrogen), and the construct was transformed into Top10F cells. The nucleotide sequence of cloned cDNAs was determined using a 3730xl DNA analyzer (Macrogen, Seoul, Republic of Korea).

Stable transfection of CHO cells. Fc γ RIIb cDNAs were inserted into the pEGFP-C1 expression vector (Clontech, Mountain View, CA). These constructs were transfected into CHO-K1 cells by using a Lipofectamine 2000 kit (Invitrogen) according to a protocol supplied by the manufacturer. In brief, CHO-K1 cells were seeded in 10-cm culture dishes and grown at 37°C with 5% CO₂ until they were 60-70% confluent ($3-8 \times 10^5$) (1 or 2 days). The cells were then incubated for 3.5 h with premixed DNA and Lipofectamine added to Opti-MEM. After 24-48 h, the cells were harvested and analyzed for expression of the transfected cDNAs by flow cytometry and confocal microscopy.

Flow cytometry. The binding of the Fc portion of anti-mouse IgE was examined using flow cytometry. The CHO-stable cell line expressing mouse Fc γ RIIb or the mock-transfected cell line was stained with either FITC-conjugated 2.4G2 mAb (1 μ g/ml) or the FITC-conjugated Fc portion of anti-IgE mAb (1 μ g/ml) that was diluted with FACS buffer (PBS, 0.1% BSA, and 10 mM sodium azide, pH 7.4) at 4°C for 30 min. The cells were then washed and analyzed using a FAC-Scan flow cytometry (Becton Dickinson, Mountain View, CA).

Preparation of Fab and Fc fragments. Fc fragments of anti-IgE mAb were prepared using the papain digestion

(24). Papain (4 mg/ml) was activated in 100 mM Tris-HCl (pH, 8.0) solution containing 2 mM EDTA and 1 mM DTT for 5 min at 37°C. IgG (1 mg) was dissolved in 1 ml of 2 mM EDTA and 1 mM DTT buffer, and activated papain was added at 16 μ g/ml. The mixture was incubated for 1 h at 37°C, and Fab and Fc fragments were separated in a Protein G-Sepharose column. The purity of Fab and Fc fragments was confirmed by SDS-PAGE.

Statistical analysis. Data are expressed as mean \pm SD.

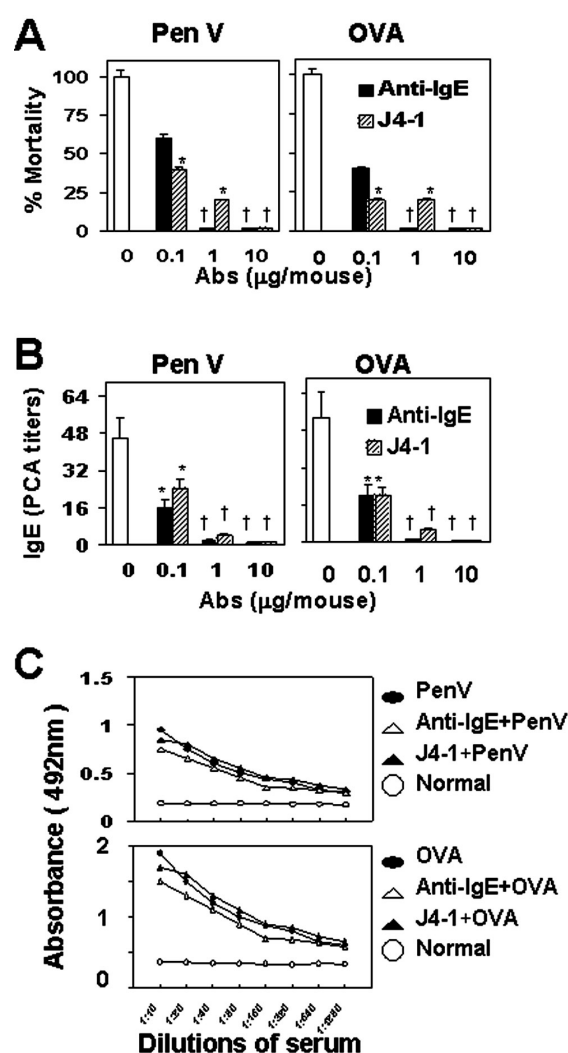


Figure 1. Effects of anti-IgE mAb on Pen V- and OVA-induced active fatal anaphylaxis (A) and antigen-specific IgE (B) and IgG (C) responses. Varying concentrations of Abs were administered s.c. 2 days prior to sensitization, and challenge injections were given 5 weeks after sensitization. Partial bleeding was performed 1 day before the challenge to assess Ab responses. Each group was comprised of 8-14 animals from two or three separate experiments. Values are expressed as mean \pm S.E. * p < .05; \dagger p < .01 vs. control group.

Statistical comparison was performed using one-way ANOVA followed by the Fisher test. Significant differences between the groups were determined using the unpaired Student's t-test. A value of $p < 0.05$ was accepted as an indication of statistical significance.

Results

Anti-IgE mAb prevents both Pen V- and OVA-induced active fatal anaphylaxis. The preventive effect of anti-IgE mAb on Pen V- and OVA-induced active fatal anaphylaxis was examined. When varying doses of anti-IgE mAb were administered 2 days prior to sensitization, anti-IgE mAb prevented Pen V-induced fatal anaphylaxis (Fig. 1A). At a concentration of 1 or 10 μ g, anti-IgE mAb prevented 100% of fatal reactions. Anti-IgE mAb also effectively prevented fatal OVA-induced anaphylaxis (Fig. 1A). Anti-IgE mAb blocked IgE responses to both Pen V and OVA in a dose-dependent manner (Fig. 1B), but it failed to suppress

IgG responses (Fig. 1C). Unexpectedly, the control Ab, J4-1, also displayed similar inhibitory effects to those of anti-IgE mAb in terms of anaphylactic symptoms (Fig. 1A) and Ab responses (Fig. 1B).

Anti-IgE mAb is also effective for preventing established Pen V- or OVA-induced anaphylaxis. The therapeutic efficacy of anti-IgE mAb on established Pen V- or OVA-induced anaphylaxis was also examined. Anti-IgE mAb showed a prompt protective effect against both types of anaphylaxis, significantly blocking mortality induced by Pen V as well as OVA up to one week after the administration of Abs (Fig. 2A), and caused a rapid reduction in the established antigen-specific IgE responses (Fig. 2B). J4-1 was also comparable to anti-IgE mAb in terms of protection of animals and IgE-suppressing activity (Fig. 2A and B).

Anti-IgE mAb prevents IgG-induced passive systemic anaphylaxis. The finding that anti-IgE mAb prevented OVA-induced fatal anaphylaxis suggests that anti-IgE

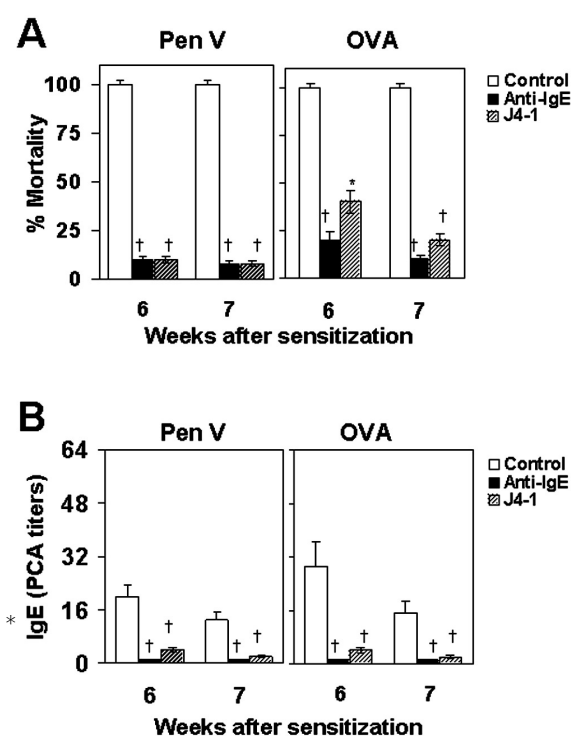


Figure 2. Anti-IgE mAb suppresses the established anaphylactic reactions (A) and IgE responses (B) to Pen V and OVA. Anti-IgE mAb or J4-1 (10 μ g each) was administered s.c. 2 days prior to challenge, which was given 5 weeks after sensitization. Partial bleeding was performed 1 day before the challenge to assess IgE responses. Each group was comprised of 8-10 animals from two separate experiments. Values are expressed as mean \pm S.E. [†] $p < .01$ vs. control group.

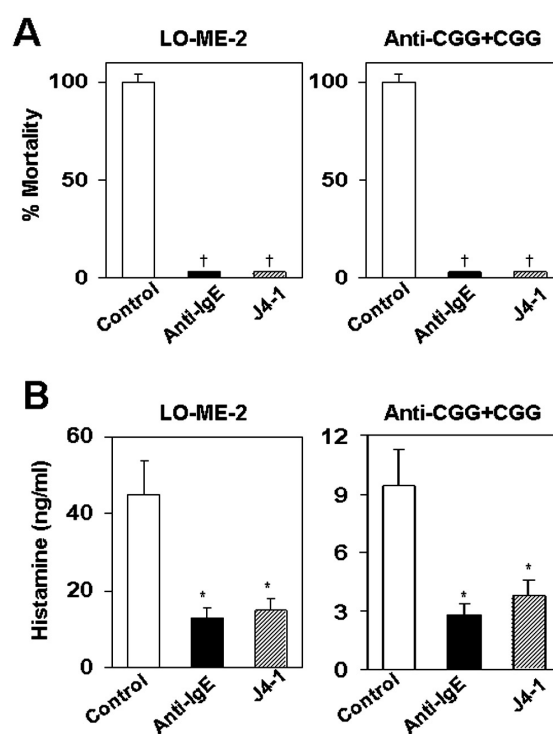


Figure 3. Anti-IgE mAb suppresses IgE- and IgG-induced passive systemic fatal anaphylaxis (A) and plasma levels of histamine (B). Anti-IgE mAb or J4-1 (10 μ g each) was administered s.c. Two days later, either anaphylactogenic rat anti-mouse IgE (LO-ME-2, 20 μ g) or the mixture of anti-CGG mAb (2.0 mg) and CGG (500 μ g) was given i.v. Plasma levels of histamine were measured 10 min after the injections. Each group was comprised of 8-10 animals from two separate experiments. Values are expressed as mean \pm S.E. ^{*} $p < 0.05$; [†] $p < .01$ vs. control group.

mAb is capable of inhibiting IgE- and IgG-induced anaphylactic reactions. Therefore, we investigated such a possibility by examining the effects of anti-IgE mAb on IgE- and IgG-induced passive systemic anaphylaxis. Pretreatment with anti-IgE mAb prevented the IgE- and IgG-induced passive fatal reactions (Fig. 3A), which was associated with a significant reduction in circulating histamine levels (Fig. 3B). J4-1 also displayed a similar inhibitory activity compared to anti-IgE mAb (Fig. 3, A and B). These data strongly suggest that anti-IgE mAb and J4-1 prevent anaphylaxis through their nonspecific abilities to suppress IgE synthesis and to induce mast cell inactivation.

Evidence indicates that anti-IgE mAb exerts its inhibitory activity via Fc γ RIIb engagement. Four classes of receptors for IgG Fc portion (Fc γ R) have been identified, Fc γ RI (CD64), Fc γ RII (CD32), Fc γ RIII (CD16), and the recently described Fc γ RIV (also termed FcRL3 and CD16-2) (25,26). Among them, Fc γ RIIb has single transmembrane domain and an intracytoplasmic domain that contains an immunoreceptor tyrosine- based inhibition motif (ITIM), which is conserved in all murine and human isoforms (27,28); thus, murine and human Fc γ RIIb negatively regulate cell activation. Fc γ RIIb inhibits B cell receptor-mediated B cell activation (29,30), T cell receptor-mediated T cell activation (31), and Fc γ RI-mediated mast cell activation (32). Based on these findings, anti-IgE mAb and J4-1 appear to display nonspecific inhibitory activities via Fc γ RIIb engagement.

OVA-induced active fatal anaphylaxis was induced in Fc γ RIIb^{-/-} mice and their littermates. In littermates, approximately 30%, 50%, and 20% of the

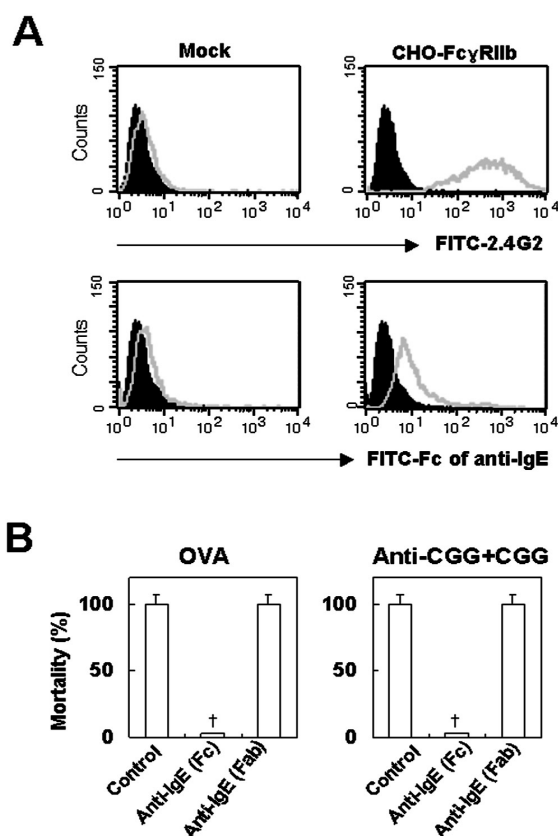


Figure 4. The Fc portion of anti-IgE mAb binds to the Fc γ RIIb-expressing CHO cell line and suppresses IgG-induced passive systemic anaphylaxis. (A) The mock-transfected or Fc γ RIIb-expressing CHO cell line was stained with 1 μ g/ml of either FITC-conjugated 2.4G2 mAb or the FITC-conjugated Fc portion of anti-IgE mAb at 4°C for 30 min, and was then analyzed by flow cytometry. The closed histogram indicates staining with FITC-conjugated isotype rat IgG, and the open histogram shows staining with FITC-conjugated 2.4G2 or the Fc protein of anti-IgE mAb. (B) The Fab or Fc portion of anti-IgE (10 μ g each) was administered s.c. OVA-induced active and anti-CGG + CGG-induced passive systemic anaphylaxis were induced two days later. Each group was comprised of 8 animals from two separate experiments. Values are expressed as mean \pm S.E. [†]p<0.01 vs. control group.

Table I. Anti-IgE prevents neither active anaphylaxis nor IgE response in Fc γ RIIb^{-/-} mice

Mice	Group	No. of mice	No. of Animals Showing following Shock Score ^a					IgE response PCA titer ^b
			0	1	2	3	4	
Fc γ RIIb ^{+/+}	No Ab	13	0	0	4	6	3	50 \pm 18
	Anti-IgE	12	0	8	3	1	0	6 \pm 2 ^c
	J4-1	12	0	9	3	0	0	5 \pm 2 ^c
Fc γ RIIb ^{-/-}	No Ab	13	0	0	2	3	8	63 \pm 22
	Anti-IgE	12	0	0	2	4	7	56 \pm 19
	J4-1	12	0	0	3	2	7	61 \pm 16

Anti-IgE or J4-1 (10 μ g each) was administered 2 days before OVA sensitization and challenge injections were given 5 weeks after sensitization. ^aShock Score; 0, no sign; 1, mild; 2, moderate; 3, severe; 4, death. ^bValues are expressed as mean \pm S.E. ^cp<0.01 vs control

mice undergoing anaphylaxis showed moderate symptoms, severe symptoms, and death, respectively, whereas pretreatment with anti-IgE mAb reduced the severity of anaphylactic symptoms so that 70% of the mice showed mild symptoms; the protective activity of anti-IgE mAb was not observed in Fc γ RIIB^{-/-} mice in any form (Table I). In parallel to the anaphylactic symptoms, anti-IgE mAb and J4-1 significantly suppressed the IgE response to OVA, but failed to suppress the IgE levels in Fc γ RIIB^{-/-} mice (Table I). In passive systemic anaphylaxis induced by either IgG or anaphylactogenic anti-IgE, the inhibitory effects on anaphylactic symptoms seen in littermates were no longer observed in Fc γ RIIB^{-/-} mice (data not shown). These data suggest that Fc γ RIIB is involved in the inhibitory activities of anti-IgE mAb and J4-1.

To confirm the involvement of Fc γ RIIB, we digested anti-IgE mAb with papain and purified Fab and Fc portions of the antibody. Flow cytometric analysis showed that FITC-conjugated 2.4G2 mAb, which is specific for mouse Fc γ RIIB and Fc γ RIIC (33), clearly bound to a CHO-stable cell line expressing Fc γ RIIB (CHO-Fc γ RIIB), but not to a mock-transfected cell line. This indicated that the stable cell lines are appropriate for use in examining the binding of Fc protein to Fc γ RIIB. As expected, FITC-conjugated Fc protein of anti-IgE mAb also bound to CHO-Fc γ RIIB, but not to the mock-transfected cell line (Fig. 4A). The purified Fc portion, but not the Fab portion, of anti-IgE mAb prevented OVA-induced active fatal anaphylaxis as well as IgG-induced passive fatal anaphylaxis (Fig. 4B). Taken together, these data indicate that anti-IgE mAb displays its inhibitory activity by binding to Fc γ RIIB via its Fc.

Discussion

In this study, we examined the therapeutic efficacy of anti-IgE mAb using various murine models of systemic anaphylaxis. Our study provides direct in vivo evidence that anti-IgE mAb can prevent anaphylaxis through Fc γ RIIB engagement rather than functioning to target IgE. This conclusion came from the following findings that: 1) anti-IgE mAb blocked IgG-induced anaphylaxis as well as histamine release; 2) anti-IgE mAb neither prevented anaphylaxis nor suppressed the antigen-specific IgE response in Fc γ RIIB^{-/-} mice; 3) the FITC-conjugated Fc portion of anti-IgE mAb

bound to CHO-Fc γ RIIB, and the Fc portion prevented OVA-induced active fatal anaphylaxis as well as IgG-induced passive fatal anaphylaxis.

Fc γ RIIB receptors suppress cellular activation by promoting dephosphorylation reactions resulting from the recruitment of SHIP to the ITIM (34). SHIP decreases the cellular levels of phosphatidylinositol-3,4,5-triphosphate (PIP3), resulting in the prevention of an influx of extracellular Ca²⁺ (35,36). PTEN also blocks the action of PI3K by dephosphorylating the PIP3. Ultimately, these phosphatases abrogate signaling by activating receptors, thereby preventing cellular immune functions such as cytotoxicity or proliferation.

In this regard, this study probably provides the mechanism by which anti-IgE mAb exerts various inhibitory activities, i.e., anti-IgE mAb likely prevents allergen-induced activation signals occurring in Fc γ RIIB-bearing cells by blocking the downregulation of Fc γ RIIB-associated phosphatases. Fc γ RIIB receptor isoforms are preferentially expressed in B lymphocytes, and are involved in the negative regulation of antibody production and B cell proliferation (37,38). In mast cells and basophils, co-clustering of Fc γ RIIB with activating Fc γ R causes an inhibition of degranulation (39). Based on these findings, anti-IgE mAb seems to provide negative signals to B cells and mast cells, thereby downregulating not only IgE synthesis from B cells, but also the release of chemical mediators from mast cells, which results in the prevention of anaphylaxis induced by either IgE or IgG. This concept could provide a possible explanation as to how anti-IgE mAb exerts inhibitory activities that cannot be explained as simply the targeting of free IgE, such as the prevention of IgG-induced passive anaphylaxis and histamine release as well as the prevention of active anaphylaxis without suppression of the IgE response.

Treatment with omalizumab in patients with asthma showed clinical benefits in terms of inhibition of allergen-induced early and late bronchoconstrictor responses (39,40), reduction in asthma exacerbations and corticosteroid requirements (41), and improvements in asthma-related quality of life (42-44). The underlying mechanism of these effects of omalizumab has been thought to be due to its ability to reduce serum levels of circulating free IgE by binding free IgE (45,47) and decreasing IgE production by the B cells (47), as well as its activity in downregulating Fc γ RI on basophils

and mast cells (48-51). These effects render the inflammatory cells insensitive to allergen stimulation. Hence, this therapeutic anti-IgE represents a new class of mast cell-stabilizing agents (52). However, omalizumab has produced a number of questions that remain unanswered despite massive investigations and numerous clinical trials (53). These include: i) an incompletely understood mechanism of action of omalizumab related to the downregulation of Fc γ RI in the presence of low levels of free IgE; ii) a lack of the ability to account for possible antigen sweeping by the complexes of IgG-IgE; and iii) skin-prick tests that may remain positive in omalizumab-treated patients for much longer than previously reported. In summary, we have provided evidence here to demonstrate an important role for the inhibitory IgG Fc receptor, Fc γ RIIb, in anti-IgE mAb-mediated inhibition of murine anaphylaxis. Currently, there is no evidence to indicate whether Fc γ RIIb is involved, even partially, in the inhibitory activity of omalizumab. However, the similar findings between this study and human studies in terms of suppression of IgE production and mast cell inactivation by anti-IgE mAb suggest such a possibility.

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