



Contents lists available at SciVerse ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio



An anti-IgE monoclonal antibody that binds to IgE on CD23 but not on high-affinity IgE.Fc receptors

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ARTICLE INFO

Article history:

Received 25 May 2011

Accepted 18 November 2011

Keywords:

Allergy

Anti-IgE

Basophils

CD23

FcεRI

IgE

Omalizumab

ABSTRACT

A new monoclonal antibody (mAb), specific for human IgE, the central mediator of immediate-type hypersensitivity reactions, has been shown to possess a unique set of binding specificities. The mAb, 8D6, binds to a conformational epitope on the CH3 domain of human e immunoglobulin and can compete with omalizumab for binding to IgE. Like omalizumab, it does not bind to IgE bound by the high-affinity IgE.Fc receptor (FcεRI) on basophils and mast cells. It also does not cause activation and degranulation of IgE-pulsed, human FcεRI-expressing rat basophilic leukemic cells (RBL SX-38). The mAb can inhibit IgE binding to recombinant α chain of human FcεRI in ELISA and to human FcεRI-expressing RBL SX38 cells in fluorescence flow cytometric analysis. However, unlike omalizumab, 8D6 can bind to IgE already bound by the low-affinity IgE.Fc receptors (FcεRII, or CD23), as revealed in ELISA with recombinant CD23 and in flow cytometric analysis with human B cells. Since earlier investigators have shown that anti-CD23 mAbs can inhibit the synthesis of IgE in lymphocyte culture *in vitro* and can down-regulate IgE production in treated patients, 8D6 may offer pharmacological mechanisms in addition to those mediated by omalizumab, for controlling IgE in patients with allergic diseases.

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Introduction

Allergic diseases, such as allergic asthma, allergic rhinitis, and food allergy, are very prevalent among populations living a Westernized lifestyle (Gold and Wright 2005; Isolauri et al. 2004). Numerous clinical studies of the humanized anti-IgE antibody, omalizumab, in many allergic diseases have shown that omalizumab is efficacious and safe in treating patients with those allergic diseases (Lanier et al. 2003; Milgrom et al. 1999; Rafi et al. 2010). These results have not only confirmed that IgE is a central mediator in the pathogenesis of many allergic diseases but have also validated IgE as a good molecular target for therapeutic intervention (Holgate et al. 2005). The widely recognized pharmacologic mechanisms of omalizumab are its ability to neutralize free IgE and to inhibit IgE binding to FcεRI on basophils and mast cells (Chang 2000; Chang et al. 2007). Moreover, the depletion of free IgE in the blood and in interstitial spaces causes a down-regulation of FcεRI on basophils and mast cells over time (Beck et al. 2004; Chang

and Shiung 2006). While the ability of the antibodies to bind to membrane-bound IgE (mIgE) (Davis et al. 1993), which forms part of the B cell receptor complex (Niir and Clark 2002), and to cause the lysis of IgE-expressing B cells and hence inhibit IgE production, was considered in the original design of the anti-IgE therapeutic antibodies, this property has not been confirmed for omalizumab in patients.

Therapeutic approaches aiming at suppressing IgE production, such as anti-interleukin 4 administration (Hart et al. 2002), TH2/TH1 modulation (Stokes and Casale 2004), and anti-CD23 treatments (Bonnefoy et al. 1990; Poole et al. 2005), have drawn much interest among researchers attempting to develop treatments for IgE-mediated allergic diseases. CD23 expressed on the surface of B cells, which binds to IgE with an association constant K_a of about 10^8 – 10^9 M⁻¹ (Gould and Sutton 2008), is known to be involved in the regulation of IgE production (Conrad et al. 2007). A mature CD23 molecule is formed by three 45-kDa type II transmembrane subunit proteins that belong to the C-type lectin family (Conrad 1990). Each subunit contains an α-helical stalk region between the extracellular lectin domain and the transmembrane domain, which bundles to form coiled-coil trimers (Beavil et al. 1992; Hibbert et al. 2005). Membrane-bound CD23 is cleaved by a metalloproteinase, ADAM10, releasing the secreted form of CD23 (Lemieux et al. 2007). It has been shown in mice *in vivo* that when the lectin domain of membrane-bound CD23 is bound

Abbreviations: ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; IgE, immunoglobulin E; mAb, monoclonal antibody; PEI, polyethylenimine; RT, room temperature.

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by IgE or by an anti-CD23 antibody, CD23 becomes more stable and is less susceptible to the cleavage by ADAM 10, which signals the B cell to reduce IgE synthesis (Conrad et al. 2007; Ford et al. 2006). Several chimeric anti-CD23 mAbs were shown to cross-link CD23 and inhibit the transcription of ϵ gene and the production of IgE by human peripheral B cells in culture (Yabuuchi et al. 2002). An anti-CD23 mAb, lumiliximab, was shown to induce apoptosis by cross-linking CD23 on B cell lines or on chronic lymphocytic leukemia cells (Pathan et al. 2008). Furthermore, lumiliximab was able to reduce blood IgE levels in allergic patients (Rosenwasser et al. 2003) and is potentially applicable for treating patients with chronic lymphocytic leukemia (Byrd et al. 2007).

In this study, we have developed an anti-IgE mAb, 8D6, which resembles omalizumab in various aspects, including being able to bind to the same region on the CH3 domain of ϵ chain, not being able to bind to IgE on Fc ϵ RI, and not being able to induce the activation of basophils. However, mAb 8D6, unlike omalizumab, has the additional attribute of being able to bind to IgE that is already bound by CD23 and hence has the potential to down-regulate IgE synthesis.

Materials and methods

Cells

Mouse NS0 myeloma cells and hybridoma cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, 50 μ g/ml penicillin (Invitrogen), and 100 μ g/ml streptomycin (Invitrogen). The RBL SX-38 cell line, provided by Dr. J.P. Kinet of Harvard Medical School, was maintained in MEM (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM GlutaMAX (Invitrogen), 25 mM HEPES, 1 mM sodium pyruvate, 50 μ g/ml penicillin, and 100 μ g/ml streptomycin. Additionally, G418 (Merck, Nottingham, UK) was diluted in the MEM medium at 2.4 mg/ml for culture splits and 1.2 mg/ml for alternating splits. The SKW cell line was maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat-inactivated FBS with 50 μ g/ml penicillin and 100 μ g/ml streptomycin. U-266 cells were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS with 50 μ g/ml penicillin, 100 μ g/ml streptomycin, and 1 mM sodium pyruvate. All of the cells mentioned above were maintained at 37 °C in 5% CO₂. The human embryonic kidney cell line, FreeStyle 293F (Invitrogen), was cultured in FreeStyle 293 Expression Medium (Invitrogen) in Erlenmeyer flasks (Corning Glass, Corning, NY) on an orbital shaker platform at 37 °C in humidified air (with 8% CO₂).

Generation of anti-IgE mAbs

BALB/c mice were immunized twice subcutaneously with mIgE.FcL, a recombinant preparation of the membrane-bound form of human ϵ chain (Chen et al. 2010) emulsified in TiterMAX Gold adjuvant (Sigma–Aldrich, St. Louis, MO) at 2-week intervals. The final boost was administered intraperitoneally with 0.1 mg of mIgE.FcL without adjuvant. By employing a standard procedure for preparing hybridomas (Chen et al. 2010), hybridoma clones secreting anti-IgE mAbs were further examined for their ability to compete with omalizumab for binding to IgE. Briefly, aliquots of culture supernatants of hybridomas were mixed with horseradish peroxidase (HRP)-conjugated omalizumab, and the mixture was added to wells of ELISA plates coated with 1 μ g/ml human IgE and incubated for 1 h at room temperature (RT). The wells were then washed 5 times with PBS-T (1 $\times$ PBS, 0.05% Tween-20) and then incubated with peroxidase substrate NeA-Blue (Clinical Science Products, Mansfield, MA) for 2 min at RT. The reaction was

stopped by adding 1 N HCl and the optical density (OD) at 450 nm was read with a Model 680 microplate reader (BioRad Laboratories, Hercules, CA). The clones that inhibited omalizumab binding to human IgE were cultured and their secreted antibodies purified for various analyses. HRP–omalizumab used in this analysis was prepared by the sodium periodate method described (Wisdom 2005).

Purification of human IgE and anti-IgE mAb

Omalizumab was coupled to CNBr-activated Sepharose 4 Fast Flow resin (GE Healthcare, Fairfield, CT) according to the manufacturer's instruction, and the omalizumab-conjugated resin was equilibrated with 50 mM Tris–HCl, pH 8.0. To purify human IgE, the culture supernatant of U-266 cells was harvested and passed through a column packed with the equilibrated resin. Unbound contaminants were removed by flushing the resin with 50 mM Tris–HCl, pH 8.0. Captured IgE was eluted with 0.1 M glycine, pH 3.0, and the eluate was brought to neutral pH immediately with 1 M Tris–HCl, pH 8.0. For preparing the mAb, 8D6, the culture supernatant of a hybridoma clone producing 8D6 was purified using a column packed with Protein A Sepharose CL-4B resin (GE Healthcare).

Preparation of recombinant α chain of human Fc ϵ RI

The fusion protein, huFc ϵ RI α –huFc γ , linking the extracellular portion of the α chain of human Fc ϵ RI (denoted as huFc ϵ RI α) and the Fc of human γ 1 immunoglobulin (denoted as huFc γ) was prepared by expressing their fused corresponding gene segments in a mammalian expression system. For the fused gene, the respective DNA segments, encoding Ig κ leader peptide, the extracellular region of human Fc ϵ RI α (from Val²⁶ to Leu²⁰⁴), and CH2–CH3 of human γ 1, were linked as a single segment and placed in the pSec-Tag expression vector (Invitrogen). The expression vector with its insert was transfected into FreeStyle 293F cells by the polyethylenimine (PEI) method (Polysciences, Warrington, PA). Briefly, lots of 1 $\times$ 10⁷ FreeStyle 293F cells were resuspended in 0.8 ml of FreeStyle 293 Expression Medium in an Erlenmeyer flask. In a separate tube, 100 μ l of 9 g/L NaCl solution containing 18 μ g of PEI was mixed with 100 μ l of pre-warmed serum-free DMEM containing 6 μ g of the DNA to be transfected. The PEI and the DNA were immediately mixed by vortexing, incubated for 10 min at room temperature, and added to the flask containing the FreeStyle 293F cells. Four hours later, 3 ml of FreeStyle 293 Expression Medium was added per 10⁷ cells, and the transfected cells were incubated on a shaker as described above. After 4 days, the culture medium containing huFc ϵ RI α –huFc γ was harvested and passed over a column packed with Protein A Sepharose CL-4B resin. The fusion protein was purified, concentrated, and buffer-exchanged to HBSS buffer (2 mM CaCl₂ in 1 $\times$ HBSS) using Amicon Ultra tubes (Millipore, Billerica, MA).

Preparation of recombinant CD23

The vector pET5a–HuLZ, a gift from Dr. D.H. Conrad of Virginia Commonwealth University, was used as a source to amplify a gene segment encoding an isoleucine zipper (denoted as ILZ) and the extracellular region (from Asp⁴⁸ to Ser³²¹) of human CD23 (denoted as huCD23). For this, a set of PCR primers, taaGGTACcaagaatgaacagatagaggataagatcgaagagatc and gcaGAATTCtaagagtggagagggcagag, were synthesized and employed. The amplified gene segment encoding the recombinant human CD23 protein, ILZ–huCD23, was digested by restriction enzymes *Kpn*I and *Eco*RI and ligated into a pYSC expression vector with a polyhistidine tag at the 5' end of the cloning site. The expression vector with the CD23 insert was

transfected into FreeStyle 293F cells by the PEI method as described above. The culture medium of the transfected FreeStyle 293F cells was harvested and passed over an equilibrated nickel Sepharose column (GE healthcare). The polyhistidine-tagged ILZ-CD23 fusion protein was eluted with elution buffer (20 mM NaH₂PO₄, 500 mM NaCl, and 500 mM imidazole, pH 8.0) according to the manufacturer's instructions. The eluted fusion protein was concentrated and buffer-exchanged to HBSS buffer (2 mM CaCl₂ in 1 × HBSS) using Amicon Ultra tubes.

ELISA for characterization of mAbs that compete with omalizumab for binding to human IgE

The wells of ELISA plates were coated with 4 µg/ml of purified human IgE in coating buffer (15 mM Na₂CO₃, 35 mM NaHCO₃, pH 9.6) and subsequently blocked by incubating with blocking buffer (1% BSA in PBS) overnight at 4 °C. Separately, a stock solution of HRP-omalizumab was diluted 1:4000 in the blocking buffer and aliquots were mixed with unconjugated omalizumab and 8D6 serially diluted from 0.09 mg/ml in 1:3 steps. Both series of mixtures were added to the ELISA wells and incubated for 30 min at RT. The wells were then washed with PBS-T, incubated with NeA-Blue, and their OD₄₅₀ read as described above.

ELISA for determining the binding of anti-IgE mAbs to IgE fragments

The wells of ELISA plates were coated with 2 µg/ml of recombinant dimeric human IgE.Fc (designated as εCH2-3-4) and dimeric human CH3 domain (designated as Cys328-εCH3) in coating buffer at 4 °C overnight and treated with blocking buffer for 1 h at RT. The coated IgE fragments were then incubated with various antibodies, including 8D6, omalizumab, 13F7 (an anti-εCH2 mAb prepared in our lab), an anti-IgE mAb 5H2 (Serotec, Oxford, UK), mouse IgG (Sigma-Aldrich), or human IgG (Sigma-Aldrich), at 1 µg/ml in blocking buffer for 1 h at RT. After the wells were washed 3 times with PBS-T, the bound antibodies were assayed using HRP-conjugated goat anti-mouse IgG.Fc or HRP-conjugated goat anti-human IgG.Fc (both from Jackson ImmunoResearch, West Grove, PA) and peroxidase substrate NeA-Blue.

ELISA for measuring the binding of anti-IgE mAbs to recombinant FcεRIα and CD23

ELISA was performed to examine the binding of mAbs to IgE bound by recombinant α chain of human FcεRI and to IgE bound by recombinant CD23 on the solid phase. For the former, ELISA strip plates (Nunc, Denmark) were coated with purified huFcεRIα-huFcγ fusion protein at 2 µg/ml in coating buffer at 4 °C overnight and blocked by incubating with blocking buffer for 1 h at RT. The coated huFcεRIα-huFcγ was then saturated with IgE by incubating with purified human IgE at 2 µg/ml in blocking buffer for 1 h at RT, followed by rinsing the wells 3 times with 200 µl PBS-T. For the experiments with recombinant CD23, ELISA plates were coated with purified ILZ-CD23 at 5 µg/ml and blocked, and the coated ILZ-CD23 was then saturated with 5 µg/ml of purified human IgE in blocking buffer and washed. Various antibodies, including 8D6, omalizumab, 5H2, mouse IgG and human IgG, at 1, 3, and 10 µg/ml in blocking buffer were added to the wells of ELISA plates, and the plates incubated for 1 h at RT. The wells were then washed 3 times with PBS-T. Antibody binding was then measured colorimetrically by employing appropriate secondary antibodies, including HRP-conjugated goat anti-mouse IgG.Fc, HRP-conjugated goat anti-human κ light chain (GeneTex, San Antonio, TX), and HRP-conjugated goat anti-human IgG.Fc, using the peroxidase substrate NeA-Blue.

Preparation of IgE:anti-IgE immune complexes

Purified human IgE (about 190 kDa), omalizumab, 5H2, and 8D6 (IgGs about 150 kDa) were diluted to 0.1 mg/ml in 1 × PBS. Mixtures containing IgE:anti-IgE immune complexes were prepared by mixing 95 µl of 0.1 mg/ml human IgE (about 50 pmol) and 75 µl of 0.1 mg/ml anti-IgE mAbs (about 50 pmol). The mixtures were incubated for 2 h at RT and used for flow cytometry analyses.

Flow cytometry analysis

Lots of 2 × 10⁵ RBL SX-38 cells or SKW cells were washed twice with "staining buffer" (1 × PBS containing 1% FBS and 0.01% sodium azide) and resuspended in 100 µl of staining buffer containing 1 µg/ml human IgE for 30 min at 4 °C. The cells were washed twice again to remove unbound IgE, resuspended in 100 µl staining buffer containing 8D6, omalizumab, or 5H2 at 2 µg/ml, and incubated for 30 min at 4 °C. The cells were then washed twice and incubated with 100 µl staining buffer containing FITC-conjugated F(ab')₂ rabbit anti-mouse IgG (Serotec), or FITC-conjugated goat anti-human IgG.Fc (Caltag Laboratories, Burlingame, CA) diluted 1:400 for 30 min at 4 °C. After another two washes, the stained cells were analyzed on a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA). For analyzing whether anti-IgE mAbs could block the binding of IgE to FcεRI on RBL SX-38 cells, purified human IgE at 1 µg/ml was mixed with 0.04, 0.2, 1, or 5 µg/ml of anti-IgE mAbs in 1 × PBS for 1 h at RT. The mixtures were added to 2 × 10⁵ RBL cells diluted in the staining buffer. IgE binding to RBL SX-38 cells was measured by incubating these cells with 100 µl of staining buffer containing 0.1 µg/ml of the FITC-conjugated anti-IgE mAb IgE21 (eBiosciences, San Diego, CA). To analyze whether IgE:anti-IgE immune complexes would bind to RBL SX-38 cells or SKW cells, IgE and anti-IgE mAbs were mixed at a molar ratio of 1:1 and the mixtures were then analyzed similarly, except that the cells were not pre-saturated with IgE.

Sensitization of RBL-SX38 cells and β-hexosaminidase release assay

RBL SX-38 cells were seeded in a 96-well plate at 4 × 10⁴ cells/well in 200 µl culture medium containing G418 overnight in a 37 °C incubator. On the next day, the medium was removed and 100 µl of pre-warmed culture medium containing 1 µg/ml purified human IgE was added to each well. After 2 h of incubation in a 37 °C incubator, each well was washed twice with 200 µl Tyrode's buffer (135 mM NaCl, 5 mM KCl, 5.6 mM glucose, 1.8 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and 0.5 mg/ml BSA, pH 7.3) and 100 µl of pre-warmed Tyrode's buffer containing 0.04, 0.2, 1, or 5 µg/ml of antibodies and 1% Triton X-100 (Merck) added. After a 30-min incubation in a 37 °C incubator, the plate was centrifuged at 300 × g for 10 min at RT and 50 µl supernatant was transferred from each well to that of a new 96-well black OptiPlate (Perkin-Elmer, Wellesley, MA). 50 µl of assay solution (0.1 M citric acid, pH 4.5, containing 80 µM of 4-MUG (4-methylumbelliferyl-N-acetyl-β-D-glucosaminide)) was added into each well and the plate was incubated at 37 °C and 8% CO₂ with continuous shaking for 1 h. The reaction was terminated by adding 100 µl glycine buffer (0.2 M glycine, 0.2 M NaCl, pH 10.7). The resulting fluorescence (excitation 355 nm; emission 460 nm) was measured at the top of each well by a Victor 3 fluorescence reader (Perkin-Elmer). Measured values were expressed as percentages relative to the value of release (100%) obtained by lysing cells with 1% Triton X-100.

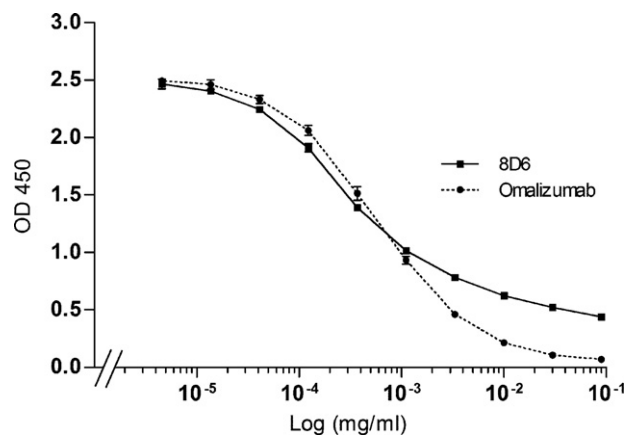


Fig. 1. MAb 8D6 competes with omalizumab effectively for binding to human IgE. The competitive ELISA, which examines 8D6 and unlabeled omalizumab in competing with HRP–omalizumab in binding to human IgE, is described in “Materials and methods”. Each point represents the mean $\pm$ SD of triplicate measurements. In a related analysis, the experimental data were used to generate best-fitted curves, from which IC₅₀ with R -square values > 0.99 under the experimental conditions were obtained; for omalizumab, IC₅₀ = 0.56 μ g/ml and for 8D6, IC₅₀ = 0.31 μ g/ml.

Results

Generation of anti-human IgE mAbs that bind to IgE on Fc ϵ RII but not Fc ϵ RI

To obtain anti-human IgE mAbs with the desired set of binding specificities as described in the introduction, BALB/c mice were immunized with human mIgE.FcL, and hybridoma clones were prepared. In the primary screening procedure, the clones specific for human IgE in the initial ELISA were tested for their ability to compete with omalizumab for binding to IgE. Those that did compete with omalizumab were then screened for their ability to bind to human IgE-pulsed CD23-expressing SKW cells by fluorescence flow cytometry. After these primary binding features were determined, the mAbs were then tested for their inability to bind to IgE bound by Fc ϵ RI on basophils and to stimulate basophil sensitization and the discharge of pharmacological mediators. A number of mAbs that met the primary screening criteria were identified. One mAb, 8D6,

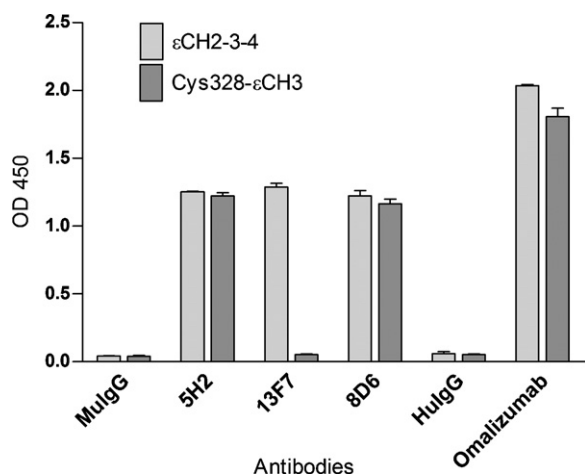


Fig. 2. MAb 8D6 binds to the CH3 domain of the human ϵ chain. The wells of ELISA plates were coated with recombinant human IgE.Fc (ϵ CH2-3-4) or dimeric CH3 (Cys328- ϵ CH3) at 2 μ g/ml and treated with blocking buffer. The mAbs to be tested or control antibodies were then added at 1 μ g/ml. The amounts of bound antibodies were measured by reaction with HRP-conjugated goat anti-mouse IgG.Fc or goat anti-human IgG.Fc, as described in “Materials and methods”. Data represent the means $\pm$ SD of triplicate measurements.

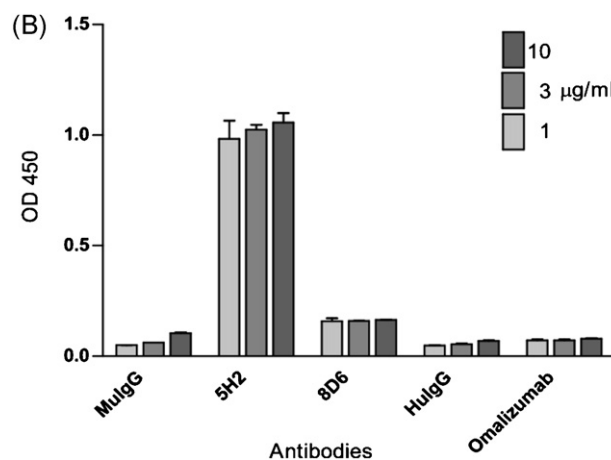
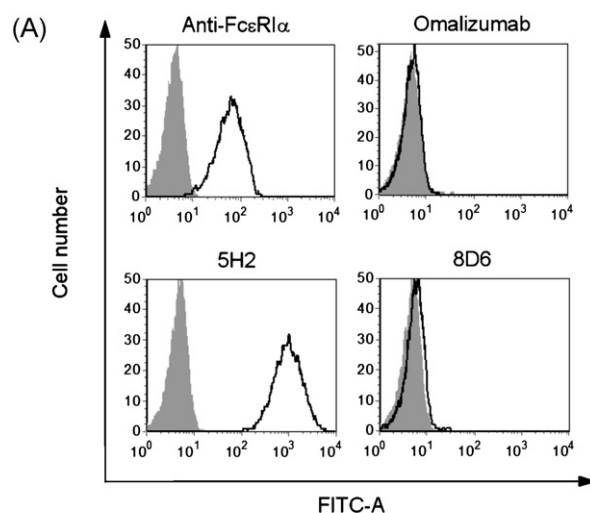


Fig. 3. MAb 8D6 is unable to bind to IgE on Fc ϵ RI. (A) In the flow cytometry analysis, 2×10^5 RBL SX-38 cells were preincubated with 1 μ g/ml human IgE. The cells were then washed and incubated with the mAbs to be examined at 1 μ g/ml. The binding of the mAbs was then measured by FITC-conjugated F(ab')₂ rabbit anti-mouse IgG or goat anti-human IgG.Fc, as described in “Materials and methods”. (B) In the ELISA, wells of ELISA plates were coated with recombinant α chain of human Fc ϵ RI (huFc ϵ RI α -huFc γ fusion protein) at 2 μ g/ml and the coated α chain was subsequently saturated with human IgE at 2 μ g/ml. After washing, the wells were incubated with the mAbs to be tested or control antibodies, and the amounts of bound antibodies were measured by reaction with HRP-conjugated goat anti-mouse IgG.Fc or goat anti-human κ light chain, as described in “Materials and methods”. Data represent the means $\pm$ SD of triplicate measurements.

belonging to γ 2a heavy chain subclass and κ light chain (γ 2a, κ), was further characterized and found to be able to bind to IgE on Fc ϵ RII (CD23) on B cells.

The mAb 8D6 competes with omalizumab for binding to IgE

To determine the relative locations of the binding sites of 8D6 and of omalizumab on human IgE, competitive ELISA tests were performed to compare the effectiveness of the two mAbs to compete with HRP-conjugated omalizumab in binding to human IgE. 8D6 did compete with HRP-omalizumab for binding to IgE with an IC₅₀ of 0.31 μ g/ml, the IC₅₀ of unconjugated omalizumab in the same assay was 0.56 μ g/ml (Fig. 1). The mAb 5H2, which binds to another site on CH3 of human ϵ chain, competed with HRP-omalizumab much less effectively. The near-equal efficacy of 8D6 and omalizumab competition for HRP-omalizumab binding suggests that 8D6 probably binds to a site very close to omalizumab's binding site on the CH3 domain of human ϵ chain. In

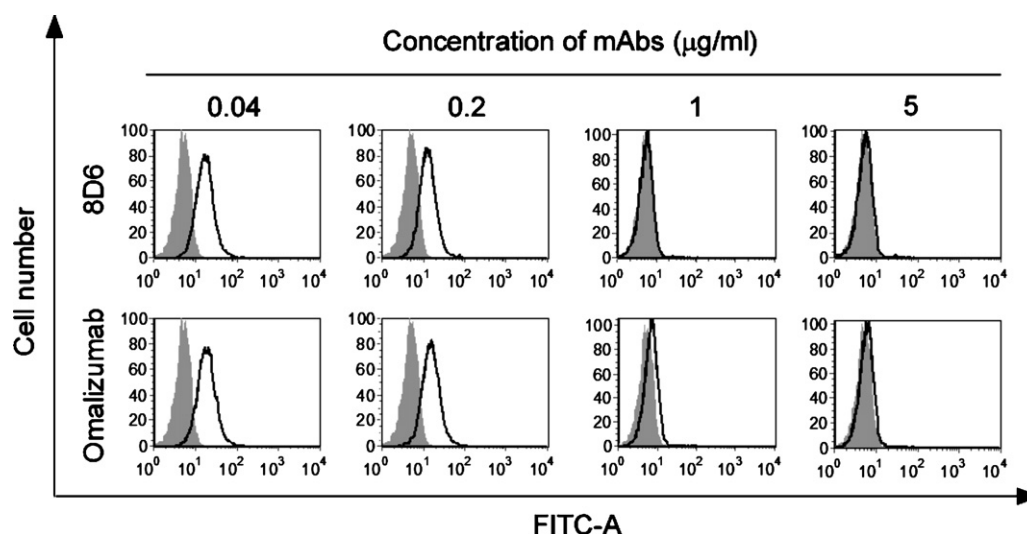


Fig. 4. MAbs 8D6 blocks the binding of IgE to FcεRI. In flow cytometric analysis, 1 µg/ml of human IgE was mixed with 0.04, 0.2, 1, or 5 µg/ml of 8D6 or omalizumab. The mixture was added to 2×10^5 RBL SX-38 cells. After the cells were washed, IgE binding to the cells was measured by FITC-conjugated anti-IgE mAb (IgE21).

an ELISA test, 8D6, omalizumab, and mAb 5H2, but not mAb 13F7 (which binds to the CH2 domain of human ϵ chain) bound to a recombinant human ϵ chain fragment, Cys328– ϵ CH3, containing the CH3 domain and the cysteine residue (#328) at the C-terminal of the CH2 domain (Fig. 2). These results confirm that 8D6 binds to the CH3 domain of human ϵ chain.

Inability of 8D6 to bind to IgE on basophils

To determine whether or not 8D6 is capable of binding to IgE bound by FcεRI on the cell surface, cells of the RBL SX-38 cell line, a rat basophilic leukemia cell line transfected with genes encoding the α , β , and γ chains of human FcεRI (Wiegand et al. 1996), were used as a pool of cell surface FcεRI. 8D6, omalizumab, or 5H2 were added to RBL SX-38 cells that had been pre-incubated with human IgE, and the binding of the mAbs to the cells was measured with FITC-conjugated secondary antibodies (Fig. 3A). Both 8D6 and omalizumab were unable to bind to IgE-saturated RBL SX-38 cells, while 5H2 demonstrated substantial binding.

Inability of 8D6 to bind to IgE-saturated recombinant α chain of FcεRI

To confirm 8D6's inability to bind to IgE bound by FcεRI, ELISA was used to examine the binding of 8D6 to the solid-phase recombinant FcεRI α fusion protein, huFcεRI α –huFc γ , which had been pre-incubated with human IgE (Fig. 3B). Neither 8D6 nor omalizumab at 1, 3, and 10 µg/ml showed significant binding to IgE-saturated recombinant FcεRI α fusion protein, while 5H2 did so at all three concentrations.

The mAb 8D6 blocks the binding of IgE to FcεRI

To determine whether 8D6 resembles omalizumab in being able to inhibit IgE binding to FcεRI, purified human IgE was mixed with increasing amounts of 8D6 or omalizumab, and the mixtures were added to suspensions of RBL SX-38 cells. The binding of the cells by IgE, either in free form or in complex form, was then measured by FITC-conjugated anti-IgE mAb. The mAb 8D6 blocked the binding of IgE to FcεRI at concentrations equal to or higher than that of IgE (Fig. 4).

The mAb 8D6 can bind to IgE-pulsed SKW cells

To investigate whether 8D6 is capable of binding to IgE on CD23, SKW cells, a human B cell line expressing high levels of CD23 (Pathan et al. 2008) was used to pulse CD23 by incubating with purified human IgE. Subsequently, these IgE-pulsed SKW cells were incubated with 8D6, 5H2, and omalizumab. The binding of the mAbs to IgE on CD23 was then measured by FITC-conjugated secondary antibodies. At a concentration of 1 µg/ml, omalizumab was prevented from binding to IgE molecules bound by CD23, while 5H2 and 8D6 were still able to effectively bind to IgE on SKW cells (Fig. 5A).

8D6 can bind to IgE on recombinant CD23

To confirm that 8D6 can bind to IgE bound by CD23, ELISA was used to examine the binding of 8D6 to solid-phase recombinant human CD23 fusion protein, IL2–CD23, which had been pre-saturated with human IgE. Fig. 5B shows that 8D6, as well as 5H2, can bind to human IgE-saturated recombinant CD23 coated on solid surface, while omalizumab has marginal binding, comparable to the isotype-control human IgG antibodies, to human IgE-saturated CD23.

The immune complexes of 8D6 and IgE bind to B cells expressing CD23

Since we suggest that 8D6 may have potential as an IgE-targeting agent in patients with allergy, it is pertinent to examine whether the IgE:8D6 immune complexes, which would be formed in a patient treated with 8D6, can bind to FcεRI or CD23. For these experiments, 8D6, omalizumab, and 5H2 were individually mixed with human IgE at 1:1 molar ratio and then incubated with RBL SX-38 and SKW cells. The binding of the immune complexes to the cells was then measured by FITC-conjugated secondary antibodies specific for either human IgG.Fc or mouse IgG.Fc (Fig. 6A and B). The IgE:omalizumab complexes did not stain either RBL SX-38 or SKW cells, indicating that IgE:omalizumab cannot bind to FcεRI or CD23. The IgE:8D6 complexes did stain SKW cells, but not RBL SX-38 cells, indicating that IgE:8D6 complexes can bind to CD23, but not to FcεRI. The immune complexes of IgE with the control mAb 5H2 stained both cell types, indicating that the IgE:5H2 complexes bind to both FcεRI and CD23.

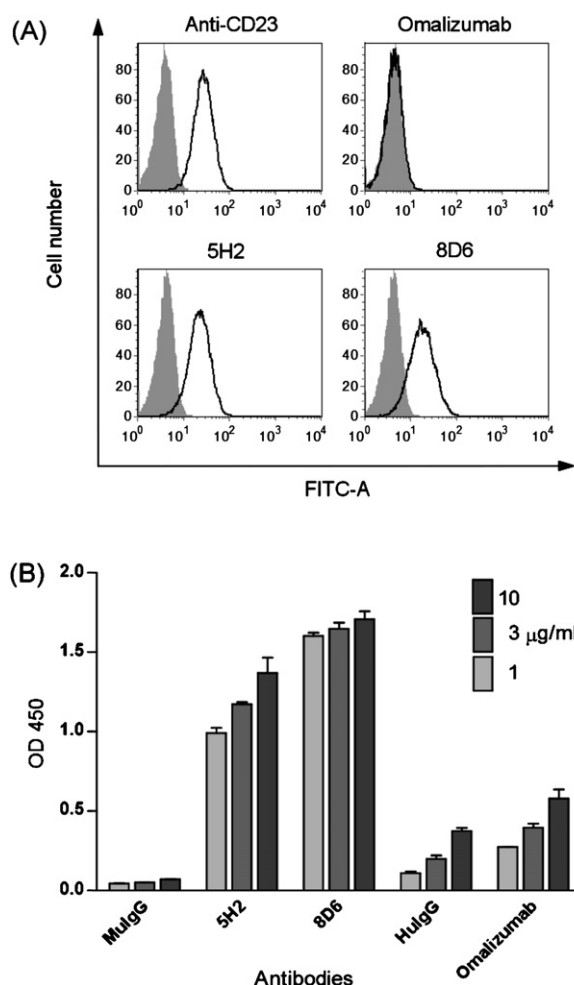


Fig. 5. MAb 8D6 is capable of binding to IgE bound by CD23. (A) In flow cytometric analysis, 2×10^5 SKW cells were preincubated with human IgE at 1 µg/ml. After washing, the wells were incubated with mAbs to be examined at 1 µg/ml. The binding of the mAbs were then measured with FITC-conjugated F(ab')₂ rabbit anti-mouse IgG or goat anti-human IgG.Fc. (B) In the ELISA, wells of ELISA plates were coated with recombinant CD23 (ILZ-CD23) at 5 µg/ml and subsequently saturated with human IgE at 5 µg/ml. After washing, the wells were incubated with the mAbs to be examined or control antibodies and the binding of the antibodies measured with HRP-conjugated goat anti-mouse IgG.Fc or goat anti-human IgG.Fc. Data are the means $\pm$ SD of triplicate measurements. Note that in the present ELISA, human IgG exhibits some degree of binding to the solid-phase proteins.

Inability of 8D6 to sensitize RBL SX-38 cells

The mAb 8D6 was further examined for its inability to activate basophils and cause their degranulation. For this, 8D6 and other control mAbs, at various concentrations, were added to cultures of RBL-SX38 cells pre-incubated with human IgE. The extent of degranulation was assessed by measuring the release of β -hexosaminidase, a lysosomal enzyme stored in granules, into the culture medium (Fig. 7). The mAb 5H2 did induce the degranulation of IgE-sensitized RBL-SX38 cells, while both omalizumab and 8D6 did not.

Discussion

The anti-IgE therapeutic antibody, omalizumab, has been approved in the United States since 2003 for the treatment of moderate to severe allergic asthma and in the European Union since 2005 for the treatment of severe allergic asthma (Chang et al. 2007). Many patients have been given the same omalizumab dosage continuously for several years without a clearly defined treatment

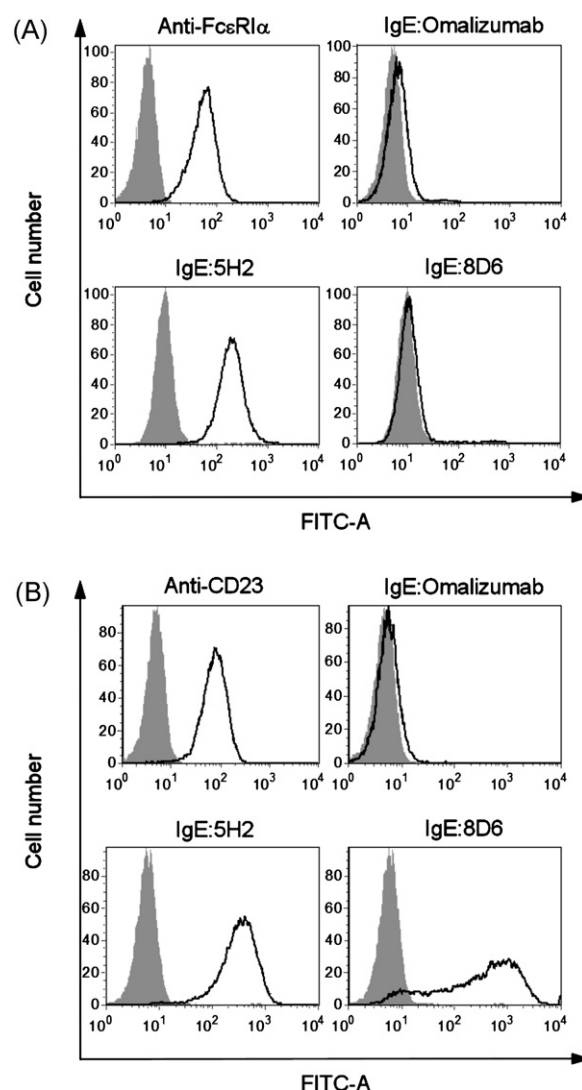


Fig. 6. The different ability of IgE:8D6 immune complexes to bind to FcεRI and to CD23. IgE and anti-IgE mAbs were mixed in PBS at a molar ratio of 1:1 to form immune complexes. The mixtures of immune complexes were then used to stain (A) RBL SX-38 cells and (B) SKW cells. The degree of staining was then measured as described in Figs. 3A and 5A.

period (Lanier 2006). The general understanding among the clinicians prescribing omalizumab is that IgE is continuously being synthesized in the treated patients and that the same anti-IgE regimen should hence be continued. This continual dosing requirement consumes large medical resource per patient and causes the health care system to erect high thresholds for approving the use of omalizumab for allergic patients. Patients with severe allergic asthma, whose serum IgE levels are higher than about 700 international units per ml, are currently precluded from using omalizumab by the health care systems (Holgate et al. 2009).

It would be desirable to develop an antibody therapeutic that possesses all the pharmacological attributes of omalizumab, but in addition, the ability to inhibit IgE production in the treated patient. A number of therapeutic approaches that aim to reduce IgE production have been proposed and examined (Stokes and Casale 2004). A major line of research is to employ mAbs specific for CD23 for down-regulating IgE production (Rosenwasser and Meng 2005). For example, lumiliximab, a chimeric macaque/human mAb specific for the lectin domain of CD23, was shown to be capable of depressing blood IgE levels in patients in Phase I human clinical trials (Rosenwasser et al. 2003). It has been reported that anti-CD23

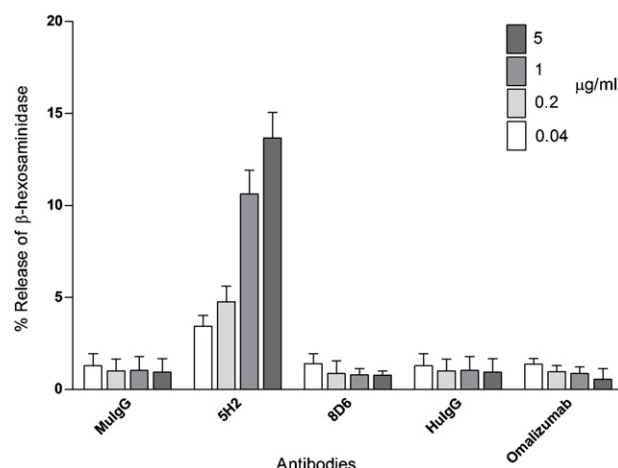


Fig. 7. MAb 8D6 cannot induce the activation and degranulation of RBL SX-38 cells. RBL SX-38 cells were preincubated with human IgE at 1 $\mu\text{g/ml}$. After washing, the cells were incubated with the mAbs to be tested and control antibodies in Tyrode's buffer at various concentrations for 30 min at 37 $^{\circ}\text{C}$. The release of β -hexosaminidase into the Tyrode's buffer was then measured with 4-MUG, a substrate of the enzyme, as described in "Materials and methods". Data are the means $\pm$ SD from triplicate measurements.

mAbs can cross-link CD23 on human B cells and induce feedback inhibitory signals for IgE production (Bonnefoy et al. 1990; Conrad et al. 2007; Nakamura et al. 2000).

In the present study, we have developed a new mAb, 8D6, with a set of binding specificities not previously reported. The mAb 8D6 competes effectively with omalizumab in binding to the CH3 domain of human ϵ chain. The antigenic epitope of 8D6, like that of omalizumab, is conformational (data not shown). Like omalizumab, 8D6 cannot bind to IgE bound by Fc ϵ RI, but unlike omalizumab, 8D6 can bind to IgE bound by CD23. We thus propose that the mAb 8D6 possesses not only the major pharmacologic properties of omalizumab to neutralize IgE without sensitizing mast cells and basophils but also the property of an anti-CD23 mAb to cross-link CD23 on B cells. Our rationale for a therapeutic approach employing an 8D6-like antibody is supported by the recent report (Griffith et al. 2011) that anti-IgE and anti-CD23 can induce B cells preloaded with IgE to undergo activation and subsequent signal transduction and that the B cells can be preloaded with antigen-specific IgE and triggered to undergo activation process by the antigen to which the IgE is specific.

It has been suggested that by binding to IgE, CD23 on B cells becomes stabilized and induces negative feedback signals for IgE synthesis. Consequently, when IgE is high, CD23 molecules are mostly occupied by IgE and hence suppress IgE synthesis, and when IgE is low, CD23 molecules are mostly unoccupied and therefore enhance IgE synthesis (Conrad et al. 2007; Gould and Sutton 2008). It is possible that 8D6 may contribute to the negative feedback in IgE synthesis at high and low IgE concentrations. Conceptually, after a period of treatment with 8D6, which behaves like omalizumab, the free IgE in blood and extracellular fluids would be depleted, the immune complexes of IgE:8D6 accumulated to elevated levels, and the Fc ϵ RI on basophils and mast cells gradually down-regulated. During this period, the immune complexes of IgE:8D6, unlike those of IgE:omalizumab, should bind to the bare CD23 on B cells and cause their cross-linking, which should still have a negative feedback effect on IgE synthesis. We are currently exploring the development of a mouse model to investigate whether 8D6 can modulate B cells and thus regulate IgE production.

Acknowledgements

We thank Dr. Bing-Hung Chen for helping us to obtain experimental materials from Dr. D.H. Conrad. This study was supported by a grant, NSC99-2320-B-001-006-MY3, from the National Science Council, Taiwan.

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