

Monoclonal Antibodies against the C ϵ mX Domain of Human Membrane-Bound IgE and Their Potential Use for Targeting IgE-Expressing B Cells

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Key Words

IgE · mIgE · C ϵ mX · IgE-expressing B cells · Monoclonal antibody · Fc fusion protein

Abstract

Background: IgE mediates immediate-type hypersensitivity reactions responsible for various allergic symptoms. It is secreted by IgE-producing plasma cells, which differentiate from B cells expressing membrane-bound IgE (mIgE) on their surface. The ϵ -chain of human mIgE contains a membrane-anchoring peptide and an extra 52-amino-acid (a.a.)-long domain (referred to as C ϵ mX) between the membrane anchor and the CH4 domain.

Objective: The study was designed to evaluate the effects of C ϵ mX-specific monoclonal antibodies (mAbs) to target IgE-expressing B cells and decrease IgE production. **Methods:** A C ϵ mX-containing IgG1.Fc fusion protein was produced in CHO cells and used to immunize mice; five hybridoma clones secreting C ϵ mX-specific mAbs were obtained. **Results:** Characterization of the mAbs using ELISA, immunoprecipitation, and immunoblotting methods showed that they could bind to both native and denatured forms of C ϵ mX. The mAbs exhibited mutual inhibition of binding to mIgE. Epitope map-

ping using synthetic peptides revealed that all five mAbs recognize the same epitope, RADWPGPP, located near the C-terminus of C ϵ mX. Binding of one of the mAbs to mIgE on SKO-007 cells induced the cross-linking of mIgE molecules on the cell surface, resulting in their patching and capping. In vitro functional analysis revealed that mAbs are able to cause complement-mediated cytotoxicity on transfectants expressing the Fc portion of mIgE.

Conclusion: We have prepared several human mIgE-specific mAbs. The potential of the mAbs on targeting mIgE+ B cells was demonstrated by CDC analysis.

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Introduction

The symptoms of most allergic reactions result from the cross-linking of IgE-armed IgE.Fc receptors on mast cells and basophils by multivalent allergens and the subsequent release of mediators by those allergen-sensitized cells. Since IgE plays a central role in those allergic responses, devising treatments to control IgE levels in the body and regulating IgE synthesis has been of great interest to immunologists. The membrane immunoglobulin on mature B cells is essential for B cell functions [1] and can

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transduce signals for resting B cells to differentiate into activated lymphoblasts and Ig-secreting plasma cells [2–4]. Membrane-bound IgE (mIgE) is expressed on B cells at certain stages during the process of class switching from mIgM+ mIgD+ immunocompetent B cells to IgE-expressing B cells and subsequent differentiation to plasma cells. In genetically engineered mice lacking the membrane-anchoring segment of the ϵ -chain of mIgE (m ϵ -chain), the serum IgE level is only 5% of that of wild-type mice [2], suggesting the importance of mIgE in regulating IgE levels. The functions proposed for mIgE include class switching [5], affinity maturation [6, 7], memory maintenance [8], and the differentiation of IgE-expressing B cells at the later stages [2].

Several strategies have been proposed to treat IgE-mediated allergic diseases by downregulating IgE levels [9–14], one of them being the use of unique anti-IgE antibodies, which bind to epitopes in the CH3 domain of the ϵ -chain of an IgE molecule in or near the IgE.Fc receptor site [15]. These antibodies are designed to neutralize free IgE and target IgE-expressing B cells [9, 11, 12]. In clinical trials, allergic patients treated with anti-IgE antibodies showed nearly complete depletion of free IgE in the blood and improved allergic symptoms [16–19]. One therapeutic anti-IgE antibody, Xolair, is now pending for marketing approval from the FDA of the USA and regulatory agencies in other countries. The proposed effects of anti-IgE antibodies include the neutralization of free IgE, the downregulation of IgE.Fc receptors on basophils and mast cells [20, 21], and the sweeping of incoming allergens by rapidly accumulating, elevated IgE-anti-IgE complexes [12]. It has also been proposed that the anti-IgE antibodies may downregulate or lyse IgE-expressing B cells although the results from the clinical trials do not confirm this hypothesis.

The ‘membrane-anchoring peptide’ of mouse or human m ϵ -chain, which extends beyond the CH4 domain, contains an intracellular segment, a transmembrane segment, and an extracellular segment (referred to as migis- ϵ peptide) between the transmembrane segment and CH4 domain of the ϵ -chain [10, 22]. An isoform of the human m ϵ -chain contains an extra domain of 52 amino acid (a.a.) residues (named C ϵ mX) between the CH4 domain and the migis- ϵ peptide [23]. This isoform has been identified in a human IgE-secreting myeloma, SKO-007, and normal blood lymphocytes [23, 24]. A C ϵ mX-like domain has not been found in several rodent species studied. At both the mRNA and protein levels, the isoform containing C ϵ mX is the dominant one, whereas the conventional form without the C ϵ mX domain exists in minute or unde-

tectable amounts [23, 25, 26]. The ϵ m67 peptide, which is expressed in recombinant fusion proteins in the present studies, is composed of the contiguous 52-a.a. C ϵ mX domain and the 15-a.a. migis- ϵ peptide, both of which are unique to human mIgE.

The C ϵ mX-specific mAbs prepared in the present studies, which target mIgE and not secreted IgE, can be used to resolve the issue whether the therapeutic anti-IgE antibodies, like Xolair mentioned above, cause the downregulation of IgE-expressing B cells. If anti-C ϵ mX mAbs can downregulate the levels of IgE in the body, they should have the potential for treating patients with very high IgE levels, for whom anti-IgE antibodies would not be practical. Positive results may also encourage a therapeutic approach based on developing an immunogen using ϵ m67 peptide, which can induce antibody response against the C ϵ mX domain and hence mIgE-expressing B cells.

Materials and Methods

Construction of Plasmids and Procedure for Transfection

C ϵ mX-migis- ϵ (ϵ m67) was amplified by RT-PCR from the total RNA of an IgE-secreting cell line, SKO-007 (ATCC CRL-8033–1), which was originally derived from U266 myeloma cell line [27], using aagGGATCCgggctggctggcgccct and ggcCTCAGGccacggcgccctcgccct as primers. The amplified product was digested by restriction enzymes, *Bam*HI and *Xho*I, and ligated between the genomic DNA sequence of CH2-CH3 domains of human IgG1 and a leucine zipper fragment (sequence: LEAKIEALEAKIEALEAKIEALEAKIEALEAK) in a plasmid derived from pcDNA3 (Invitrogen, Carlsbad, Calif., USA). Another stable transfectant used was a CHO cell line transfected by an Fc portion of mIgE (mIgE.Fc). The cDNA sequence of m ϵ -chain containing the segment from CH2 domain through the membrane anchor peptide was inserted into pcDNA3 vector. The recombinant plasmid was transfected into CHO cells using a liposome reagent DMIRE-C (GIBCO BRL, Rockville, Md., USA) according to the manufacturer’s instructions. Briefly, 10 μ g of plasmid DNA and 10 μ g of DMIRE-C were added to 6×10^5 cells in a 10-cm petri dish and incubated at 37°C for 5 h in fresh IMDM (GIBCO). Afterwards, 10 ml of medium containing 10% fetal bovine serum (FBS) was added and the cells were incubated at 37°C for an additional 48 h. The cells were then resuspended in a medium containing G418 (Calbiochem, San Diego, Calif., USA) and distributed into the individual wells of 96-well plates.

Screening of Transfectants

To identify clones expressing ϵ m67-Fc fusion protein, the expressed protein in the culture medium was measured by ELISA. Briefly, the wells of 96-well microtiter plates were coated with 1 μ g/ml goat anti-human IgG (KPL, Gaithersburg, Md., USA) in coating buffer (100 mM sodium carbonate, pH 9.6) at 37°C for 1 h, followed by 3% nonfat milk in PBS. The plates were then washed with 0.05% Tween-20 in PBS (PBST) 5 times. The culture medium was applied to the wells and the plates were placed at 37°C for 1 h. After five

washes as above, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (0.3 µg/ml, KPL) was added to the wells, followed by peroxidase substrate, *o*-phenylenediamine (OPD), in sodium acetate buffer (pH 4.5). The reaction was stopped by 1 M H₂SO₄, and the optical density was read with a SpectraMax 250 microplate reader (Molecular Device, Sunnyvale, Calif., USA). The screening of mIgE.Fc-expressing CHO transfectants was performed in two steps. The first was with ELISA as described above, using goat anti-human IgE as the solid phase and HRP-conjugated goat anti-human IgE as the detecting antibody. Equal portions of the cells in the wells of each individual transfectant clone were made into Triton X-100 lysates for detecting the expressed product contained therein. The second step was with fluorescence flow cytometric analysis using FITC-goat anti-IgE (ε) (Caltag, Burlingame, Calif., USA). The stained cells were analyzed by FACScan (Becton Dickinson, Franklin Lakes, N.J., USA).

Purification of Recombinant Proteins

Selected recombinant cell lines were cultured in IMDM containing 10% of protein-G-filtered FBS in roller bottles. Recombinant proteins in conditioned medium were purified with protein-A-conjugated Sepharose CL-4B (Pharmacia, Piscataway, N.J., USA). The purified recombinant protein was analyzed by 12.5% SDS-PAGE.

Preparation of Anti-εm67 Hybridomas and mAbs

6- to 8-week old BALB/c mice were injected intraperitoneally (one site) and subcutaneously (two sites) with 50 µg purified recombinant protein in complete Freund's adjuvant (Sigma, Saint Louis, Mo., USA). After 3 weeks, the mice were injected similarly with the same amount of antigen in incomplete Freund's adjuvant (Sigma). Hereafter, an additional 3–4 booster immunizations were performed similarly every 2 weeks. The anti-εm67 titers of the sera from the immunized mice were determined by ELISA. Briefly, 96-well plates were coated with 1 µg/ml goat anti-human IgE at 4°C overnight and then blocked with 3% nonfat milk in PBS 50 µl of SKO-007 cell lysate, prepared by lysing 4 × 10⁶ SKO-007 cells in 1 ml lysis buffer (0.01 M Tris-HCl, 0.15 M NaCl, 0.02% NaN₃, 1.0% Triton X-100, and 5.0 mM EDTA, pH 8.0), was added to each well. The procedure allowed the mIgE and secreted IgE from the SKO-007 cell lysate to be bound to the plates. Serial dilutions of the sera from the immunized mice were then applied to the wells for titrating anti-εm67 activities.

For preparing hybridomas secreting anti-εm67 mAbs, the spleen cells from the immunized mice were fused with mouse NS0 myeloma cells to form hybrids using the routine procedure with polyethylene-glycol [28]. The hybrids were selected by culturing in IMDM containing hypoxanthine, aminopterin, and thymidine (HAT, GIBCO) and then screened by the same ELISA as described above. The hybridomas secreting anti-εm67 mAbs were subcloned. Chosen clones were grown as ascitic tumors in BALB/c mice. The antibodies in collected ascitic fluids were purified using protein G-Sepharose CL-4B (Pharmacia).

Immunoprecipitation and Immunoblotting Analyses

1 × 10⁷ SKO-007 cells were lysed with 1 ml of lysis buffer and the resultant lysate was mixed with 5 µg of goat anti-human IgE, anti-εm67 mAb, or control anti-DNP IgG2b [29] at 4°C for 1 h. 80 µl protein G-Sepharose CL-4B resin was added to absorb the formed complexes. After the beads were washed with lysis buffer, the bound complexes were eluted by boiling in sample buffer, subjected to SDS-PAGE, and analyzed by incubation with HRP-conjugated goat anti-human IgE (fig. 2, lanes 1–3). For the analysis of binding of anti-

εm67 to mIgE, the lysate of SKO-007 cells was subjected to SDS-PAGE, the proteins in the gel were electro-transferred to polyvinylidene fluoride membrane (Millipore, Bedford, Mass., USA) and analyzed with anti-IgE (ε) (lane 4) or anti-εm67 (lane 5).

Competitive Binding of mAbs to mIgE

Microtiter plates were coated with various anti-εm67 mAbs (1 µg/ml) at 4°C overnight and then blocked with 3% nonfat milk in PBS. SKO-007 cell lysate from 4 × 10⁶ cells was mixed with serial dilutions of an mAb, which inhibitory activity was to be examined, and the mixture was added to individual wells. The mIgE bound to the mAb coated on the wells was measured by HRP-conjugated goat anti-human IgE in an ELISA as described above.

Epitope Mapping of Anti-εm67 mAbs Using Synthetic Peptides

Fourteen peptides (designated p1–p14) of 15-a.a. residues, which overlapped 11-a.a. residues in a stepwise fashion, along the entire 67-a.a. length of the εm67 segment were synthesized at La Jolla Institute for Allergy and Immunology (San Diego, Calif., USA). Two methods were employed to map the epitopes of the mAbs. In the first method, plates were coated with one of the peptides at 10 µg/ml and then blocked by 3% nonfat milk in PBS. An mAb at 1 µg/ml was applied and the bound mAb was detected as described above. In the second method, plates were first coated with anti-IgE and SKO-007 cell lysate was followed to allow mIgE to be captured. Anti-εm67 at 1 µg/ml mixed with one of the peptides at 5 µg/ml was added to the individual wells and the bound mAb was detected by HRP-conjugated goat anti-mouse IgG.

Fluorescence Flow Cytometric Analysis

1 × 10⁶ SKO-007 (mIgE+) cells or transfected CHO cells (mIgE.Fc+) were washed with PBS containing 2% FCS and 0.1% sodium azide. Cells were resuspended in 100 µl buffer containing 10 µg/ml anti-CεmX mAb or anti-DNP IgG2b in the presence or absence of p10 peptide (for which the anti-CεmX mAbs are specific) at 100 µg/ml for 45 min at 37°C. Cells were washed once with 3.5 ml PBS containing 2% FCS and 0.1% sodium azide and then incubated with 100 µl 10 µg/ml FITC-conjugated F(ab')₂ goat anti-mouse IgG.Fc (Jackson Laboratory, West Grove, Penn., USA) at 4°C for 30 min. Cells were washed with PBS and analyzed by FACScan (Becton Dickinson, Franklin Lakes, N.J., USA).

Fluorescence Microscopy

SKO-007 cells were incubated with 10 µg/ml anti-CεmX at 37°C for 2 h, FITC-conjugated goat F(ab')₂ anti-mouse IgG was then added and the cells were incubated at 4°C for 30 min. The cells were washed once and fixed with 1% paraformaldehyde/PBS. The fluorescence-labeled cells were mounted on slides and observed with a confocal microscope (Bio-Rad, Hercules, Calif., USA).

Mass-Spectrometric Analysis

The lysate from 1 × 10⁸ SKO-007 cells was mixed with 100 µg anti-CεmX mAb and 500 µl of protein G-Sepharose CL-4B. After washing with lysis buffer, the beads were boiled in sample buffer, and the dissolved material was subjected to 10% SDS-PAGE. The separated proteins were stained by Coomassie blue and the band corresponding to mε-chain (confirmed by immunoblotting) was cut off and analyzed by mass spectrometry at The Scripps Research Institute (La Jolla, Calif., USA). The procedure consisted of digestion of the protein by trypsin, extraction of the digested peptides, analysis of the

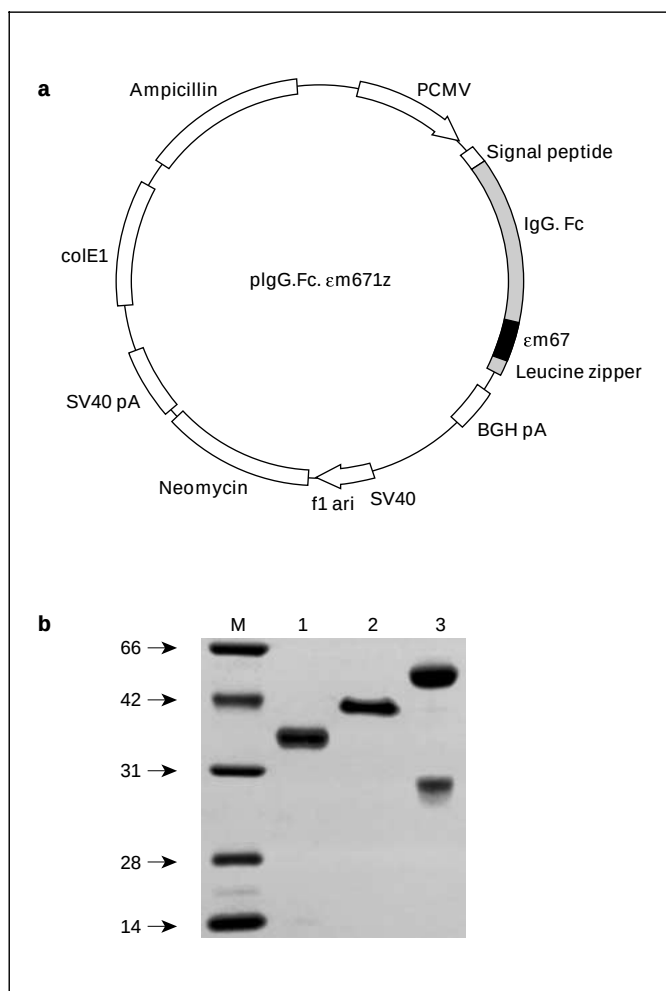


Fig. 1. Construction of the plasmid and analysis of IgG.Fc fusion protein. a Recombinant plasmid was constructed by inserting ϵ m67 into the 3' end of the IgG.Fc genomic sequence. b The purified recombinant protein was analyzed by SDS-PAGE. M = Molecular weight markers; lane 1 = IgG.Fc; lane 2 = ϵ m67-Fc; lane 3 = bovine IgG.

masses of the peptides by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), and analysis of the sequences of peptides by liquid chromatography-mass-mass (tandem mass) spectrometry.

Complement-Mediated Cytotoxicity

4×10^4 CHO cell transfectants expressing mIgE.Fc or SKO-007 cells were placed in individual wells of 96-well plates and the cells were cultured overnight. Twofold serial dilutions (final concentration 0.25–4 μ g/ml) of an anti-C ϵ mX mAb or a control mAb (anti-DNP IgG2b) were added in the presence of 15-fold-diluted baby rabbit complement (Serotec, Raleigh, N.C., USA). After incubation for 2 h at 37°C in 5% CO₂ incubator, live cells were determined using WST-1 assay (Dojindo, Kumamoto, Japan).

Results

Preparation of ϵ m67-Fc Fusion Protein and Anti- ϵ m67 mAbs

The expression level of the recombinant Fc fusion protein containing ϵ m67 peptide (C ϵ mX-migis- ϵ segment) by the transfectants described in Materials and Methods was approximately 10 μ g/1 $\times 10^6$ cells/day. Several milligrams of the recombinant protein were obtained from 1 liter of culture medium by using affinity purification with a protein A-Sepharose CL-4B column. Analysis of the purified fusion protein showed that it was not contaminated by bovine IgG (fig. 1). Probably because ϵ m67 represents a small portion of the fusion protein, it was necessary to screen large numbers of hybrids for those secreting ϵ m67-specific mAbs. From several thousands of hybridomas screened, five secreting anti- ϵ m67 antibodies were obtained. They were designated as a10, a23, a25, and a31. The heavy-chain isotypes of the mAbs secreted by those hybridomas are γ l for a10, a20, a23, a25, and a31 clones and γ 2b for a20 clone, whereas the light-chain isotypes for all five clones are κ .

Immunoprecipitation and Immunoblotting of mIgE in mIgE + Cell Lysates by Anti- ϵ m67 mAbs

To characterize the binding specificity of the anti- ϵ m67 mAbs, their reactivity with the Triton (detergent) lysate of SKO-007 cells, which should contain mIgE and secreted IgE, was analyzed. In one set of experiments, the SKO-007 cell lysate was mixed with either goat anti-IgE antibodies or anti- ϵ m67 mAbs and the immune complexes formed were isolated by protein G-Sepharose CL-4B and subjected to SDS-PAGE. Subsequently, the gels were stained with goat anti-IgE antibodies coupled to colorimetric second antibodies. The results of lanes 1–3 in figure 2 show that three ϵ -related molecular species were pulled down by the goat anti-IgE antibodies [26], while among them, only the largest species was pulled down by anti- ϵ m67 mAb, a20. In the other experiments, the SKO-007 cell lysate was subjected to SDS-PAGE and the resultant gels reacted with either goat anti-IgE antibodies or anti- ϵ m67 mAbs coupled to colorimetric second antibodies. The results of lanes 4 and 5 in figure 2 also show that among the three ϵ -related molecular species, anti- ϵ m67 mAb, a20, could only react with the largest one. The results indicate that the largest ϵ -related chain contains C ϵ mX domain and can be recognized by anti- ϵ m67 mAbs.

Mutual Inhibition of Binding of Anti- ϵ m67 mAbs to mIgE

To analyze the relationship among the epitopes recognized by these mAbs, the effects of one mAb on the binding of another mAb to mIgE were examined. A constant amount of mIgE-containing SKO-007 cell lysate was mixed with increasing concentrations of an mAb and the mixtures were added to microtiter wells coated with another mAb. If the epitope of the mAb in the solution overlaps or is in the close vicinity of that of the mAb on the solid phase, lower amounts of mIgE binding to the plate result. The data show that the binding of any of the anti- ϵ m67 mAbs to mIgE was inhibited by all other anti- ϵ m67 mAbs. Figure 3 illustrates the results of experiments using a20 as the solid-phase mAb. Thus, all five mAbs bind either to the same epitope on ϵ m67 or to epitopes that are in close enough proximity to cause steric hindrance of one another. Among the mAbs tested, a10, a20 and a31 inhibited the binding of other mAbs more efficiently than a25. A control mAb, a16, which is specific for human IgG.Fc, did not inhibit the binding of mIgE to any of the five mAbs coated on the solid phase.

Epitope Mapping of Anti- ϵ m67 mAbs

Since the anti- ϵ m67 mAbs could bind to the denatured mIgE in SDS gel (fig. 2), they might bind to linear epitopes that correspond to peptide segments of contained ϵ m67. We synthesized fourteen peptides of 15-a.a. residues that sequentially overlap 11-a.a. residues in a stepwise fashion and employed them to determine the epitopes of the anti- ϵ m67 mAbs. In one set of ELISA using plates coated with different peptides, it was found that all these anti- ϵ m67 mAbs bound to peptides p10, p11 and p12, which represent overlapping sequences containing RADWPGPP located near the C-terminal of the C ϵ mX domain (fig. 4). In another set of ELISA analyzing the inhibitory effects of the peptides on the binding of anti- ϵ m67 mAbs to solid-phase mIgE, it was found that p10, p11 and p12 could inhibit all five mAbs binding to mIgE, with the exception that p10 inhibited a31 poorly (fig. 5). In summary, the five anti- ϵ m67 mAbs are all specific to a linear epitope containing RADWPGPP on the C ϵ mX domain and not to the 15-a.a. migis- ϵ peptide.

Binding of Anti-C ϵ mX mAbs to the Surface of mIgE+ Cells

To examine whether the anti-C ϵ mX mAbs could bind to mIgE on the cell surface of B cells, flow cytometric analysis was performed. The results show that a20 could bind to SKO-007 cells (fig. 6a) and to mIgE-Fc-transfected

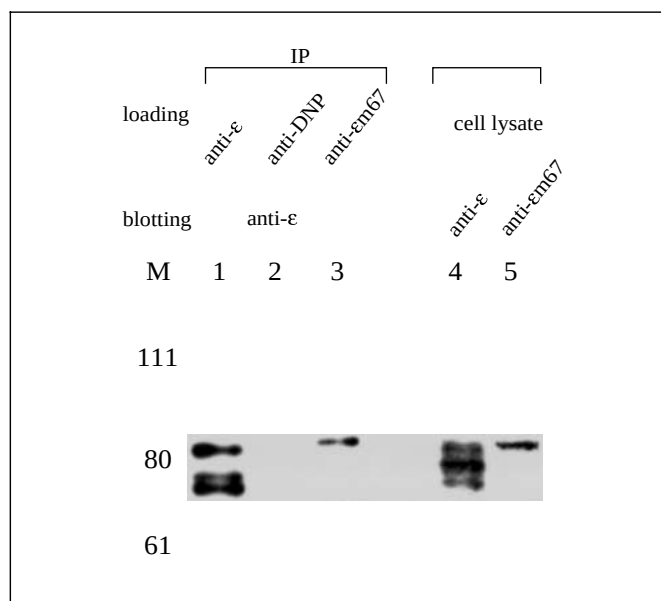


Fig. 2. Immunoprecipitation and immunoblotting analysis of binding of anti- ϵ m67 to mIgE. SKO-007 cell lysate was treated with goat anti-human IgE (lane 1), anti-DNP IgG2b (lane 2) or anti- ϵ m67 a20 (lane 3) followed by incubation with protein G-Sepharose CL-4B beads. The absorbed proteins were eluted and loaded onto individual lanes. Alternatively, the lysate was loaded directly, subjected to electrophoresis, and electro-transferred to polyvinylidene membrane (lanes 4 and 5). IgE-related proteins were detected by using HRP-conjugated goat anti-IgE (lanes 1–3), or anti- ϵ m67 a20 (lanes 4 and 5) followed by HRP-conjugated anti-mouse IgG.

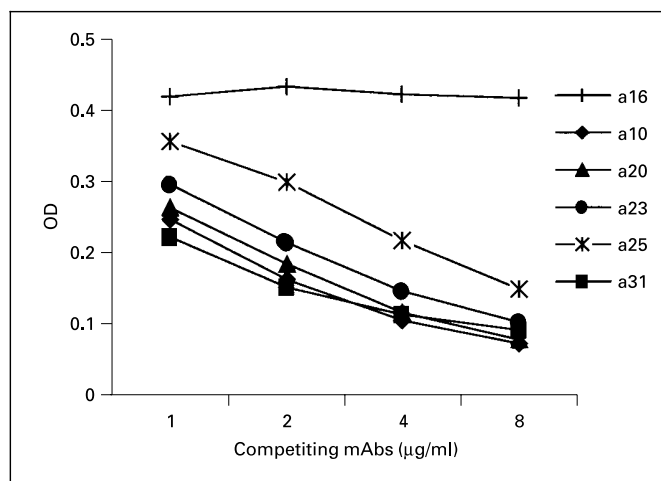


Fig. 3. Mutual inhibition of binding of anti- ϵ m67 mAbs to mIgE. ELISA plates were coated with a20 mAb. Equal aliquots of SKO-007 cell lysate were mixed with various concentrations of anti- ϵ m67 mAb, a10, a20, a23, a25, or a31, or a negative control mAb, a16, before addition to a20-coated wells. Bound mIgE was detected by HRP-conjugated goat anti-human IgE.

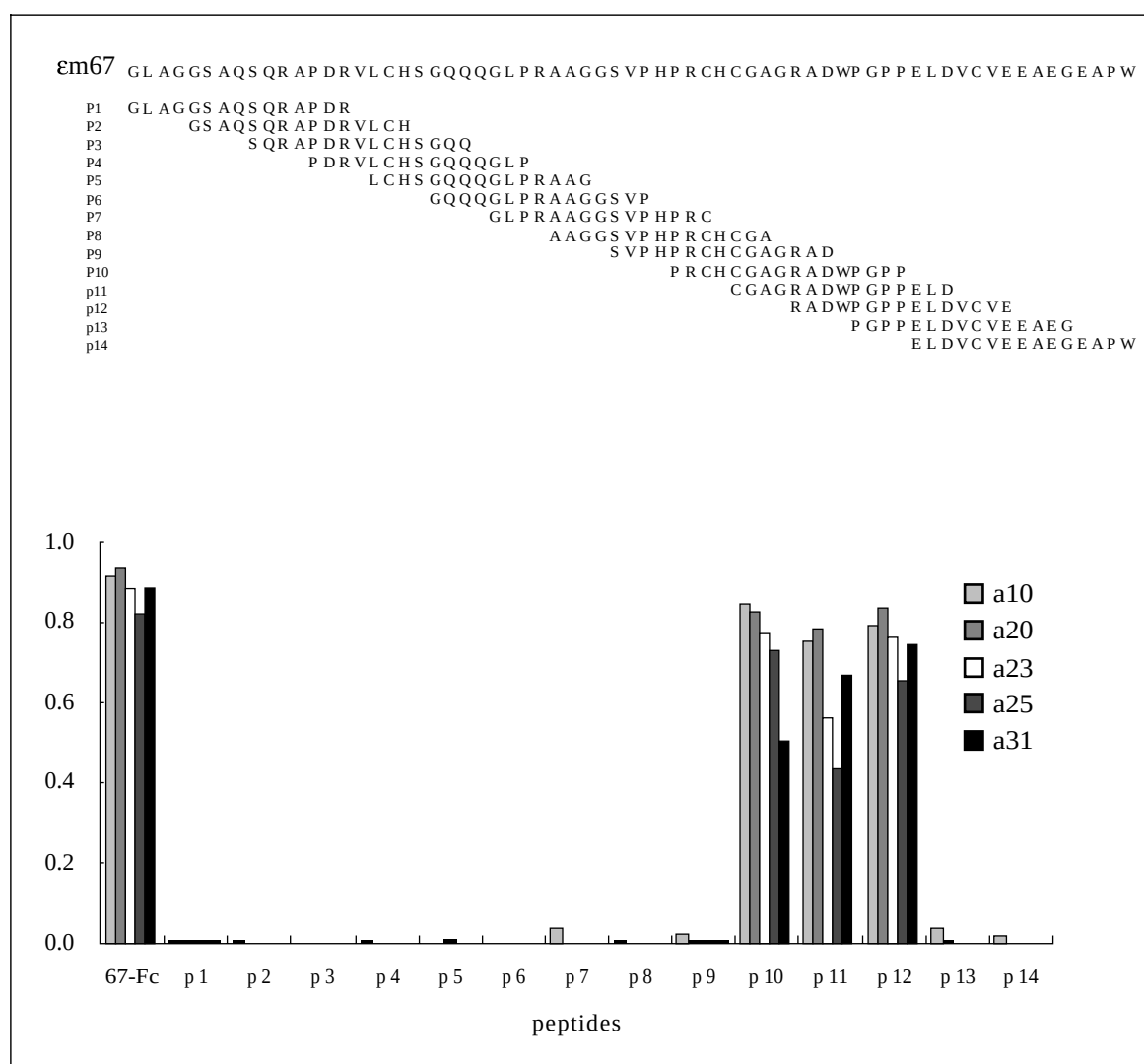


Fig. 4. Epitope mapping of mAbs using synthetic peptides. Plates were coated with 10 µg/ml of peptides or em67-Fc (67-Fc). Aliquots of various anti-em67 mAbs at 1 µg/ml were added and the bound mAb was detected by HRP-goat anti-mouse IgG.

CHO cells (fig. 6c), and such binding could be inhibited by p10 peptide (fig. 6b, d). Results with other mAbs were similar (data not shown). The binding of a20 to SKO-007 cells was also examined by confocal immunofluorescence microscopy. As shown in figure 7, patching of the reacted antigen on the cell membrane (fig. 7a) occurred on most of the cells, and capping (fig. 7b) was observed on some of the cells. It should be noted that the fluorescent second antibody, FITC-conjugated F(ab')₂ goat anti-mouse IgG, was added at 4°C, and the cells were swiftly washed with cold medium and fixed with 1% paraformaldehyde/PBS. This procedure prevented patching and capping observed

due to cross-linking induced by the second antibody. The results suggest that a20 could induce cross-linking of mIgE+ on the cell surface.

Analysis of Anti-CemX-Precipitated mIgE by Mass Spectrometry

To verify that the mIgE recognized by the anti-CemX mAbs contains CemX, one of the mAbs, a20, was used to immunoprecipitate mIgE from SKO-007 cell lysate and the isolated protein was analyzed by mass spectrometry. Trypsin-digested fragments were analyzed by MALDI-TOF and sequenced by tandem mass spectrometry. The

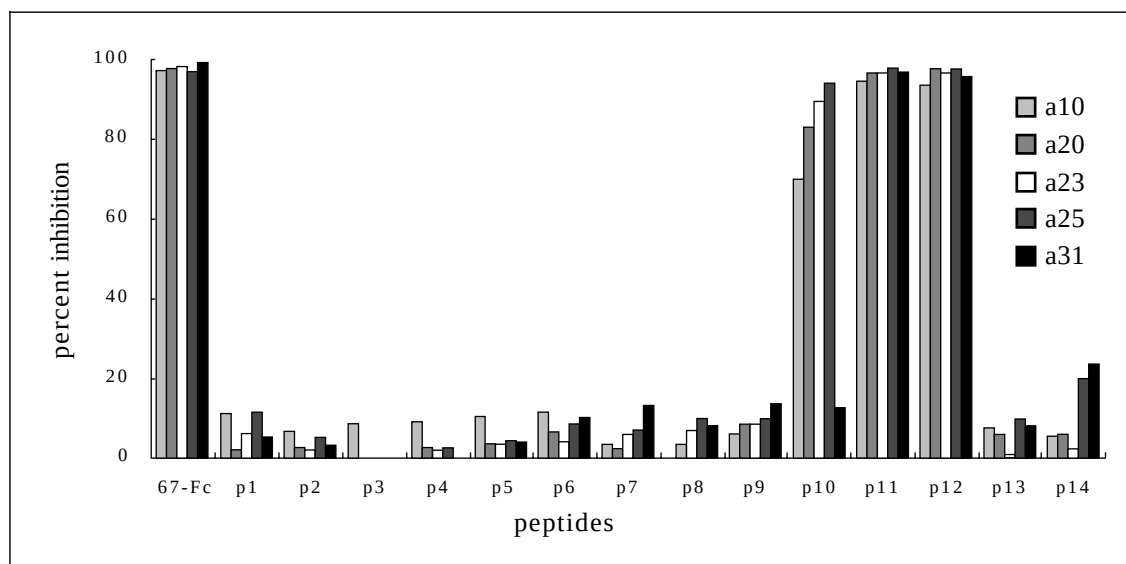


Fig. 5. Inhibition of binding of anti-em67 mAbs to mIgE by synthetic peptides. Plates were first coated with goat anti-IgE and SKO-007 cell lysate was applied to allow mIgE to be captured (Materials and Methods). Aliquots of mAbs at 1 μ g/ml mixed with 5 μ g/ml of the inhibitors, peptides or em67-Fc (67-Fc), were added and the bound mAb was detected by HRP-goat anti-mouse IgG. The inhibitory effect was expressed as the percentage of reduced OD compared to maximum binding.

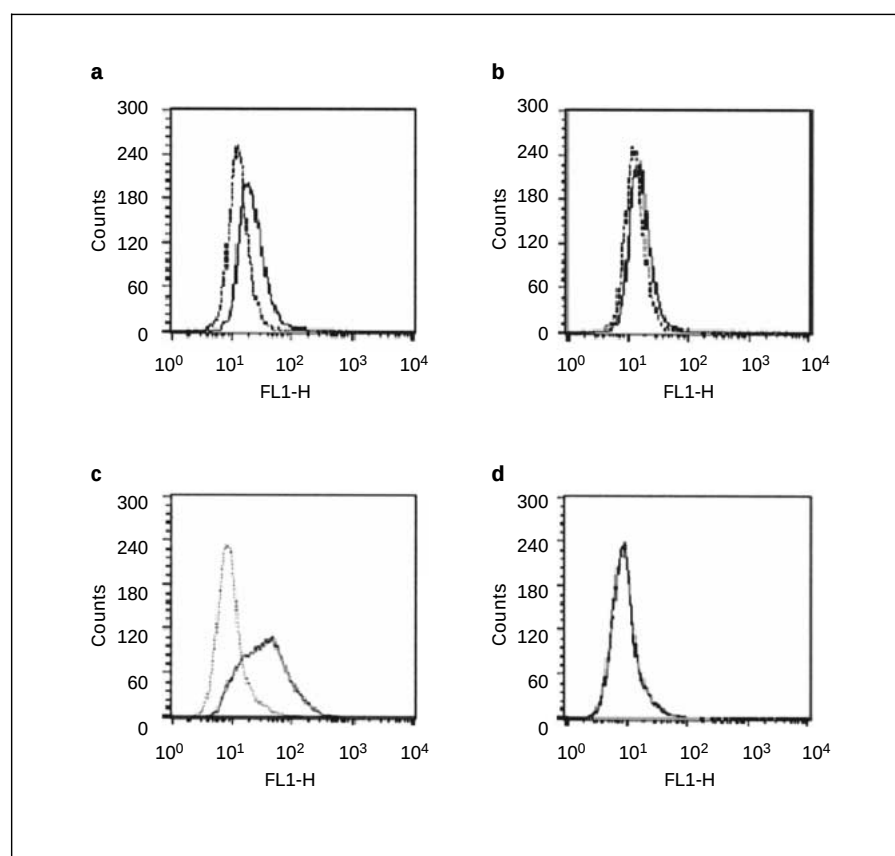


Fig. 6. Binding of anti-CemX mAb on the surface of mIgE+ cells. SKO-007 (a, b) and CHO transfectant (c, d) cells were incubated with either control (anti-DNP IgG2b, dotted line) or anti-CemX a20 (solid line) at 10 μ g/ml in the absence (a, c) or presence of an inhibiting peptide (b, d). After incubation for 30 min at 37°C, the cells were labeled with FITC-conjugated F(ab')₂ goat anti-mouse IgG and analyzed by fluorescence flow cytometry.

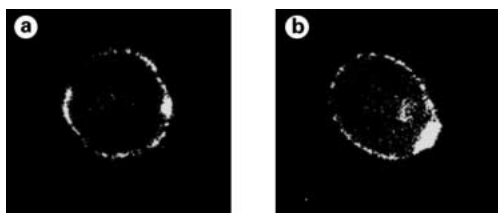


Fig. 7. Induction of patching (a) and capping (b) on mIgE+ cells by an anti-C ϵ mX mAb. SKO-007 cells were incubated with 10 μ g/ml of anti-C ϵ mX a20 mAb for 2 h at 37°C. After washing, the cells were labeled with FITC-conjugated F(ab')₂ goat anti-mouse IgG at 4°C for 30 min and observed with a confocal microscope.

results of MALDI-TOF reveal that 14 of the 27 detected peptides matched fragments of the m ϵ -chain (data not shown). The results of tandem mass spectrometry resolved 13 peptide sequences that match to the m ϵ -chain, and two of them, a 17-a.a. residue and a 10-a.a. residue, were found to be identical to peptide segments of the C ϵ mX domain that had previously been derived from a nucleotide sequence of cDNA (data not shown).

Induction of Complement-Mediated Cytotoxicity of mIgE.Fc Transfectants by Anti-C ϵ mX mAb

To study whether anti-C ϵ mX mAbs could be used to target mIgE+ B cell, mIgE.Fc-transfected CHO cells were examined for their susceptibility to complement-mediated cytotoxicity in the presence of anti-C ϵ mX a20 mAb and baby rabbit complement. The results show that over 50% of the target cells could be lysed, as compared to the result with an irrelevant control antibody under the same condition (fig. 8). The anti-C ϵ mX mAb a20 was effective at a concentration as low as 0.25 μ g/ml.

Discussion

In this study, an immunogen in the form of an Fc fusion protein containing ϵ m67 unique to human mIgE was expressed in a mammalian expression system. By employing this fusion protein as an immunogen, murine mAbs specific for the C ϵ mX domain were generated. The mAbs bind to a linear epitope in the C ϵ mX domain and recognize both the native and denatured forms of mIgE. One of the mAbs, which was studied more extensively, was shown to be capable of inducing the cross-linkage of

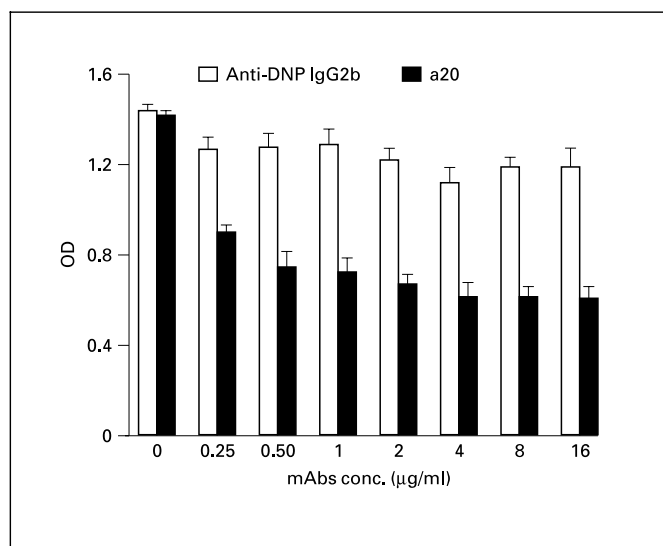


Fig. 8. Induction of complement-mediated cytotoxicity by anti-C ϵ mX on mIgE.Fc transfectants. 4×10^4 cells were cultured in a 96-well plate overnight, and 0–16 μ g/ml of anti-C ϵ mX a20 (■) or control, anti-DNP IgG2b (□) were added together with 15-fold-diluted baby rabbit complement and the mixtures were cultured at 37°C for 2 h. Live cells were determined by a WST-1 assay.

mIgE on B cells as well as complement-mediated cytotoxicity of transfectants expressing mIgE.Fc on the surface.

The rationale for employing the Fc of human IgG1 as a fusion partner for ϵ m67 is as follows [30, 31]: (1) to provide T-cell-reactive peptidic segments for eliciting T cell help; (2) to possibly preserve the native conformation of the C ϵ mX domain through dimer formation, and (3) to simplify the purification of the expressed product by employing protein A affinity purification. mAbs against the linear peptide epitope may also be prepared with ϵ m67 immunogens in other forms of molecular constructs although we have not been successful in attempts at using ϵ m67 peptide in a fusion protein with bacterial maltose binding protein.

The mAbs were characterized by various assays. The results indicate that the mAbs can detect the existence of C ϵ mX on B cells by immunoblotting and immunofluorescent methods, and hence can be employed to detect, enumerate, and isolate mIgE-expressing B cells. These mAbs do not bind to Fc ϵ RII-bound IgE and should not recognize Fc ϵ RII-expressing B cells, which do not express mIgE, or other Fc ϵ RII-expressing cells such as monocytes.

Epitope mapping of the five anti-C ϵ mX mAbs showed that they bind to a linear peptide, RADWPGPP, located near the C-terminus of the C ϵ mX domain. These results have several implications. First, the conformation of this sequence in the ϵ m67-Fc fusion protein and mIgE is similar to that in the synthetic peptide. Alternatively, synthetic peptides containing the epitope may be induced to fit the binding site of the Fv of the mAbs. Second, this peptide segment in C ϵ mX is exposed on the surface of the protein. The RADWPGPP sequence indicates that, owing to its proline and glycine residues in the last four residues, it possibly bears a turn structure on the surface of a folded protein. Third, this peptide region is possibly a dominant immunogenic epitope site.

Epitope mapping has also proven that the peptide sequences previously deduced from cDNA sequencing exist in the C ϵ mX domain. Tandem mass analysis provided convincing evidence that C ϵ mX is an integral part of the human m ϵ -chain. The results on the peptide masses yielded by MALDI-TOF also indicate that there is no posttranslation modification on these peptides in the m ϵ -chain.

In this study, the ability of anti-C ϵ mX mAbs on targeting and lysing mIgE⁺ B cells was examined. The mAb a20 could induce patching and capping of mIgE on the surface of SKO-007 cells although such cross-linking of mIgE did not affect cell growth [unpubl. obs.]. This is possibly due to the low expression level of mIgE on SKO-007 cells or to the fact that the cells are of a terminally differentiated myeloma cell line. The low expression level of mIgE on SKO-007 cells was also shown by the low level of fluorescence staining by the anti-C ϵ mX mAbs in flow cytometric analysis. The results are consistent with the finding that the anti-C ϵ mX mAbs did not mediate complement-dependent cytotoxicity on SKO-007 cells (data not shown), probably because a certain expression level of surface molecules is required for effective cytotoxicity. In contrast, anti-C ϵ mX mAb a20 was able to cause complement-dependent cytotoxicity on the mIgE-Fc-expressing transfectants, which expressed relatively higher levels of mIgE-Fc on the surface than SKO-007 cells.

The desired pharmacological targets for anti-C ϵ mX antibodies are mIgE-bearing memory B cells involved in secondary immune responses that generate IgE. The mIgE levels on memory B cells responsible for secondary IgE responses should be higher than those on IgE-secreting plasma cells. The effects of anti-C ϵ mX mAbs on lysis or downregulation of primary mIgE⁺ B cells and mIgE⁺ memory B cells remain to be investigated. Studies are now in progress to investigate the potential of anti-C ϵ mX anti-

bodies in causing the reduction of mIgE-bearing B cells and hence serum IgE levels in transgenic mice (KM mouseTM developed by Kirin Brewery. Co., Ltd and Medarex, Inc.), which harbor human immunoglobulin genes.

In light of the probably successful development of an anti-IgE antibody (Xolair) for the treatment of asthma and allergic rhinitis [12, 16–19], the pharmaceutical development of anti-C ϵ mX mAbs may not seem convincing. However, studies on C ϵ mX antibodies will be valuable for the following reasons. The clinical investigations on anti-IgE antibodies have not resolved the question whether the antibodies inhibit or lyse mIgE-expressing B cells and hence downregulate IgE production. The primary reasons for failing to attain a conclusion are that anti-IgE antibodies deplete all free IgE in the blood and that IgE-secreting plasma cells, which do not express mIgE and are not targets of anti-IgE antibodies, are very long-lived in the bone marrow, secreting IgE into the blood, and thus obscuring observation [12]. As anti-C ϵ mX mAbs target only mIgE and not secreted IgE, they should help resolve the question whether mIgE-expressing B cells are inhibited or downregulated.

If anti-C ϵ mX mAbs can indeed inhibit or downregulate IgE synthesis *in vivo*, these antibodies, after being properly humanized, may be used to treat allergic patients with very high IgE levels because treatment with anti-IgE antibodies is not practical. In patients with high blood IgE levels, the total amount of mIgE on the relatively small numbers of mIgE-expressing B cells should be much less than that of free IgE. Therefore, much less anti-C ϵ mX mAb than anti-IgE mAb will be required for treating those patients. Perhaps more intriguingly, positive results on the effects of anti-C ϵ mX mAbs on inhibiting mIgE-expressing B cells and decreasing IgE synthesis *in vivo* should support a therapeutic approach that develops an immunogen based on C ϵ mX peptide or ϵ m67 peptide in combination with a foreign peptide for eliciting T cell help. Such an immunogen, if successfully developed, will be able to induce specific autologous antibodies in patients that target mIgE-expressing B cells and hence achieve pharmacological effects similar to those rendered by anti-IgE antibodies or anti-C ϵ mX mAbs.

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