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Unique Epitopes on C ϵ mX in IgE–B Cell Receptor Are Potentially Applicable for Targeting IgE-Committed B Cells

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Membrane-bound IgE (mIgE) is part of the IgE–BCR and is essential for generating isotype-specific IgE responses. On mIgE⁺ B cells, the membrane-bound ϵ -chain (m ϵ) exists predominantly in the long isoform, m ϵ_L , containing an extra 52 aa C ϵ mX domain between CH4 and the C-terminal membrane-anchoring segment; the short isoform of m ϵ , m ϵ_S , exists in minor proportions. C ϵ mX thus provides an attractive site for immunologic targeting of mIgE⁺ B cells. In this study, we show that nine newly prepared C ϵ mX-specific mAbs, as well as the previously reported a20, bound to mIgE.Fc ϵ L-expressing CHO cells, while only 4B12 and 26H2 bound to mIgE.Fc ϵ L-expressing B cell line Ramos cells. The mAb 4B12 bound to the N-terminal part, 26H2 the middle part, and all others the C-terminal part of C ϵ mX. Expression of Ig α and Ig β on the mIgE.Fc ϵ L-CHO cells reduces the binding of a20 to C ϵ mX as compared with that of 4B12 and 26H2. The chimeric mAbs c4B12 and c26H2, when cross-linked by secondary antibodies, lysed mIgE.Fc ϵ L-Ramos cells by apoptosis through a BCR-dependent caspase pathway. Using PBMCs as the source of effector cells, c4B12 and c26H2 demonstrated Ab-dependent cellular cytotoxicity toward mIgE.Fc ϵ L-Ramos cells in a dose-dependent fashion. In cultures of PBMCs from atopic dermatitis patients, c4B12 and c26H2 inhibited the synthesis of IgE driven by anti-CD40 and IL-4. These results suggest that 4B12 and 26H2 and an immunogen using the peptide segments recognized by these mAbs are potentially useful for targeting mIgE⁺ B cells to control IgE production. *The Journal of Immunology*, 2010, 184: 000–000.

Immunoglobulin E mediates type-I hypersensitivity reactions responsible for various allergic diseases, including allergic asthma, allergic rhinitis, and atopic dermatitis. In such a reaction, allergens cross-link allergen-specific IgE molecules, which are already bound by Fc ϵ RI on basophils and mast cells, causing the release of a host of preformed and newly-synthesized mediators from these inflammatory cells (1). Because IgE plays a central role in allergic reactions, several strategies aiming to reduce free IgE levels or modulate IgE production have been pursued for the treatment of IgE-mediated allergic diseases (2, 3).

Omalizumab, a humanized mAb with a set of specificities binding to human IgE (4), has been approved in the United States, the European Union, and many other countries to treat adults and adolescents with severe or moderate-to-severe persistent allergic asthma. Omalizumab causes a rapid decrease in free IgE levels and gradually downregulates Fc ϵ RI on basophils, dendritic cells, and mast cells (5–7). Whether omalizumab can target membrane-bound IgE (mIgE⁺) B cells and hence inhibit the generation of new IgE-secreting plasma

cells in patients in vivo is not well understood, complicated in part by the fact that the injected omalizumab is largely bound by the free IgE in blood and in interstitial fluid. Therefore, an approach that targets mIgE⁺ B cells directly and inhibits the synthesis of IgE without binding to free IgE would be attractive (8–10). Membrane-bound IgE is part of the IgE–BCR on IgE-committed lymphoblasts and memory B cells. Several lines of evidence indicate that the membrane-bound Ig-expressing B cell is essential for generating isotype-specific responses (11, 12). For example, in transgenic mice lacking transmembrane and cytoplasmic segments of m ϵ , the generation of IgE-secreting plasma cells and IgE synthesis is impaired (11).

Our group first proposed that the extracellular portions of the membrane-anchoring segment of membrane-bound Ig [referred to as mIg isotype-specific (migis) segment (8); or extracellular membrane-proximal domain or “EMPD” (13)] may be used for mAb-based, isotype-specific targeting of B cells. Our group also discovered that a new isoform of m ϵ (i.e., m ϵ_L) contains an extra segment of 52 aa residues (referred to as C ϵ mX; Fig. 1) between the CH4 domain and the migis segment (14). C ϵ mX is resulting from an alternative splicing of the ϵ transcript, using an acceptor site 156-bp upstream of the previously known site used by m ϵ_S . The isoform m ϵ_L is predominantly expressed on human IgE-expressing myeloma cells and primary B cells, whereas m ϵ_S exists in minute or undetectable amounts at both mRNA and protein levels (14, 15). C ϵ mX is found only in humans and other primates examined, and not in other species; its sequence is unique in the existing DNA and protein databases.

Our group previously reported the development of several mouse anti-C ϵ mX mAbs, including a20, which bound to an 8-aa peptidic segment, RADWPGPP, at the C-terminal end of C ϵ mX (9). In the current study, we further investigate this peptidic segment and other parts of C ϵ mX to evaluate their accessibility by Abs, and hence their applicability for Ab- or immunogen-based targeting of mIgE⁺ B lymphocytes and memory B cells.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: ADCC, antibody-dependent cellular cytotoxicity; mIgE, membrane-bound IgE; migis, mIg isotype-specific; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PS, phosphatidylserine; TM, transmembrane segment; UDM, *n*-undecyl- β -D-maltopyranoside.

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Materials and Methods

Cells

The Ramos cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FBS (Invitrogen), 4 mM L-glutamine, 25 mM HEPES, and 1 mM sodium pyruvate (Invitrogen; complete RPMI medium). Stable transfectants of Ramos cells expressing mIgE.Fc_L or mIgE.Fc_S were maintained in complete RPMI 1640 medium supplemented with 400 µg/ml Zeocin (Invitrogen). Mouse NS0 myeloma cells (ATCC) and hybridoma lines were cultured in DMEM medium (Invitrogen) supplemented with heat-inactivated 10% FBS, 4 mM L-glutamine, 50 µg/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen). All cells were grown at 37°C, 5% CO₂. The human embryonic kidney cell line, FreeStyle 293F (Invitrogen), was cultured in FreeStyle 293 Expression medium (Invitrogen). Stable transfectants of CHO cells expressing mIgE.Fc_L or mIgE.Fc_S, derived from the CHO dhfr⁻ cell line (ATCC), were adapted to suspension culture in CD CHO medium (Invitrogen) supplemented with 8 mM GlutaMax (Invitrogen, complete CD CHO medium) and 50 µM methotrexate (Sigma-Aldrich, St. Louis, MO). A stable transfectant of CHO cells expressing mIgE.Fc_L, Igα, and Igβ were maintained in complete CD CHO medium supplemented with 50 µM methotrexate and 200 µg/ml Zeocin. All suspension cells were cultured in disposable Erlenmeyer flasks (Corning Glass, Corning, NY) at 133 rpm on an orbital shaker platform under humidified conditions (37°C and 8% CO₂).

Preparation of mIgE.Fc recombinant proteins

Groups of 10⁹ mIgE.Fc_L- or mIgE.Fc_S-expressing CHO cells were collected by centrifugation at 400 × g and resuspended in 30 ml of resuspension buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM PMSF), followed by homogenization at 1500 ψ with a French Press Cell Disrupter (Thermo Scientific, Waltham, MA). The homogenate was centrifuged at 40,000 × g at 4°C for 1 h to discard supernatants. The resultant pellet was dissolved in 20 ml solubilizing buffer (1% *n*-undecyl-β-D-maltopyranoside [UDM], 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM PMSF) for 16–18 h with gentle agitation at 4°C. The solution was centrifuged at 12,000 × g for 30 min at room temperature; the resultant supernatant was collected and diluted with resuspension buffer to a final concentration of 0.05% UDM. The diluted supernatant was passed over an equilibrated nickel Sepharose column (GE Healthcare, Fairfield, CT), and then polyhistidine-tagged mIgE.Fc_L or mIgE.Fc_S recombinant proteins were eluted with elution buffer (500 mM imidazole, 0.05% UDM, 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM PMSF). The proteins were further purified by fast protein liquid chromatography on a Superpose 12 10/300 GL column (GE Healthcare) equilibrated with 0.05% UDM, 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 1 mM PMSF.

Generation and chimerization of anti-CemX mAbs

To induce an anti-CemX immune response, BALB/c mice were immunized twice s.c. with mIgE.Fc_L emulsified in TiterMax Gold adjuvant (Sigma-Aldrich) at 2-wk intervals. A final boost was given i.p. with 0.1 mg of mIgE.Fc_L without adjuvant, and 3 d later, the spleen cells from immunized mice were used for hybridomas as described previously (9). The isotypes of anti-CemX mAbs prepared in this study were determined by an Ig isotyping ELISA kit (Caltag Laboratories, Burlingame, CA) and listed in Supplemental Table 1.

For chimerization of 4B12, 26H2, and a20, cDNA of V_H was amplified by PCR using a set of DNA primers as designed by Essono et al. (16). The V_K of 4B12 and a20 and V_λ of 26H2 were PCR-amplified using V_K-specific primers designed by Essono et al. (16) and Ig-Primers for mouse V_λ (Novagen, Madison, WI). The V_H and V_L of 4B12, 26H2, and a20 were cloned and sequenced. The cDNAs of mouse V_H and V_K from 4B12, a20, and BAT123, which is specific for a peptide segment of gp120 of HIV-1 (17), were joined to a human C-γ1 and a human C-κ of a chimeric Ab expression vector—the pIgG1(κ) vector, which was modified from the TCAE vector (18). The V_H and V_K of 26H2 and B1-8, which is specific for nitrophenyl-hapten (19) (a gift from Dr. Takeshi Tsubata, Tokyo Medical and Dental University, Tokyo, Japan), were ligated to a human C-γ1 and a human C-λ of the pIgG1(λ) vector.

Transfection of chimeric Ab genes into 293F cells was performed at the cell density of 10⁷ cells/ml in 300 ml culture in 2-L Erlenmeyer flasks using linear polyethylenimine with an average m.w. of 25 kDa (Polysciences, Warrington, PA) as a transfection reagent, according to Sun et al. (20). The transfected cells were placed on an orbital shaker in a 37°C incubator and incubated for 4 h. The cell density was then adjusted to 2.5 × 10⁶ cells/ml by addition of fresh medium. Culture supernatants were harvested after 4 d for Ab purification using protein A chromatography (GE Healthcare) according to the manufacturer's instructions.

Synthetic peptides

For epitope mapping of anti-CemX mAbs, a series of peptides of 12–21 aa residues encompassing the entire length of CemX were synthesized at the Genomics Research Center, Academia Sinica (Taipei, Taiwan). The peptides were used as solid-phase antigens in ELISA and as inhibitors in apoptosis assays.

Determination of the binding affinity of mAbs by surface plasmon resonance

The human IgG1.Fc fusion protein, IgG1.Fc-CemX-migis-ε (γ1-εm67), containing CH2-CH3 of γ1, CemX, and migis-ε segments (9) was diluted in 10 mM sodium acetate (pH 5.5) at a concentration of 20 µg/ml and coupled to a CM5 chip (GE Healthcare) according to the manufacturer's instruction. Purified mAbs at different concentrations in HBS-EP buffer (GE Healthcare) were injected into each sensor cell at a flow rate of 35 µl/min in a Biacore T100 device (GE Healthcare). Each sample was run through 10-min association and 12-min dissociation phases. After each injection, the chip was regenerated using 10 mM glycine, pH 1.75. The collected data were fitted to the 1:1 Langmuir interaction model formulated by BIAevaluation software (GE Healthcare) to calculate *k*_{on}, *k*_{off}, and *K*_D of each mAb.

Apoptosis assays

Ramos cells stably expressing mIgE.Fc_L (5 × 10⁵ cells/ml) were incubated with chimeric anti-CemX mAbs, omalizumab or control Abs in complete RPMI 1640 medium for 1 h at 37°C. The cells were then treated with the secondary Ab, goat F(ab')₂ specific for Fc of human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA), at a final concentration of 10 µg/ml and incubated for an additional 24 h at 37°C. The extent of apoptosis of the cells was analyzed in the following ways.

For assays with annexin V, the phosphatidylserine (PS) exposure was measured by resuspending cells in a staining solution for 15 min in dark at room temperature. The staining solution contained FITC-labeled annexin V (Biovision, Mountain View, CA), diluted 1/200, and 2.5 µg/ml propidium iodide (PI) in a buffer with 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 5 mM CaCl₂. The stained cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). The percentage of apoptotic cells, defined as annexin V-positive and PI-negative, was obtained in a dot-plot analysis.

For detecting the cleavage of caspase-3 and poly(ADP-ribose) polymerase (PARP), cell lysates were prepared in ice-cold lysis buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton-X 100, 0.5% deoxycholate, 0.1% sodium SDS, 5 mM EDTA, and protease inhibitor mixture (Sigma-Aldrich)]. Each sample was normalized for total protein content and subjected to SDS-PAGE followed by transfer to PVDF membranes (GE Healthcare). Rabbit polyclonal Abs to caspase-3 and PARP (Cell Signaling Technology, Beverly, MA), were applied to PVDF membranes and further developed with HRP-conjugated goat anti-rabbit IgG and its substrate.

For examining the effect of the caspase inhibitor, z-VAD-fmk (R&D Systems, Minneapolis, MN), on the apoptotic process, the inhibitor was present in the cell culture at 1, 10, or 100 µM during the 1-h incubation with the tested mAbs and the subsequent 24-h incubation with secondary Abs. The measurement of apoptosis with annexin V was then performed.

Ab-dependent cellular cytotoxicity assay

PBMCs were purified from buffy coats of blood samples from healthy donors (Taiwan Blood Service Foundation) by centrifugation over a Ficoll-Paque PLUS (GE Healthcare) density gradient and cryopreserved in 90% FBS/10% DMSO. Prior to use, the PBMCs were thawed and cultured at 2 × 10⁶ cells/ml overnight in IMDM medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 4 mM L-glutamine, 25 mM HEPES, 50 µg/ml penicillin, and 100 µg/ml streptomycin (complete IMDM medium). mIgE.Fc_L-expressing Ramos cells were labeled with CFSE (Invitrogen) in PBS/0.1% BSA for 10 min at 37°C. After three washes with cold complete RPMI 1640 medium, cells were adjusted to 10⁵ cells/ml. Aliquots of 20,000 labeled cells in 200 µl complete RPMI medium were coated with mAbs for 30 min at 37°C, and then combined with PBMCs at specified E/T ratios. After 24 h incubation, the total cells were stained with 2.5 µg/ml 7-amino actinomycin D (Invitrogen) for 15 min on ice and then analyzed on a Becton Dickinson FACSCanto II flow cytometer (BD Biosciences). Living target cells were defined as CFSE-positive and PI-negative in a dot-plot analysis. The percentage of lysed target cells at a given E/T ratio was: 100 × [(percentage of live target cells in Ab-independent control – percentage of live target cells in the sample) / percentage of live target cells in the Ab-independent control].

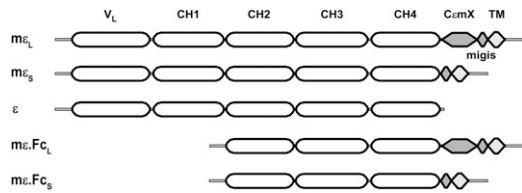


FIGURE 1. Schematic representation of human ϵ -chain, the long and short isoforms of $m\epsilon$ chain, and the long and short $m\epsilon$.Fc constructs used to transfect CHO and Ramos cells. CemX contains 52 aa and migis- ϵ 15 aa. TM, transmembrane segment.

IgE production by stimulated PBMCs in vitro

The protocol for the collection of blood samples from atopic dermatitis patients was approved by the institutional review boards of National Taiwan University Hospital and Academia Sinica. Fifteen atopic dermatitis patients (5 females, 10 males) with serum IgE levels ranging 932–25,891 IU/ml were enrolled in this study. The isolated PBMCs were suspended at 10^6 cells/ml in complete IMDM with 100 ng/ml human IL-4 (PeproTech, Rocky Hill, NJ) and 100 ng/ml mouse anti-CD40 mAb (ATCC, G28-5), by adopting protocols used by earlier investigators (21–23). Each tested mAb was added into the culture at a concentration of 10 μ g/ml. After the cells were cultured for 12 d, cell-free supernatants were harvested and stored at -20°C . IgE, IgA, and IgM released into the supernatants were measured by ELISA using human IgE, IgA, and IgM Quantitation Kits (Bethyl Laboratories, Montgomery, TX) according to the manufacturer's instructions.

Results

Generation of anti-CemX mAbs

To obtain anti-CemX mAbs specific for various parts of CemX, BALB/c mice were immunized with recombinant $m\text{IgE.Fc}_L$ protein, which contains the CH2 domain through the cytoplasmic end of $m\epsilon_L$ chain, and hybridoma clones were prepared. Of the more than 4000 hybridoma clones screened, nine clones with binding activity with $m\text{IgE.Fc}_L$ but not with $m\text{IgE.Fc}_S$, were obtained. The nine clones were further tested for binding to $m\text{IgE.Fc}_S$ -expressing CHO, $m\text{IgE.Fc}_L$ -expressing CHO, $m\text{IgE.Fc}_S$ -expressing Ramos, and $m\text{IgE.Fc}_L$ -expressing Ramos cells (Fig. 1) by fluorescence flow cytometric analysis. Ramos, a human Burkitt's lymphoma cell line, is known to express membrane-bound IgM in a complete BCR complex (24). All nine clones showed binding to $m\text{IgE.Fc}_L$ -expressing CHO cells, but not to $m\text{IgE.Fc}_S$ -expressing CHO and $m\text{IgE.Fc}_S$ -expressing Ramos cells, indicating that they recognized CemX. Among the nine anti-

CemX clones, only two (4B12 and 26H2) bound to $m\text{IgE.Fc}_L$ -expressing Ramos cells. The other seven clones (1A8, 1G2, 3A6, 3H11, 6H12, 18A11, 23B8) did not bind to $m\text{IgE.Fc}_L$ -expressing Ramos cells. The anti-CemX mAb a20, which was prepared by our group earlier (9), bound to $m\text{IgE.Fc}_L$ -expressing CHO, but did not bind to $m\text{IgE.Fc}_S$ -expressing CHO, $m\text{IgE.Fc}_S$ -expressing Ramos, and $m\text{IgE.Fc}_L$ -expressing Ramos cells (Fig. 2). In addition, a20 did not bind to both $m\text{IgE.Fc}_L$ - and $m\text{IgE.Fc}_S$ -expressing mouse mature B cell line A20 cells (data not shown). These results suggested that only 4B12 and 26H2, and not the other mAbs, including a20, bound to IgE-BCR.

Epitope mapping

All nine anti-CemX mAbs bound to denatured $m\text{IgE.Fc}_L$ in a Western blot analysis (data not shown), indicating that they can recognize unfolded singular peptidic segments in CemX. Because a CemX segment contains three cysteine residues, of which one is involved in forming an interchain disulfide bond and the other two in forming an intrachain disulfide bond (25), our initial experiments examined whether the anti-CemX mAbs could bind to linear segments between the cysteine residues. We synthesized three peptides: P1 peptide of 21 aa residues contained 4 aa residues corresponding to the C-terminal 4 residues of CH4 and the N-terminal 17 residues of CemX; P2 peptide corresponded to the middle 20 aa residues of CemX; and P3 peptide of 15 aa residues contained the CemX C-terminal 11 aa residues and 4 aa residues corresponding to the first four residues of the migis- ϵ segment (Fig. 3A). In ELISA using those peptides as solid-phase antigens, 4B12 and 26H2 reacted strongly with P1 and P2, respectively, whereas 1A8, 1G2, 3A6, 3H11, 6H12, 18A11, 23B8, and a20 reacted with P3 (Fig. 3A).

To further define the epitopes recognized by newly prepared anti-CemX mAbs, we constructed overlapping, sequential peptides covering the N-terminal, middle, and C-terminal regions of CemX. The mAb 4B12 did not react with P1.1; it reacted weakly with P1.2 and P1.3, but reacted with P1.4 nearly as strongly as with P1 (Fig. 3B). The mAb 26H2 did not react with P2.3; it reacted with P2.1, P2.2, and P2.4 nearly as well as with P2 (Fig. 3C). The mAb a20 reacted with P3 and P3.1, both of which contain RADWPGPP. The mAbs 1A8, 1G2, 3A6, 3H11, 6H12, 18A11, and 23B8 reacted strongly with P3, but not with P3.1, suggesting that their epitope, encompassed in GAGRADWPGPPPELDV, must contain ELVDV from the N-terminal region of migis- ϵ peptide (Fig. 3D). The mAb 4B12 recognizes an epitope in GLAGGSAQSQRAPDRV and 26H2, an epitope in GQQQGLPRAAG. The mAbs 4B12, 26H2, and a20, which

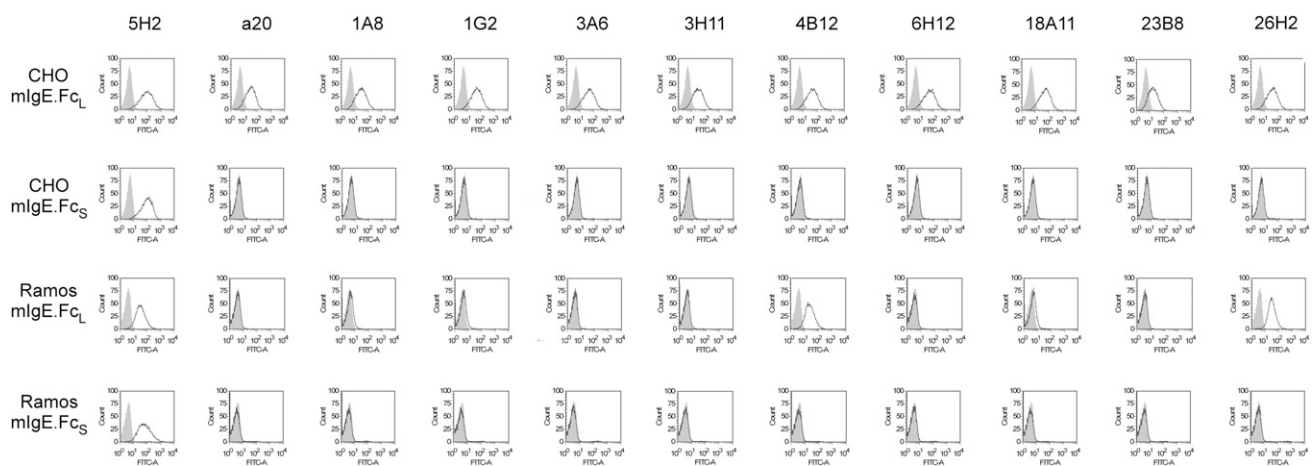


FIGURE 2. The binding of anti-CemX mAbs to CHO or Ramos lines that express $m\text{IgE.Fc}_L$ or $m\text{IgE.Fc}_S$. $m\text{IgE.Fc}_S$ -expressing CHO, $m\text{IgE.Fc}_L$ -expressing CHO, $m\text{IgE.Fc}_S$ -expressing Ramos, or $m\text{IgE.Fc}_L$ -expressing Ramos cells were incubated with 10 μ g/ml mouse anti-IgE mAb (5H2), anti-CemX mAbs (a20, 1A8, 1G2, 3A6, 3H11, 4B12, 6H12, 18A11, 23B8, and 26H2), or mouse nonimmune IgG (shaded histograms), followed by staining with FITC-labeled rabbit F(ab')₂ specific for mouse IgG.

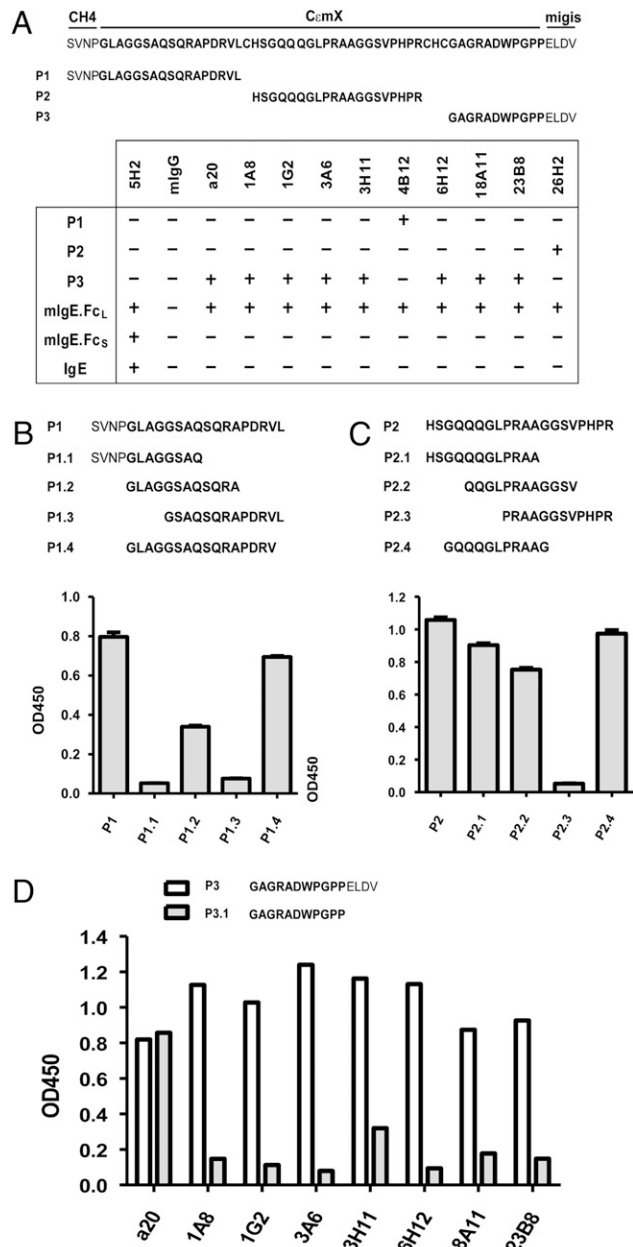


FIGURE 3. The reactivity of anti-CcmX mAbs with peptides representing consecutive segments of CcmX. **A**, Ninety-six-well ELISA plates were coated with 10 μ g/ml peptides or 1 μ g/ml human IgE, mIgE.Fc_L, or mIgE.Fc_S. The anti-IgE mAb, anti-CcmX mAbs, and mouse nonimmune IgG (mIgG) were tested at 1 μ g/ml. The secondary Ab was peroxidase-conjugated goat anti-mouse IgG. **B**, Epitope mapping of 4B12. **C**, Epitope mapping of 26H2. Data are averages of triplicate measurements.

recognize the three discrete parts of CcmX, were chosen for further affinity analysis and functional characterization.

Binding affinity of 4B12, 26H2, and a20 to CcmX

The binding affinity of anti-CcmX mAbs to γ 1-em67 was studied by SPR analysis using a Biacore T100 (GE Healthcare). γ 1-em67 was chosen as the analyte to couple to the CM5 chip, because purified mIgE.Fc_L-recombinant protein formed precipitates when the detergent UDM contained in the preparation was removed. In addition, purified IgE.Fc-CcmX-migis- ϵ and IgE.Fc-CcmX-migis- ϵ -leucine zipper recombinant proteins were unstable and underwent degradation at room temperature. Conversely, purified γ 1-em67 was stable and recognized by all nine anti-CcmX mAbs prepared in this study as

well as by a20. Solutions of each of 4B12, 26H2, and a20 were injected at different concentrations (6.25–400 nM), and the SPR was recorded as described in *Materials and Methods*. The mAb 4B12 had a K_D of 2.54 ± 0.58 nM, 26H2 had a K_D of 4.66 ± 0.27 nM, and a20 had a K_D of 1.68 ± 0.09 nM in binding to γ 1-em67 (Table I). Note that because the K_D constants of these three mAbs binding to γ 1-em67 are all in the nanomolar range, the ability of 4B12 and 26H2 and the inability of a20 to bind to mIgE.Fc_L-expressing Ramos cells (Fig. 2) should not be due to a difference in their binding affinity to CcmX.

The binding of anti-CcmX mAbs to mIgE.Fc_L-CHO cells that simultaneously express Ig α and Ig β

It was previously reported that both long and short isoforms of membrane-bound IgE (mIgE_L and mIgE_S) can assemble with Ig α and Ig β to form functional IgE-BCRs (13). To address whether the failure of a20 to bind to mIgE.Fc_L-expressing Ramos is due to the interference by Ig α and Ig β , we tested the binding of a20 to mIgE.Fc_L-CHO cells that also expressed Ig α and Ig β . The cDNAs of Ig α and Ig β were placed in an expression vector with two respective promoters and transfected into mIgE.Fc_L-expressing CHO cells. Fig. 4A shows that the CHO transfectants expressed mIgE.Fc_L and Ig α and Ig β . Fig. 4B shows that a20, 4B12, and 26H2 bound equally well to mIgE.Fc_L-expressing CHO cells, which did not express Ig α and Ig β , and that a20 bound more weakly than 4B12 and 26H2 to mIgE.Fc_L-expressing CHO cells that also expressed Ig α and Ig β . Note that mIgE.Fc_L was expressed at a much higher level in the CHO cells, which simultaneously expressed Ig α and Ig β , than in the CHO cells that did not express Ig α and Ig β . Because a20, 4B12, and 26H2 bound to γ 1-em67 with comparable affinity, as revealed in SPR assays (Table I), and equally well to mIgE.Fc_L-expressing CHO cells that did not express Ig α and Ig β (Figs. 2, 4B), the reduction of the binding of a20 to mIgE.Fc_L-expressing CHO cells which express Ig α and Ig β is probably related to the expression of Ig α and Ig β . Whether Ig α and Ig β do so by rendering steric hindrance over the accessibility of CcmX by a20 requires further investigation (see *Discussion*).

Construction and expression of chimeric forms of 4B12, 26H2, and a20 mAbs

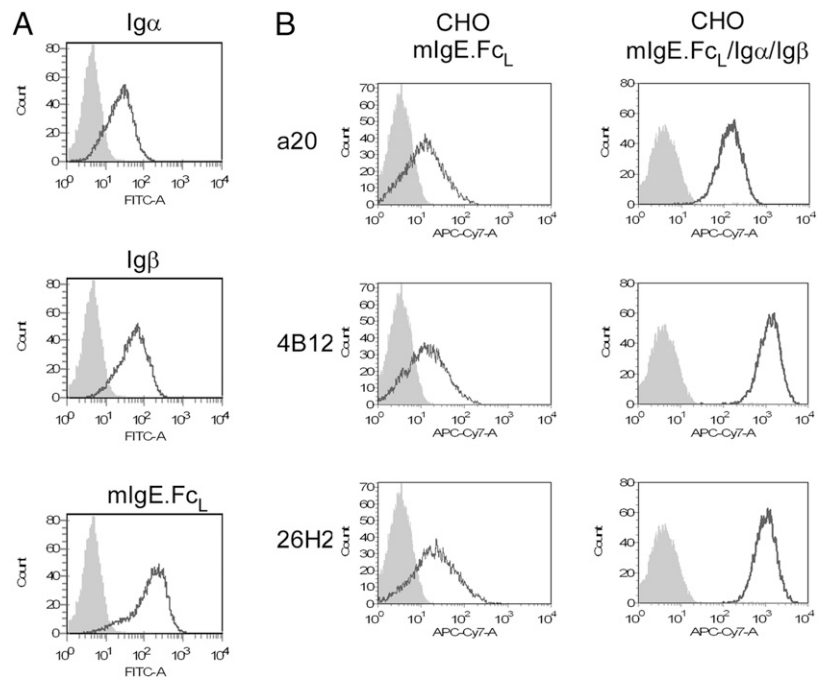
Because the immunopharmacologic activities of anti-CcmX mAbs may be mediated by human effector cells through the interaction of their IgG Fc receptors (Fc γ Rs) with the mAbs, chimeric forms of 4B12, 26H2, and a20 using human γ 1 constant regions were constructed. We prepared V_H and V_L cDNAs from the hybridoma clones producing the respective mAbs, constructed chimeric γ 1 heavy and κ or λ L chain genes, expressed the chimeric Ab genes in 293F cells, and purified the secreted recombinant Abs, as described in *Materials and Methods*. The V_H of 4B12, 26H2, and a20 belong to mouse V_H subgroups III, I, and V, respectively, whereas their V_L belong to mouse κ -chain subgroup I, λ -chain subgroup III, and κ -chain subgroup I, respectively. Two control chimeric mAbs, of cBAT123 (human γ 1 κ) and cB1-8 (human γ 1 λ), were also prepared. The mAb cBAT123 retained specificity for gp120 of HIV-1, and cB1-8 retained specificity for nitrophenyl-hapten. The specificity of the chimeric c4B12, c26H2, and ca20 for CcmX was confirmed by ELISA and fluorescent flow cytometric analysis. In the ELISA, the three mAbs bound to mIgE.Fc_L and not to mIgE.Fc_S, whereas omalizumab, which binds to the CH3

Table I. Surface plasmon resonance analysis of anti-CcmX mAbs

Ab	k_{on} ($\times 10^5$, M ⁻¹ s ⁻¹)	k_{off} ($\times 10^{-4}$, s ⁻¹)	K_D (nM)
4B12	0.543 ± 0.034	1.36 ± 0.23	2.54 ± 0.58
26H2	0.802 ± 0.068	3.75 ± 0.52	4.66 ± 0.27
a20	0.994 ± 0.039	1.67 ± 0.15	1.68 ± 0.09

Values are means $\pm$ SD of measurements from three experiments.

FIGURE 4. The binding of anti-CemX mAbs to CHO cells that express mIgE.Fc_L with and without coexpressed Igα and Igβ. **A**, Expression of mIgE.Fc_L, Igα, and Igβ of CHO cells transfected with mIgE.Fc_L, Igα, and Igβ. The cells were stained with FITC-labeled anti-IgE, anti-Igα, or anti-Igβ. **B**, The relative binding of 4B12, 26H2, and a20 to CHO cells that express mIgE.Fc_L without and with Igα and Igβ. The mIgE.Fc_L-CHO cells without Igα and Igβ were stained with biotin-labeled a20, 4B12, or 26H2 (histograms with solid contours), or isotype control mAbs (shaded histograms), followed by staining with APC-Cy7-labeled streptavidin. The mIgE.Fc_L-CHO cells with Igα and Igβ were stained additionally with FITC-labeled anti-Igα and PE-labeled anti-Igβ to allow the gating of cells positive for Igα and Igβ.



domain of IgE, bound to both mIgE.Fc_L and mIgE.Fc_S (Supplemental Fig. 1A). The mAbs c4B12, c26H2, and ca20 bound to mIgE.Fc_L-expressing CHO cells, but not to mIgE.Fc_S-expressing CHO and mIgE.Fc_S-expressing Ramos cells; c4B12 and c26H2 bound to mIgE.Fc_L-expressing Ramos cells, but ca20 did not (Supplemental Fig. 1B). These results indicate that c4B12, c26H2, and ca20 retain the binding specificity observed with their respective parental mouse mAbs.

Chimeric anti-CemX mAbs mediated apoptosis of mIgE.Fc_L-expressing B cells

To test whether c4B12 or c26H2 can induce apoptosis *in vitro*, mIgE.Fc_L-expressing Ramos cells were incubated with the chimeric mAbs with or without the addition of secondary Abs. In the same experiment, ca20 and omalizumab were also compared. The extent of apoptosis was assessed 24 h later using a flow cytometric-based assay that measured PS exposure, an early marker for cells undergoing apoptosis, using Annexin V. In the absence of secondary Abs, c4B12, c26H2, or omalizumab did not induce apoptosis (Fig. 5A). In the presence of secondary Abs, c4B12, c26H2, or omalizumab at 1 μg/ml caused, respectively, 83.2%, 84.8%, and 85.2% of mIgE.Fc_L-expressing Ramos cells to undergo apoptosis, whereas ca20 caused ~3.2%, suggesting that the cross-linking of IgE-BCR by these three mAbs required enhancement with secondary Abs, which by itself did not induce apoptosis (Fig. 5A). The apoptotic effects of c4B12, c26H2, or omalizumab on mIgE.Fc_L-expressing Ramos cells were dose dependent, reaching maximum levels at ~1 μg/ml (Fig. 5B). The observed apoptosis was not due to a possible crosslinking of FcγRIIB-bound anti-CemX mAb by the secondary Ab, because Ramos cells do not express FcγRIIB (26). Approximately 3–4% of apoptotic cells were observed in the presence of negative control mAbs, cBAT123, and cB1-8, at 2 μg/ml (Fig. 5B).

Anti-CemX-induced apoptosis of mIgE.Fc_L-expressing B cells is caspase-dependent

Apoptosis induced by many agents is associated with the activation of caspases and their subsequent cleavage of various cellular components. For example, caspase-3 cleavage has been observed 2–24 h after BCR ligation in various human B cell lines (27). To examine whether the apoptosis of mIgE.Fc_L-expressing Ramos cells by c4B12, c26H2, and omalizumab also involved caspase activation, immunoblot analysis of caspase-3 cleavage was performed (Fig.

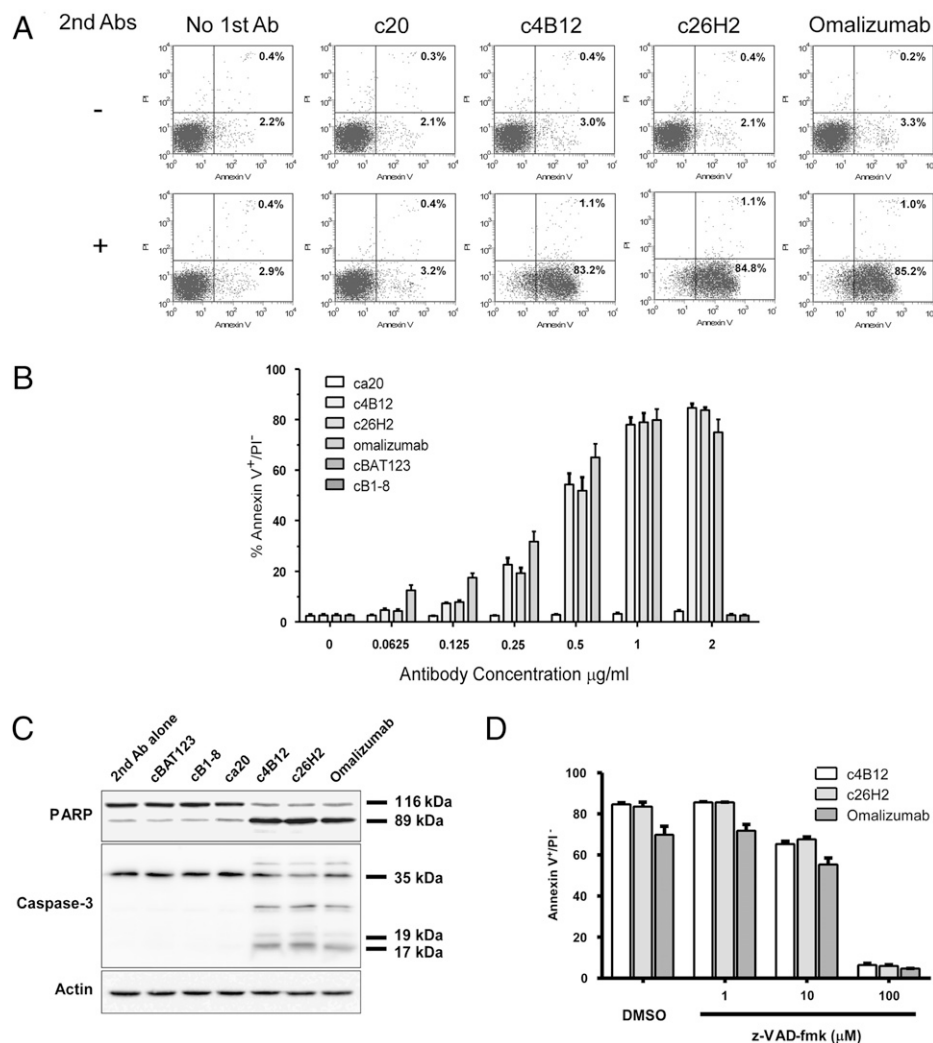
5C). The full length of caspase-3 was significantly decreased and cleaved into *M_r* 19- and 17-kDa fragments 24 h after mIgE.Fc_L-expressing Ramos cells were incubated with c4B12, c26H2, or omalizumab, but not with ca20, cBAT123, or cB1-8 in the presence of secondary Abs. In addition, another frequently studied substrate of caspases, PARP with intact *M_r* of 116 kDa, was also decreased and cleaved into a product of *M_r* 89 kDa (Fig. 5C). Secondary Abs alone had no effect on the cleavage of caspase-3 and PARP (Fig. 5C).

We further examined the effects of a broad-range caspase inhibitor, z-VAD-fmk, on the anti-CemX-induced apoptosis of mIgE.Fc_L-expressing Ramos cells. Fig. 5D shows that z-VAD-fmk inhibited the PS exposure of mIgE.Fc_L-expressing Ramos cells upon incubation with c4B12, c26H2, or omalizumab in a dose-dependent fashion. At 100 μM, z-VAD-fmk inhibited the apoptosis induced by these mAbs by ~90%. These results further confirm that the apoptosis of mIgE⁺ B cells induced by anti-CemX mAbs and omalizumab proceeds through the caspase pathway.

Anti-CemX mAbs induce Ab-dependent cellular cytotoxicity against mIgE.Fc_L-expressing B cells

To examine whether c4B12 and c26H2 can recruit effector cells and cause Ab-dependent cellular cytotoxicity (ADCC) upon IgE-BCR-expressing cells, mIgE.Fc_L-expressing Ramos cells were used as the target cells. The mAbs ca20 and omalizumab were also compared and rituximab, which binds to CD20, was used as a positive control for the ADCC assay (18). The ADCC activity of c4B12, c26H2, and omalizumab was examined at multiple E:T ratios. After 24 h incubation, c4B12, c26H2, omalizumab, and rituximab caused, respectively, 57%, 56.2%, 67.1%, and 67.6% specific lysis of targeted cells, while ca20 produced 17.2% lysis, at a maximum E:T ratio of 50 (Fig. 6A). Significant ADCC was observed when the concentrations of c4B12 and c26H2 were >0.01 μg/ml, compared with 0.001 μg/ml for omalizumab, probably owing to a higher binding affinity of omalizumab to mIgE.Fc_L than that of c4B12 and c26H2. At the maximum concentration of 10 μg/ml, the mean amount of specific lysis of target cells caused by c4B12, c26H2, omalizumab, and rituximab were 84.4%, 81.4%, 75.0%, and 88.4%, respectively, and ~50% with ca20 (Fig. 6B). These results indicated that c4B12, c26H2, ca20, and omalizumab, mediated ADCC of mIgE⁺ B cells in a dose-dependent fashion and that c4B12 and c26H2 are more effective than ca20 in this process.

FIGURE 5. Induction of apoptosis by chimeric anti-CemX mAbs. **A**, Induction of apoptosis by c4B12 or c26H2 in the absence or presence of secondary Abs. mIgE.Fc_L-expressing Ramos cells (5×10^5 cells/ml) were incubated with 1 μ g/ml c4B12, c26H2, ca20, or omalizumab for 1 h at 37°C, followed by incubation with or without 10 μ g/ml goat F(ab')₂ specific for Fc of human IgG (2nd Abs), or 2nd Abs alone for 24 h. **B**, Induction of apoptosis by increasing concentrations of c4B12 or c26H2. The staining with annexin V-FITC and PI and analysis of stained cells with FACSCanto II flow cytometer are described in *Materials and Methods*. Data are means $\pm$ SD from three experiments. **C**, The cleavage of caspase 3 and PARP in mIgE.Fc_L-expressing Ramos cells upon treatment with c4B12 or c26H2. Western blot analysis of 30 μ g of whole cell lysates with Abs specific for caspase 3 and PARP was described in *Materials and Methods*. **D**, The effects of z-VAD-fmk on anti-CemX-induced apoptosis. The staining with annexin V-FITC and PI and analysis with FACSCanto II flow cytometer were described in *Materials and Methods*. The mAbs, cBAT123 and cB1-8, were control Abs. Data are means $\pm$ SD from three experiments.



Anti-CemX mAbs inhibit IgE synthesis by stimulated PBMCs *in vitro*

The above results led to the question of whether anti-CemX mAbs can potentially suppress IgE production by B cells from human subjects. PBMCs prepared from blood samples from atopic dermatitis patients were cultured in the presence of IL-4 and anti-CD40 for 12 d to promote IgE production. In those cultures, IgE levels in the medium were mostly in the range measurable by the ELISA used in the current study. Rituximab, which is known to lyse B cells through apoptosis (28) and/or ADCC (18), reduced culture IgE by 63.6% (Fig. 7A) and IgM by 62.9% (Fig. 7B), but did not reduce IgA levels (Fig. 7C). The mAbs c4B12, c26H2, or omalizumab at 10 μ g/ml in the initiation of cultures reduced culture IgE levels by 42.2%, 51.8%, and 44.7%, respectively, compared with their isotype control Abs (Fig. 7A), but did not reduce IgM (Fig. 7B) or IgA levels (Fig. 7C). The mAb ca20 did not significantly affect the amount of IgE, IgM, or IgA. These results indicate that c4B12 and c26H2, as well as omalizumab, target primary mIgE⁺ B cells in an isotype-specific fashion.

Discussion

The goal of this study was to evaluate whether the various segments of CemX are accessible by Abs and can provide antigenic epitopes for Ab or immunogen-based targeting of IgE-BCR on B cells. Passive administered anti-CemX mAbs or endogenous anti-CemX Abs actively induced by a CemX-containing immunogen should

activate BCR signaling, leading to anergy or apoptosis of the mIgE⁺ B cells in the absence of T cell assistance (29). The differentiation of IgE-secreting plasma cells must go through the mIgE⁺ lymphoblast stage. Therefore, if the generation of plasma cells is abolished at the mIgE⁺ B cell stage by an immunologic agent targeting CemX, the synthesis of IgE by newly generated plasma cells will be interrupted. Because the existing IgE-secreting plasma cells will gradually die, anti-CemX treatment should result in a long-term attenuation of IgE synthesis and eventually a desensitized state in treated allergic patients.

The mAb, a20, previously developed by our group (9) was found to bind fairly well to mIgE.Fc_L-expressing cell lines, such as CHO or NS0 cell lines. However, a20 bound weakly to mIgE.Fc_L-expressing CHO cells that expressed Ig α and Ig β , when compared with 4B12 and 26H2, and did not bind to mIgE.Fc_L-expressing Ramos and A20 B cell lines. Furthermore, the chimeric a20 mAb did not lyse mIgE.Fc_L-expressing Ramos cells by either apoptosis or ADCC, nor did it inhibit IgE secretion of PBMCs driven by anti-CD40 and IL-4. As a consequence, the C-terminal segment of CemX is not a suitable epitope for developing immunologic agents for targeting mIgE⁺ B cells.

The structure and function of CemX, in particular the possible association of Ig α or Ig β or other proteins with CemX on B cells and the functional consequence of such molecular interaction, warrant further investigation. Several lines of evidence showed that the migis peptides of mIgM and mIgD were associated with Ig α of different glycosylation patterns in a BCR complex (30, 31).

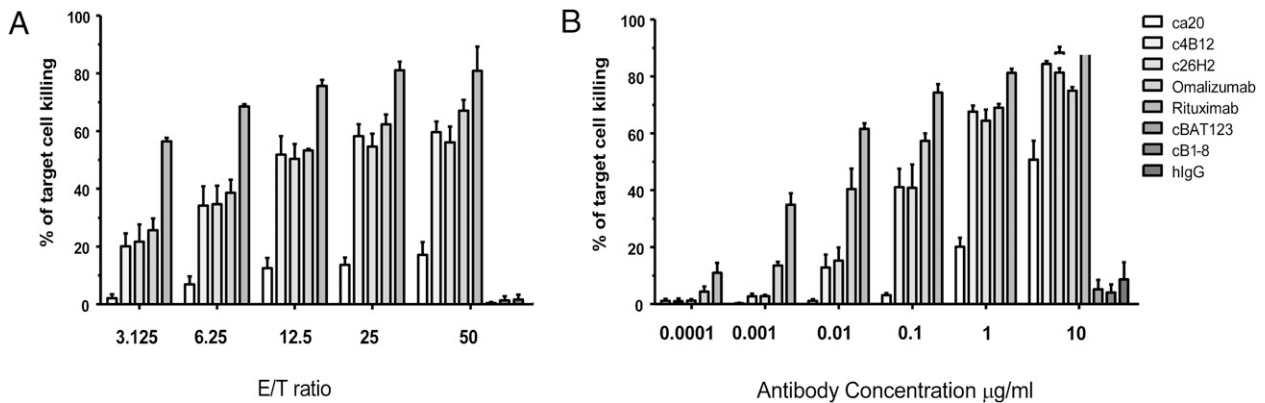


FIGURE 6. Chimeric anti-CemX-mediated ADCC of mIgE.FcL-expressing B cells. *A*, Induction of ADCC of mIgE.FcL-expressing Ramos cells by c4B12 or c26H2. CFSE-labeled cells were treated with specified Abs at 1 μ g/ml, mixed with PBMCs at different E:T ratios, incubated for 24 h, and stained with 7-amino actinomycin D for fluorescent flow cytometric analysis as described in *Materials and Methods*. Data are means $\pm$ SD of measurements from five experiments using PBMCs isolated from different donors. *B*, Induction of ADCC of mIgE.FcL-expressing Ramos cells by various concentrations of c4B12 or c26H2. CFSE-labeled cells were treated with specified Abs at different concentrations, and then PBMCs were added at an E:T ratio of 25. Human IgG, cBAT123, and cB1-8 were control Abs. Data are means $\pm$ SD of measurements from four experiments.

Batista et al. (13) showed that human mIgE_L and mIgE_S, when expressed on a mouse immature B cell line WEHI cells, were associated with different glycoforms of Ig α and with different amounts of ϵ BAP41. The expression of Ig α and Ig β by mIgE.FcL-Ramos cells somehow abolishes α 20 to bind to CemX, whereas their expression on mIgE.FcL-CHO cells only partially reduces α 20 to bind to CemX. One possible explanation is that certain other

proteins of B cells affect the accessibility of epitopes on human CemX by anti-CemX Abs. Whether Ig α and Ig β and other proteins do so by rendering steric hindrance remains to be clarified. To make this issue even more interesting, Feichtner et al. (10) showed that an anti-migis- ϵ mAb, mAbA9, could bind to mIgE-expressing mouse B lymphoma cell line K46 cells and block IgE synthesis in mouse *in vivo*. These various findings indicate that only parts of CemX in

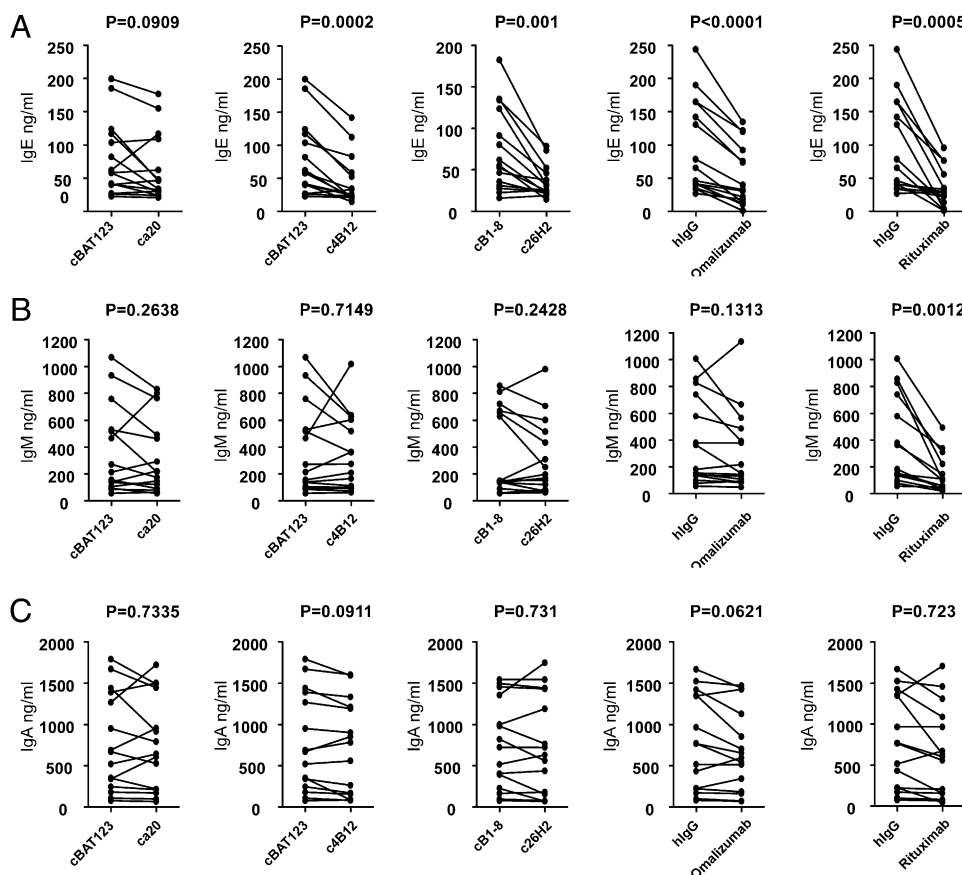


FIGURE 7. The inhibitory effects of anti-CemX mAbs on IgE synthesis *in vitro*. PBMCs from atopic dermatitis patients (10^6 cells/ml) were incubated with specified Abs at 10 μ g/ml with 100 ng/ml IL-4 and 100 ng/ml mouse anti-CD40 for 12 d at 37°C; ELISAs were performed to assay IgE, IgM, and IgA in cell-free supernatants. *A*, The effects on IgE. *B*, The effects on IgM. *C*, The effects on IgA. Human IgG, cBAT123, and cB1-8 were control Abs. Student *t* tests were used to compare data from 15 patients.

human mIgE on B cells are accessible by Abs, whereas the membrane-proximal migis- ϵ segment in mouse mIgE, which does not contain CemX, is somehow accessible by Abs.

The fact that most of the newly prepared anti-CemX mAbs (seven of nine) bound to P3 peptide (the C-terminal segment of CemX) was consistent with the report of Chen et al. (9), in which all anti-CemX mAbs generated bound to RADWPGPP. The C-terminal segment of CemX appears to be dominantly immunogenic, but not exclusive, because mAbs (4B12 and 26H2) specific for GLAGGSAQSQRA-PDRV (N-terminal of CemX) and GQQQGLPRAAG (middle segment of CemX) could still be generated. These two segments are nonetheless antigenic and readily accessible by Abs and represent unique epitopes for immunologic targeting of IgE-committed B cells.

In vitro functional characterization, c4B12 and c26H2, as well as omalizumab, were able to induce apoptosis in mIgE.FcL-expressing Ramos cells through a caspase pathway. Batista et al. (13) reported that the crosslinking by polyclonal anti-IgE serum activated a small extent of protein tyrosine phosphorylation and did not cause apoptosis of WEHI-231 cells transfected with the mIgEL isoform. Furthermore, Poggianella et al. (32) showed that mIgEL-expressing A20 cells were resistant to apoptosis when treated by polyclonal anti-idiotypic serum. It is possible that c4B12, c26H2, and omalizumab coupled with secondary Abs can induce extensive cross-linking of mIgEL and strong signal transduction to cause apoptosis, which is not attainable by polyclonal anti-IgE or anti-idiotypic Abs alone (33). Our assays examining the effects of c4B12, c26H2, and omalizumab on ADCC required a 24-h incubation rather than the more usual 4-h period, suggesting that the mAbs can possibly lyse mIgE.FcL-expressing Ramos cells via apoptosis in the presence of Fc γ R-expressing accessory cells (without secondary Abs). Shan et al. (34) showed that apoptosis of Ramos cells was induced by anti-CD20 mAb in the presence of mouse fibroblasts transfected with the human Fc γ RII/CDw32, and suggested that crosslinking of CD20 on malignant B cells by anti-CD20 mAb may be achieved by the binding of the mAb to Fc γ R on auxiliary cells in vivo. Studies are in progress in our laboratory to construct a transgenic mouse strain, whose B cells express mIgE containing human CemX. Such mice should be valuable for investigating the potential of anti-CemX mAbs, 4B12 and 26H2, in causing the reduction of mIgE⁺ B cells and serum IgE levels.

It is interesting that the amount of IgA in culture medium of PBMCs when treated with anti-CD40 and IL-4 was not affected by rituximab. CD20 is a B cell-specific marker whose expression is initiated during late pre-B cell development and lost during plasma cell differentiation (35, 36). The levels of IgA and IgE in blood were generally elevated in patients with atopic dermatitis (37). Studies have shown that high proportions (ranging 60–92%) of human blood plasma cells express IgA (38, 39). Furthermore, clinical studies have shown that rituximab-treated patients with systemic lupus erythematosus have normal IgA levels despite the marked reduction of B cells (40, 41). These findings suggest that the IgA in cultures of PBMCs from patients with atopic dermatitis is largely produced by existing IgA-secreting plasma cells, rather than by IgA-expressing lymphoblasts and plasma cells newly generated by anti-CD40 and IL-4.

Targeting IgE-committed B cells is an attractive approach for controlling IgE production, in consideration of the relative contribution of the short-lived IgE-secreting plasmablasts and plasma cells in the allergen-exposed peripheral mucosa and the long-living IgE-secreting plasma cells in the bone marrow in replenishing blood IgE. Several reports have demonstrated that mIgE⁺ B cells and IgE-secreting plasma cells are abundant in the nasal mucosa of symptomatic allergic rhinitis patients (42, 43) and that IgE is synthesized in the lung mucosa of asthmatic patients (44), suggesting that allergen-specific IgE is produced in allergen-exposed

mucosa. IgE class-switching recombination in nasal mucosa was observed after ex vivo or in vivo allergen exposure (45, 46). Although IgE may be produced in various lymphoid tissue sites, local mucosa offers not only biologic efficiency in countering allergen at its entry site, but also a microenvironment favoring class-switching to IgE (47). McMenamin et al. (48) showed that Ag-specific IgE-secreting plasma cells were not found in the bone marrow of rats that were repeatedly exposed to Ag-containing aerosols. Furthermore, a recent study in the murine system has shown that mIgE⁺ plasmablasts intrinsically do not migrate to the bone marrow and contribute to the pool of long-lived plasma cells as actively as mIgG1⁺ plasmablasts (49). Together, these studies suggest that Ag-specific IgE-committed B cells in the allergen-exposed mucosal tissues may contribute significantly to the Ag-specific IgE pool. Nonetheless, it is generally accepted that the lifespan of long-lived plasma cells in the bone marrow ranges from several weeks to several years (50). Earlier studies also showed that long-lived IgE-secreting plasma cells were persistent in rodents receiving whole body irradiation (51) or immunosuppressive treatment (52). Considering the longevity of IgE-secreting plasma cells, it is rational that a patient receiving mIgE-B cell-targeting treatment, with either an anti-CemX mAb or a CemX-based immunogen, also be given omalizumab in the initial period of treatment.

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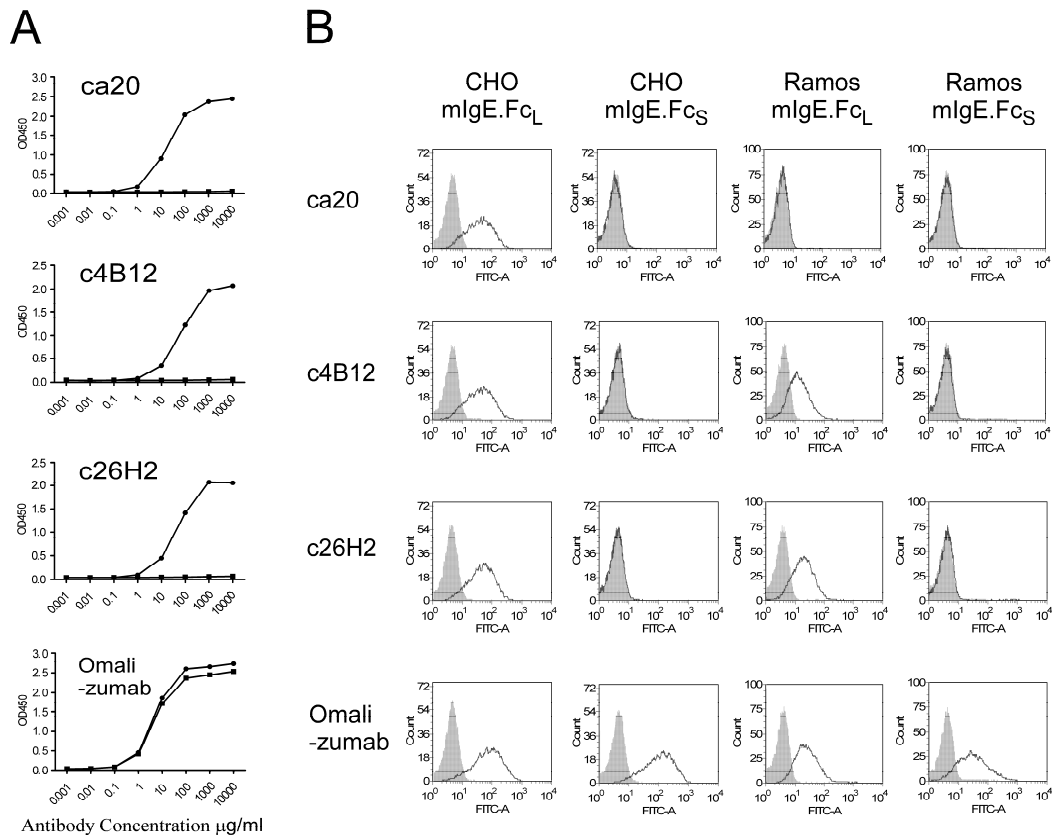
Disclosures

The authors have no financial conflicts of interest.

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Suppl. Figure 1. Specificity of chimeric anti-CemX mAbs in binding to CemX

(A) 96-well ELISA plates were coated with 1 $\mu\text{g/ml}$ of mlgE.Fc_L (●) or mlgE.Fc_S (■).

c4B12, c26H2, ca20, and omalizumab were tested at the indicated concentrations. Data are averages of triplicate measurements. (B) mlgE.Fc_S-expressing CHO, mlgE.Fc_L-expressing CHO, mlgE.Fc_S-expressing Ramos, or mlgE.Fc_L-expressing Ramos cells were incubated with c4B12, c26H2, ca20, or omalizumab (histograms with solid contours), or appropriate isotype control antibodies (filled histograms) at 10 $\mu\text{g/ml}$, followed by staining with FITC-labeled goat F(ab')₂ specific for human IgG.

Suppl. Table 1. Isotypes of anti-CemX
monoclonal antibodies studied in this paper

Antibody	Isotype
a20	IgG2b, κ
1A8	IgG1, κ
1G2	IgG2a, κ
3A6	IgG1, κ
3H11	IgG1, κ
4B12	IgG1, κ
6H12	IgG2b, κ
18A11	IgG2b, κ
23B8	IgG2b, κ
26H2	IgG1, λ