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Unique Epitopes on CεmX in IgE–B Cell Receptors Are Potentially Applicable for Targeting IgE-Committed B Cells

Jiun-Bo Chen,*† Pheidias C. Wu,‡,† Alfur Fu-Hsin Hung,§,† Chia-Yu Chu,§ Tsen-Fang Tsai,§ Hui-Ming Yu,† Hwan-You Chang,* and Tse Wen Chang†

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 (mε) exists predominantly in the long isoform, mεL, containing an extra 52 aa CεmX domain between CH4 and the C-terminal membrane-anchoring segment; the short isoform of mε, mεS, exists in minor proportions. CεmX thus provides an attractive site for immunologic targeting of mIgE+ B cells. In this study, we show that nine newly prepared CεmX-specific mAbs, as well as the previously reported a20, bound to mIgE.Fcγ1-expressing CHO cells, while only 4B12 and 26H2 bound to mIgE.Fcγ1-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 CεmX. Expression of Igε and Igβ on the mIgE.Fcγ1-CHO cells reduces the binding of a20 to CεmX as compared with that of 4B12 and 26H2. The chimeric mAbs c4B12 and c26H2, when cross-linked by secondary antibodies, lysed mIgE.Fcγ1-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.Fcγ1-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.

Immunglobulin 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 FcεRI 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 targets 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 IgE-expressing B cell is essential for generating isotype-specific responses (11, 12). For example, in transgenic mice lacking transmembrane and cytoplasmic segments of mε, 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 mε (i.e., mεL) contains an extra segment of 52 aa residues (referred to as CεmX; Fig. 1) between the CH4 domain and the migis segment (14). CεmX is resulting from an alternative splicing of the ε transcript, using an acceptor site 156-bp upstream of the previously known site used by mεS. The isoform mεL is predominantly expressed on human IgE-expressing myeloma cells and primary B cells, whereas mεS exists in minute or undetectable amounts at both mRNA and protein levels (14, 15). CεmX 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-CεmX mAbs, including a20, which bound to an 8-aa peptide segment, RADWPGPP, at the C-terminal end of CεmX (9). In the current study, we further investigate this peptidic segment and other parts of CεmX 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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Address correspondence and reprint requests to Dr. Tse Wen Chang, Genomics Research Center, Academia Sinica, Taipei, Taiwan. E-mail address: twchang@gate.sinica.edu.tw

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, phosphatidylserine; TM, transmembrane segment; UDM, α-undecy1-β-D-maltopyranoside.
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 t-glutamine, 25 mM HEPES, and 1 mM sodium pyruvate (Invitrogen; complete RPMI medium). Stable transfectants of Ramos cells expressing mIgE.FcL or mIgE.FcS 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 t-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). Sodium bicarbonate was used as the pH indicator while growing mIgE.FcL or mIgE.FcS, 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.FcL, 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 105 mIgE.FcL or mIgE.FcS-expressing CHO cells were collected by centrifugation at 400 × g and resuspended in 30 ml of suspension buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM PMSF), followed by homogenization at 15000 × g 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-decyl-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.FcL or mIgE.FcS 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 Superose 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.FcL emulsified in TiterMax Gold adjuvant (Sigma-Aldrich) at 2-wk intervals. A final boost was given i.p. with 0.1 mg of mIgE.FcL without adjuvant, and 3 d later, the spleen cells from immunized mice were used for hybridomas as described previously (9). The isotopes of anti-CemX mAbs prepared in this study were determined by an Ig isotyping ELISA kit (Caldag Laboratories, Burlingame, CA) and listed in Supplemental Table 1.

For chimerization of 4B12, 26H2, and a20, cDNA of Vk was amplified by PCR using a set of DNA primers as designed by Essono et al. (16). The Vk of 4B12 and a20 and Vk of 26H2 were PCR-amplified using specific primers designed by Essono et al. (16) and Ig-Primers for mouse Vk (Novagen, Madison, WI). The Vk and Vk of 4B12, 26H2, and a20 were cloned and sequenced. The cDNAs of mouse Vk and Vk from 4B12, a20, and BAT123, which is specific for a peptide segment of gp120 of HIV-1 (17), were joined to a human Cγ1 and a human Cε of a chimeric Ab expression vector—the plgG1i(ε) vector, which was modified from the TCAE vector (18). The Vk and Vk of 26H2 and B1-8, which is specific for nitrophenyl-hapten (19) (a gift from Dr. Takeshi Tsutaba, Tokyo Medical and Dental University, Tokyo, Japan), were ligated to a human Cγ1 and a human Cε of the plgG1(ε) vector.

Transfection of chimeric Ab genes into 293F cells was performed at the cell density of 105 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 for 4 h. The cell density was adjusted to 2.5 × 105 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-e (γ1-εm67), containing CH2-CH3 of γ1, CemX, and migis-e segments (9) was diluted in 10 mM sodium acetate (pH 5.5) at a concentration of 20 μg/ml and coupled to a CM1 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.

Apopotosis assays

Ramos cells stably expressing mIgE.FcL (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 phosphotyridylserine (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 FACS-Canto 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 detection 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 (BD Biosciences, San Jose, CA) 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 t-glutamine, 25 mM HEPES, 50 μg/ml penicillin, and 100 μg/ml streptomycin (complete IMDM medium). mIgE.FcL-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 106 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 FACS-Canto 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]).
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 μg/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 Cε constructs used to transfect CHO and Ramos cells. Cε contains 52 aa and migis-e 15 aa. TM, transmembrane segment.

IgE production by stimulated PBMCs in vitro

To obtain anti-Cε mAbs specific for various parts of CεX, BALB/c mice were immunized with recombinant mlgE.Fcε, protein, which contains the CH2 domain through the cytoplasmic end of mε chain, and hybridoma clones were prepared. Of the more than 4000 hybridoma clones screened, nine clones with binding activity with mlgE.Fcε but not with mlgE.FcS, were obtained. The nine clones were further tested for binding to mlgE.FcS-expressing CHO, mlgE.FcL-expressing CHO, mlgE.FcS-expressing Ramos, and mlgE.FcL-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 mlgE.FcL-expressing CHO cells, indicating that they recognized CεX. Among the nine anti-CεX clones, only two (4B12 and 26H2) bound to mlgE.FcL-expressing Ramos cells. The other seven clones (1A8, 1G2, 3A6, 3H11, 6H12, 18A11, 23B8) did not bind to mlgE.FcL-expressing Ramos cells. The anti-CεX mAb a20, which was prepared by our group earlier (9), bound to mlgE.Fcε-expressing CHO, but did not bind to mlgE.FcS-expressing CHO, mlgE.FcS-expressing Ramos, and mlgE.FcL-expressing Ramos cells (Fig. 2). In addition, a20 did not bind to both mlgE.FcS- and mlgE.FcS-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-CεX mAbs bound to denatured mlgE.Fcε in a Western blot analysis (data not shown), indicating that they can recognize unfolded singular pepitic segments in CεX. Because a CεX 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-CεX 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 CεX; P2 peptide corresponded to the middle 20 aa residues of CεX; and P3 peptide of 15 aa residues contained the CεX C-terminal 11 aa residues and 4 aa residues corresponding to the first four residues of the migis-e 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-CεX mAbs, we constructed overlapping, sequential peptides covering the N-terminal, middle, and C-terminal regions of CεX. 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 RADWGPP. 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 GAGRADWPGPPELDV, must contain ELDV from the N-terminal region of migis-e peptide (Fig. 3D). The mAb 4B12 recognizes an epitope in GLAGGSAQSQRAPDRV and 26H2, an epitope in GQQQGLPRAAG. The mAbs 4B12, 26H2, and a20, which recognize the three discrete parts of CεX, were chosen for further affinity analysis and functional characterization.

![FIGURE 1. Schematic representation of human ε-chain, the long and short isoforms of me chain, and the long and short me.Fc constructs used to transfect CHO and Ramos cells. Cε contains 52 aa and migis-e 15 aa. TM, transmembrane segment.](http://www.jimmunol.org/)

![FIGURE 2. The binding of anti-CεX mAbs to CHO or Ramos lines that express mlgE.Fcε, or mlgE.Fcδ, or mlgE.FcS-expressing CHO, mlgE.FcL-expressing CHO, mlgE.FcS-expressing Ramos, or mlgE.FcL-expressing Ramos cells were incubated with 10 μg/ml mouse anti-IgE mAb (5H2), anti-CεX 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.](http://www.jimmunol.org/)
Binding affinity of 4B12, 26H2, and a20 to CemX

The binding affinity of anti-CemX mAbs to γ1-em67 was studied by SPR analysis using a Biacore T100 (GE Healthcare). γ1-em67 was chosen as the analyte to couple to the CM5 chip, because purified mlgE.Fcγ-recombinant protein formed precipitates when the detergent UDM contained in the preparation was removed. In addition, purified IgE.Fc-CemX-migis-e and IgE.Fc-CemX-migis-e-leucine zipper recombinant proteins were unstable and underwent degradation at room temperature. Conversely, purified γ1-em67 was stable and recognized by all nine anti-CemX 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 γ1-em67 (Table I). Note that because the K_D constants of these three mAbs binding to γ1-em67 are all in the nanomolar range, the ability of 4B12 and 26H2 and the inability of a20 to bind to mlgE.Fcγ-expressing Ramos cells (Fig. 2) should not be due to a difference in their binding affinity to CemX.

The binding of anti-CemX mAbs to mlgE.Fcγ-CHO cells that simultaneously express Igα and Igβ

It was previously reported that both long and short isoforms of membrane-bound IgE (mlgE.Fcγ and mlgE.Fcα) can assemble with Igα and Igβ to form functional IgE–BCRs (13). To address whether the failure of a20 to bind to mlgE.Fcγ-expressing Ramos is due to the interference by Igα and Igβ, we tested the binding of a20 to mlgE.Fcγ-CHO cells that also expressed Igα and Igβ. The cDNAs of Igα and Igβ were placed in an expression vector with two respective promoters and transfected into mlgE.Fcγ-expressing CHO cells. Fig. 4A shows that the CHO transfectants expressed mlgE.Fcγ and Igα and Igβ. Fig. 4B shows that a20, 4B12, and 26H2 bound equally well to mlgE.Fcγ-expressing CHO cells, which did not express Igα and Igβ, and that a20 bound more weakly than 4B12 and 26H2 to mlgE.Fcγ-expressing CHO cells that also expressed Igα and Igβ. Note that mlgE.Fcγ was expressed at a much higher level in the CHO cells, which simultaneously expressed Igα and Igβ, than in the CHO cells that did not express Igα and Igβ. Because a20, 4B12, and 26H2 bound to γ1-em67 with comparable affinity, as revealed in SPR assays (Table I), and equally well to mlgE.Fcγ-expressing CHO cells that did not express Igα and Igβ (Figs. 2, 4B), the reduction of the binding of a20 to mlgE.Fcγ-expressing CHO cells, which express Igα and Igβ, is probably related to the expression of Igα and Igβ. Whether Igα and Igβ do so by rendering steric hindrance over the accessibility of CemX 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-CemX mAbs may be mediated by human effector cells through the interaction of their IgG Fc receptors (FcγRs) with the mAbs, chimeric forms of 4B12, 26H2, and a20 using human γ1 constant regions were constructed. We prepared V_H and V_L cDNAs from the hybridoma clones producing the respective mAbs, constructed chimeric γ1 heavy and κ or λ light 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 γ1κ) and cB1-8 (human γ1λ), 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 CemX was confirmed by ELISA and fluorescent flow cytometric analysis. In the ELISA, the three mAbs bound to mlgE.Fcγ, and not to mlgE.Fcα, whereas omalizumab, which binds to the CH3

<table>
<thead>
<tr>
<th>Ab</th>
<th>K_0 (×10^6, M^-1 s^-1)</th>
<th>K_off (×10^-4, s^-1)</th>
<th>K_D (nM)</th>
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<tr>
<td>4B12</td>
<td>0.543 ± 0.034</td>
<td>1.36 ± 0.23</td>
<td>2.54 ± 0.58</td>
</tr>
<tr>
<td>26H2</td>
<td>0.802 ± 0.068</td>
<td>3.75 ± 0.52</td>
<td>4.66 ± 0.27</td>
</tr>
<tr>
<td>a20</td>
<td>0.994 ± 0.039</td>
<td>1.67 ± 0.15</td>
<td>1.68 ± 0.09</td>
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Values are means ± SD of measurements from three experiments.
FIGURE 4. The binding of anti-CemX mAbs to CHO cells that express mIgE.FcL, with and without coexpressed Igα and Igβ. A. Expression of mIgE.FcL, Igα, and Igβ of CHO cells transfected with mIgE.FcL, 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.FcL without and with Igα and Igβ. The mIgE.FcL-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.FcL-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.

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

To test whether 4B12 or 26H2 can induce apoptosis in vitro, mIgE.FcL-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.FcL-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.FcL-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 FcyRIIB-bound anti-CemX mAb by the secondary Ab, because Ramos cells do not express FcyRIIB (26). Approximately 3–4% of apoptotic cells were observed in the presence of negative control mAbs, cBAT123, and c8B-8, at 2 μg/ml (Fig. 5B).

Anti-CemX-induced apoptosis of mIgE.FcL-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.FcL-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 M1, 19- and 17-kDa fragments 24 h after mIgE.FcL-expressing Ramos cells were incubated with c4B12, c26H2, or omalizumab, but not with ca20, cBAT123, or c8B-8 in the presence of secondary Abs. In addition, another frequently studied substrate of caspases, PARP with intact M1, of 116 kDa, was also decreased and cleaved into a product of M1, 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.FcL-expressing Ramos cells. Fig. 5D shows that z-VAD-fmk inhibited the PS exposure of mIgE.FcL-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.FcL-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.FcL-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.FcL 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.
Anti-Cε mAbs inhibit IgE synthesis by stimulated PBMCs in vitro

The above results led to the question of whether anti-Cε 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 μg/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 CεX are accessible by Abs and can provide antigenic epitopes for Ab or immunogen-based targeting of IgE-BCR on B cells. Passive administered anti-CεX mAbs or endogenous anti-CεX Abs actively induced by a CεX-containing immunogen should activate BCR signaling, leading to anergy or apoptosis of the mlgE+ B cells in the absence of T cell assistance (29). The differentiation of IgE-secreting plasma cells must go through the mlgE+ lymphoblast stage. Therefore, if the generation of plasma cells is abolished at the mlgE+ B cell stage by an immunologic agent targeting CεX, the synthesis of IgE by newly generated plasma cells will be interrupted. Because the existing IgE-secreting plasma cells will gradually die, anti-CεX 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 mlgE.FcL-expressing cell lines, such as CHO or NS0 cell lines. However, a20 bound weakly to mlgE.FcL-expressing CHO cells that expressed Igα and Igβ, when compared with 4B12 and 26H2. Western blot analysis of 30 μg 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-CεX-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 ± SD from three experiments.
Batista et al. (13) showed that human mILgEL and mILgES, when expressed on a mouse immature B cell line WEHI cells, were associated with different glycoforms of Igα and with different amounts of εBAP41. The expression of Igα and Igβ by mILgE.FcLRamos cells somehow abolishes a20 to bind to CεmX, whereas their expression on mILgE.FcL-CHO cells only partially reduces a20 to bind to CεmX. One possible explanation is that certain other proteins of B cells affect the accessibility of epitopes on human CεmX by anti-CεmX Abs. Whether Igα and Igβ 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-mILgE-ε mAb, mAbA9, could bind to mILgE-expressing mouse B lymphoma cell line K46 cells and block IgE synthesis in mouse in vivo. These various findings indicate that only parts of CεmX in

**FIGURE 6.** Chimeric anti-CεmX-mediated ADCC of mILgE.FcL-expressing B cells. A, Induction of ADCC of mILgE.FcL-expressing Ramos cells by c4B12 or c26H2. CFSE-labeled cells were treated with specified Abs at 1 μg/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 ± SD of measurements from five experiments using PBMCs isolated from different donors. B, Induction of ADCC of mILgE.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 ± SD of measurements from four experiments.

**FIGURE 7.** The inhibitory effects of anti-CεmX mAbs on IgE synthesis in vitro. PBMCs from atopic dermatitis patients (10⁶ cells/ml) were incubated with specified Abs at 10 μg/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 mlgE on B cells are accessible by Abs, whereas the membrane-proximal migis-ε segment in mouse mlgE, 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 RADWPGP. The C-terminal segment of CemX appears to be dominantly immunogenic, but not exclusive, because mAbs (4B12 and 26H2) specific for GLAGGSASQSRAPDRV (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 mlgE,FcεI-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 mlgEε isoform. Furthermore, Poggianella et al. (32) showed that mlgEε-expressing A20 cells were resistant to apoptosis when treated by polyclonal anti-idiotype serum. It is possible that c4B12, c26H2, and omalizumab coupled with secondary Abs can induce extensive cross-linking of mlgEL and strong signal transduction to cause apoptosis, which is not attainable by polyclonal anti-IgE or anti-idiotype 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 mlgE,FcεI-expressing Ramos cells via apoptosis in the presence of FcyR-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 FcyRII/Cd832, and suggested that crosslinking of CD20 on malignant B cells by anti-CD20 mAb may be achieved by the binding of the mAb to FcyR on auxiliary cells in vivo. Studies are in progress in our laboratory to construct a transgenic mouse strain, whose B cells express mlgEl containing human CemX. Such mice should be valuable for investigating the potential of anti-CemX mAbs, 4B12 and 26H2, in causing the reduction of mlgElε 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 mlgEε 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 mlgEε plasmablasts intrinsically do not migrate to the bone marrow and contribute to the pool of long-lived plasma cells as actively as mlgG1ε 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 mlgEε-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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References


