

ORIGINAL ARTICLE

EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Generating allergen-specific human IgEs for immunoassays by employing human ϵ gene knockin mice

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Keywords

allergen-specific IgE antibodies; Chimeric human IgE; IgE hybridoma; knockin mice.

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Abstract

Background: Antigen-specific human IgEs are important reagents in immunoassays to quantify antigen-specific IgEs in allergic patients, but they are not easy to prepare.

Methods: We constructed a knockin homozygous mouse strain, referred to as H ϵ KI strain, whose gene segment encoding γ 1 constant region has been replaced by that encoding human ϵ constant region and gene segment encoding κ constant region replaced by that encoding human κ constant region. The mice were tested for their ability to produce antigen-specific chimeric human IgE (with mouse variable regions) upon the immunization with ovalbumin and papain. Subsequently, the spleen cells from the immunized mice were used as the source of B cells for the preparation of hybridomas, which secreted monoclonal human IgE antibodies specific for the antigens.

Results: The H ϵ KI mice expressed human IgE (ϵ , κ) in serum at levels 10- to 30-fold higher than those of mouse IgE. Upon immunization with an antigen, the mice yielded splenic B cells for preparing hybridomas that secrete chimeric human IgE specific for the antigen. Purified IgEs from those hybridomas could activate a basophilic cell line to undergo degranulation upon the stimulation with their respective antigens.

Conclusions: We have developed a human ϵ gene and κ gene knockin mouse strain, which is useful for producing various antigen-specific chimeric human IgEs for potential use as standards in immunoassays.

Determining the allergen profile of an allergic patient has been a routine practice in the care of patients with moderate to severe allergy. The most widely used diagnostic methods for this purpose are skin prick tests or patch tests and *in vitro* serological IgE antibody assays (1), which quantify total IgE and allergen-specific IgEs (sIgEs) in the blood samples of patients. The current immunoassays for determining sIgEs in serum samples do not employ purified human sIgEs as standards or calibrators (2). A possible reason is that human sIgEs specific for various allergens are extremely difficult to

prepare. The serum concentrations of IgE in patients with allergy are generally in the range of 5 ng to 12 μ g/ml in total and rarely above this level, and sIgE typically accounts for less than a few percent in total IgE (3). Furthermore, the scarce supply of purified natural allergenic proteins would make the affinity purification of sIgEs from human serum impractical.

Herein, we explore the feasibility of preparing monoclonal human allergen-sIgEs by creating a knockin mouse strain, which produces human IgE at elevated levels, and then by performing hybridoma methodology in a similar fashion as used in preparing IgG monoclonal antibodies. Recognizing the fact that in the C57BL/6 (B6) mouse strain, IgG1 is the most abundantly expressed IgG subclass, we therefore constructed a knockin mouse strain, in which the endogenous mouse gene segments encoding mouse γ 1 chain spanning

Abbreviations

BAC, bacterial artificial chromosome; ELISPOT, enzyme-linked immunospot; IgEs, IgE antibodies; IgGs, IgG antibodies; sIgEs, allergen-specific IgE antibodies; V_H, heavy chain variable region; V_L, light chain variable region.

CH1 to the transmembrane tail and mouse κ chain constant region, respectively, were replaced by the corresponding gene segments encoding human ϵ heavy chain and κ chain. We subsequently demonstrated that mice of this knockin strain could serve as hosts for the immunization of common allergens and provide as the source of IgE-expressing B lymphoblasts for the preparation of hybridomas secreting allergen-specific chimeric IgE monoclonal antibodies (with human ϵ and κ chain constant regions and mouse variable regions).

Material and methods

The following material and methods sections are presented in Supporting Information: (i) the construction of C ϵ and C κ gene-targeting constructs, (ii) generation of chimeric C ϵ and C κ knockin mice, (iii) real-time RT-PCR for detecting human ϵ and mouse γ 1 mRNA, (iv) determination of isotype-specific immunoglobulin-secreting plasma cell numbers by ELISPOT, and (v) purification of human IgE and papain-specific chimeric human IgE mAbs.

Determination of serum immunoglobulin levels by ELISA

Serum levels of immunoglobulins were measured by goat anti-mouse IgM, or goat anti-mouse IgE, goat anti-human IgE (Bethyl Laboratories, Montgomery, TX, USA), goat anti-mouse IgG1, IgG2b, IgG2c, IgG3 (Southern Biotech, Birmingham, AL, USA) as solid-phase adsorbent antibodies and their respective horseradish peroxidase (HRP)-conjugated forms as indicator antibodies. Mouse reference serum, mouse IgE calibrator, and human IgE calibrator (Bethyl Laboratories) were used as calibration standards.

Generation of antigen-specific chimeric human IgE mAbs

Seven- to eight-week-old H ϵ KI mice were immunized with 50 μ g of papain or ovalbumin (Sigma-Aldrich) emulsified with TiterMax[®] Gold (Sigma-Aldrich, St Louis, MO, USA) injected subcutaneously twice at day 1 and day 29. A final boost was injected intraperitoneally with 100 μ g antigen in PBS 3 days before mice were killed (day 43). The spleen cells from an immunized mouse were used for preparing hybridomas as described previously (4). Culture supernatants of hybridomas were screened for antigen specificity by ELISA.

Characterization of purified antigen-specific chimeric human IgEs

The purified fully human IgE secreted from U266 human myeloma cell line and the chimeric human IgE from the IgE hybridomas were analyzed by SDS-PAGE and Western blot. In ELISA, for examining antibody binding specificity, wells of microtiter plates were precoated with 250 ng/well of ovalbumin, papain, human serum albumin, gelatin (Sigma-Aldrich), or BSA (New England Biolabs, Ipswich, MA, USA) and then applied with 50 ng chimeric human IgE in 1% BSA/PBS. The binding was measured with HRP-conjugated goat anti-human IgE.

Activation of RBL SX-38 cells and β -hexosaminidase release assay

The RBL SX-38 cell line was a gift from Dr. J.P. Kinet of Harvard Medical School. The maintenance of RBL SX-38 cells and β -hexosaminidase assay procedure was described previously (5). Human IgE and antigen-specific chimeric human IgEs at 1 μ g/ml were used to charge the RBL SX-38 cells. The antigens, papain at 1 μ g/ml and ovalbumin at 1 and 10 μ g/ml, and controls, goat anti-human IgE at 1 μ g/ml and goat IgG (Bethyl Laboratories, Inc.) at 1 μ g/ml, all in Tyrode's buffer, were, respectively, tested for their abilities to degranulate RBL SX-38 cells pre-incubated with the different IgEs.

Results

Mouse H ϵ KI strain with γ 1 and κ genes replaced by human ϵ and κ genes

Two knockin mouse strains were separately generated. In one, the mouse γ 1 gene segment spanning from the beginning of CH1 exon to the end of membrane exon 2 was replaced by human ϵ gene segment spanning from CH1 to membrane exon 2 (Fig. 1A). In the other, the mouse κ gene segment was replaced by human κ gene counterpart (Fig. 1B). After these two homozygous mouse strains were generated, they were crossed and homozygous mouse strain with the two new genetic traits, referred to as H ϵ KI strain, was obtained. At the time when this manuscript was prepared, eight generations of H ϵ KI had been generated. Southern blotting analyses of murine γ 1 and human ϵ genes and PCR analyses of murine γ 1, κ , human ϵ , and κ transcripts were performed to confirm the genotypes of the new bred (Fig. 1C,D). The levels of mRNA of human ϵ and murine γ 1 in the splenocytes were evaluated by real-time RT-PCR for the three genotypes of mice, namely, the wild-type mice (murine γ 1/murine γ 1), the heterozygous mice (murine γ 1/human ϵ), and the homozygous H ϵ KI mice (human ϵ /human ϵ). All three genotypes in this experiment bore human κ gene. The results showed that the heterozygous mice produced about a half amount of murine γ 1 mRNA as the wild-type and the homozygous H ϵ KI mice produced no murine γ 1 mRNA (Fig. 1E). The homozygous H ϵ KI mice produced approximately twice the amount of human ϵ mRNA as the heterozygous mice, and the wild-type produced no human ϵ mRNA (Fig. 1F).

Chimeric human IgEs in H ϵ KI mice were increased upon antigen immunization

The three genotypes of mice were immunized with papain at day 1 and day 29. Blood samples from the immunized mice were collected from a facial vein in the submandibular area by a lancet (MEDipoint, Inc., Mineola, NY, USA) before the immunizations at days 1 and 29, and also at days 15 and 43. Serum levels of mouse IgM, IgE, IgG1, IgG2b, IgG2c, IgG3, and human IgE were measured by ELISA. The results showed that prior to the first immunization, serum levels of

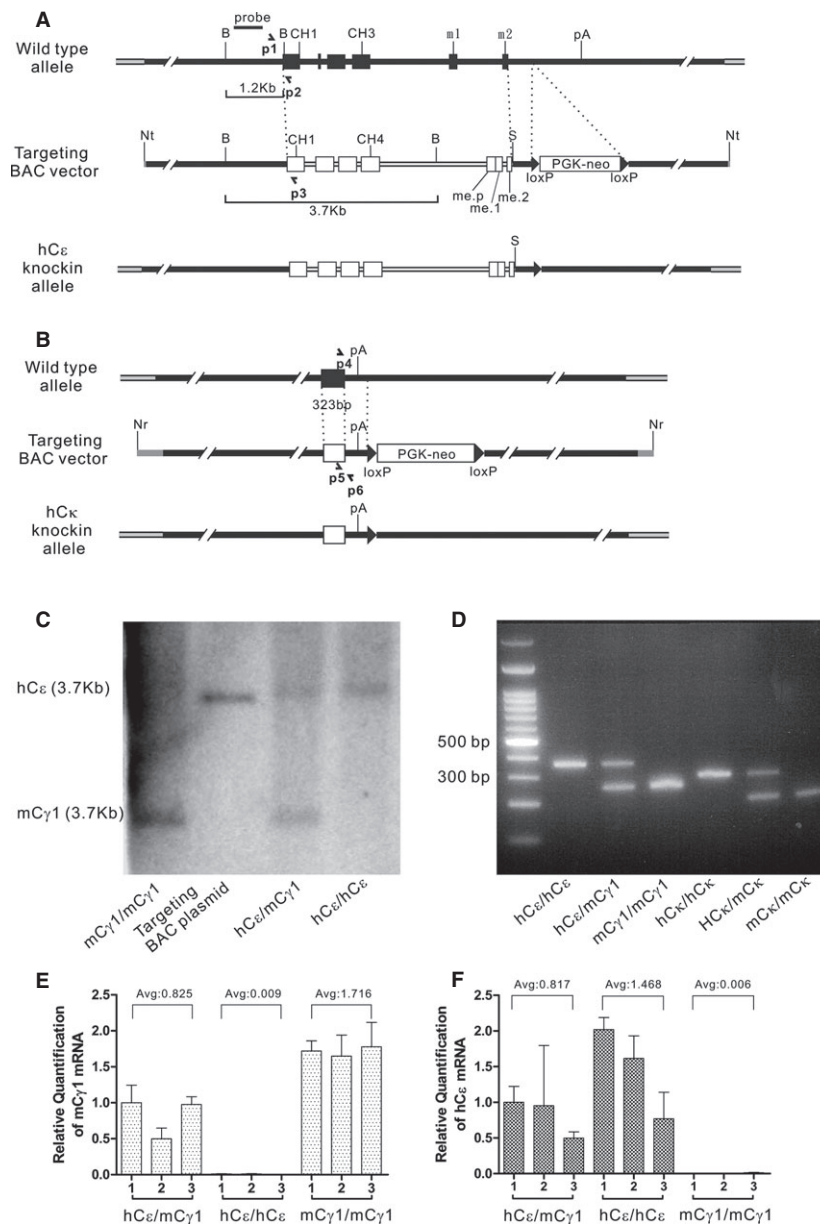


Figure 1 The generation of H ϵ K κ I mice with knockin human C ϵ and C κ . A and B, schematic diagrams of the genomic organizations of the mouse C γ 1 and C κ genes, the targeting BAC vectors and the knockin gene segments. The m1 and m2 gene segments are membrane exons for mouse C γ 1, and the mep, me1, and me2 gene segments are membrane exons for human C ϵ . Segments p1, p2, p3, p4, p5, and p6 correspond to primers used in the PCR in panel D. The restriction enzyme sites are B, BamHI; Nt, NotI; Nr, NruI; and S, SacII. pA denotes polyadenylation signal. C, Southern

blotting analysis of genomic DNA extracted from a piece of tail tissue of the indicated mice. D, Genotyping by genomic PCR with primers p1, p2, and p3 for C ϵ knockin mice (C γ 1:257 bp; C ϵ :362 bp) and p4, p5, and p6 for C κ knockin mice (mC κ :207 bp; hC κ :300 bp). E and F, relative quantification (RQ) of C γ 1 and C ϵ mRNA of three genotypes of mice performed by real-time RT-PCR (the RQ of C γ 1 and C ϵ for the 1st H ϵ K κ I/C γ 1 mouse was set as 1). Data are means $\pm$ SD of triplicate measurements.

mouse IgEs in most of the mice of all three genotypes were below the detectable level of 16 ng/ml. The limit of detection for the assay was 3.9 ng/ml, and the sera were diluted four-fold for the assay. The serum levels of human IgE in the homozygous H ϵ K κ I mice were in the range of 10–150 ng/ml. After the immunization with papain, the serum levels of

human IgE in the homozygous H ϵ K κ I mice were increased to 300–920 ng/ml (with an average of 595 ng/ml) at day 43. The serum levels of mouse IgE of wild-type mice were 7–75 ng/ml (with an average of 35 ng/ml) at day 43 (Fig. 2A). Thus, the serum levels of human IgE in immunized homozygous H ϵ K κ I mice were about 10- to 30-fold higher than

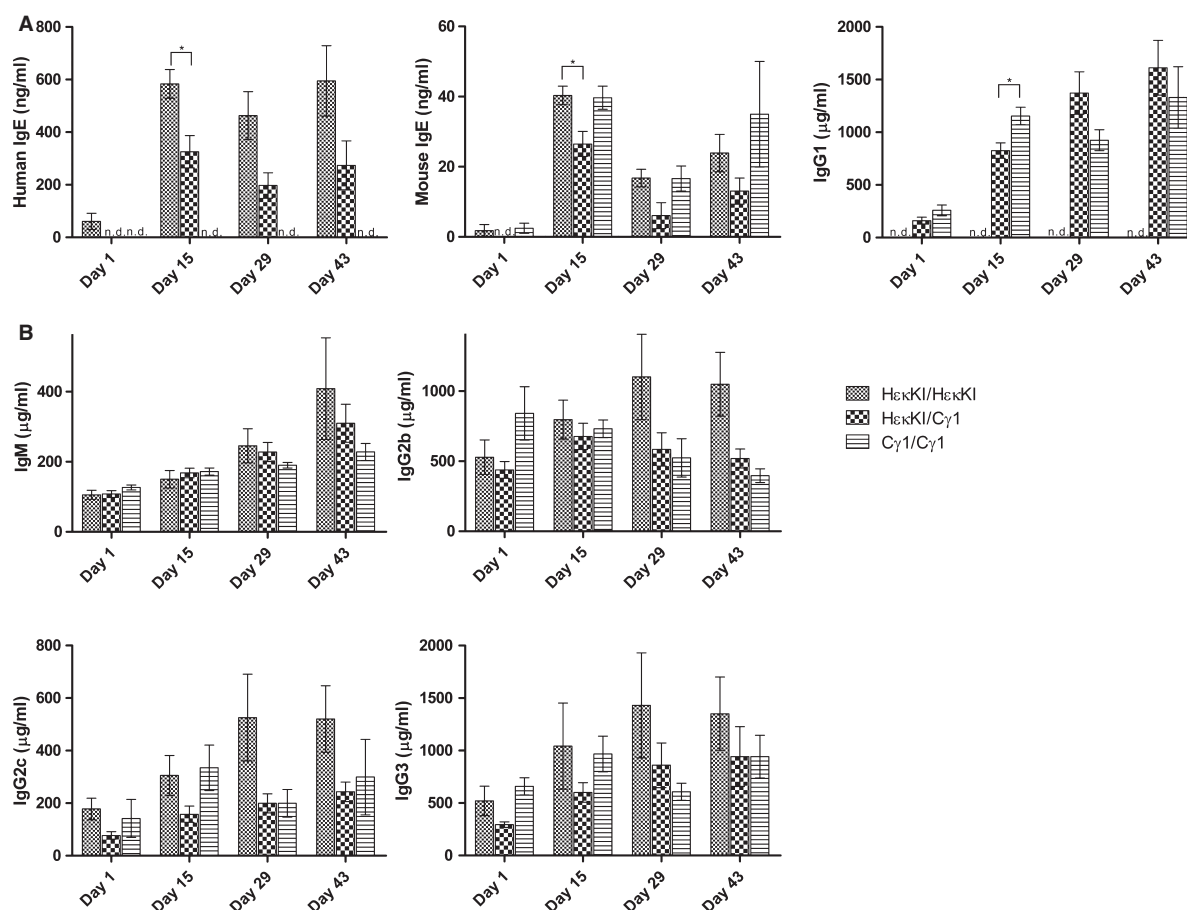


Figure 2 Serum levels of immunoglobulins of different isotypes in the three genotypes of mice. The mice were immunized with 50 μ g papain emulsified with TiterMax[®] Gold immunization at day 1 and day 29. A, serum levels of human IgE, mouse IgE and

mouse IgG1. B, serum levels of mouse IgM, IgG2b, IgG2c, and IgG3. Data are means $\pm$ SEM of duplicates from four mice. n.d. = not detectable, * P < 0.05, Student's t -test.

those of mouse IgE in the wild type. Serum levels of mouse IgG1 were undetectable in homozygous H ϵ κKI mice before and after papain immunization. The serum levels of mouse IgM, IgG2b, IgG2c, and IgG3 were similar among the three genotypes of mice (Fig. 2B). The quantitation of human IgE-secreting plasma cells in the splenocytes of papain-immunized H ϵ κKI mice is presented in Supporting Information.

Hybridomas secreting allergen-specific monoclonal chimeric IgEs were prepared

After the immunization with papain or ovalbumin, H ϵ κKI mice producing the highest titers of human IgE were killed and their spleens were taken for preparing hybridomas. Several papain-specific and ovalbumin-specific chimeric human IgE-secreting hybridoma clones were identified, and two clones, each of papain-specific (1C6, 15G10) and ovalbumin-specific ones (8G9, 18F11), were chosen for further characterization. Human IgE prepared from myeloma cell line U266 was used as a control or a standard in immunoassays. The culture media of the papain and ovalbumin-specific hybrid-

oma clones yielded 2–7 mg/l of chimeric human IgE. The molecular sizes of the chimeric sIgEs were similar to those of U266 IgE as revealed by reducing SDS-PAGE and Western blotting analyses (Fig. 3A,B). The purified chimeric IgEs exhibited high specificity in binding to their respective antigens (Fig. 3C).

Antigen-specific chimeric IgEs degranulated a basophilic cell line upon antigen stimulation

The chimeric sIgEs were subsequently examined for their ability to charge the high-affinity IgE-Fc receptors (Fc ϵ RI) on basophils and mast cells and to sensitize those cells as human IgE, by employing RBL SX-38 cell line, a rat basophilic leukemia cell line transfected with genes encoding the α , β , and γ chains of human Fc ϵ RI (6). Cells of RBL SX-38 cell line were incubated with the respective chimeric IgEs, and the antigen papain or ovalbumin was then added, and the release of an indicator enzyme β -hexosaminidase from the activated RBL SX-38 cells into the medium was measured, as described in the Material and methods. The results

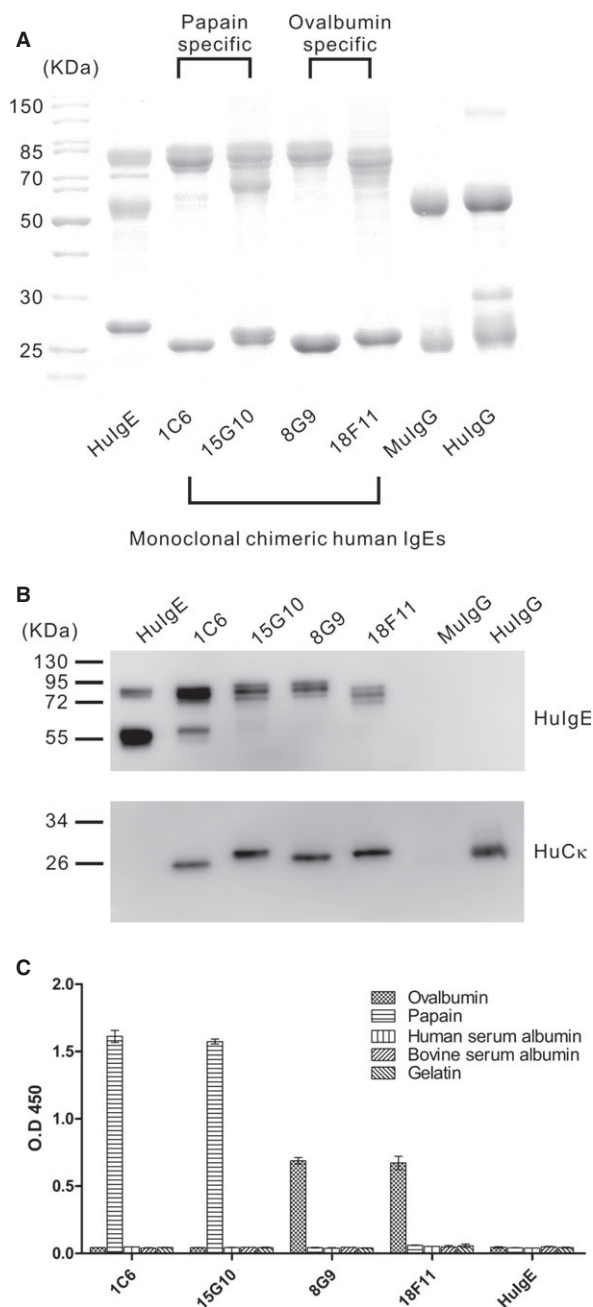


Figure 3 Characterization of the monoclonal antigen-specific chimeric human IgE antibodies generated with H ϵ KI mice. A, SDS-PAGE in reducing condition of purified IgEs. B, Western blotting analysis. C, binding specificities performed by ELISA, with data representing the means $\pm$ SD from duplicate measurements.

showed that chimeric IgEs, upon the interaction with their antigens, could induce the activation and degranulation of RBL SX-38 cells (Fig. 4). The variability of the degrees of degranulation among the four chimeric IgEs might be due to the affinity of the antibodies for their antigens or the locations of the binding epitopes of the antibodies on the antigens, which might affect the degrees of cross-linking of

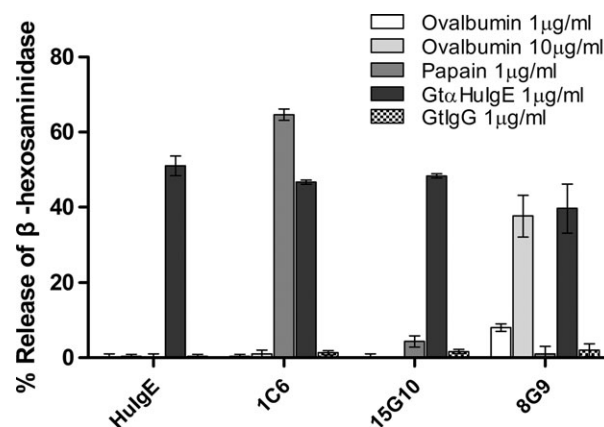


Figure 4 The induction of degranulation of basophilic cell line RBL SX-38 cells by monoclonal IgEs upon the incubation with their respective antigens. The positive control was U266 IgE, with polyclonal anti-human IgE antibodies as its inducer. Data are the means $\pm$ SD from triplicate measurements.

IgE-Fc ϵ RI on the RBL SX-38 cells. This explanation is supported by that polyclonal goat anti-human IgE antibodies could similarly induce degranulation of chimeric IgE or U266 IgE-charged RBL SX-38 cells.

Discussion

In a wild-type laboratory mouse, IgG1 is present in blood at about 100 μ g/ml and IgE at about 10 ng/ml, a difference of 10 000-fold. The H ϵ KI mice developed in this study express about 100 ng/ml of human IgE and about 10 ng/ml of mouse IgE in blood, a difference of about 10-fold. Serum levels of mouse IgM and other mouse IgG subclasses were similar in the wild-type, heterozygous, or homozygous H ϵ KI mice. As the H ϵ KI mouse strain has retained the mouse γ 1 switch region and the polyadenylation signal site, one would have expected H ϵ KI mice to produce higher human IgE than 100 ng/ml. One explanation for the low levels of human IgE in H ϵ KI mice is that IgE has much shorter half-life in the blood circulation than IgG1, and therefore, IgE cannot be accumulated to high levels like IgG1. In the (CBA $\times$ BALB/c) F1 mouse, mouse IgE has a half-life of about 12 h, while IgG1 has a half-life of 6–8 days (7). In humans, IgE has a half-life of 2.5 days and IgG about 21 days (8). We do not know the half-life of human IgE in H ϵ KI mice. If one assumes that the chimeric IgE in H ϵ KI mice also has a half-life of 12 h, an existing amount of it would have gone through about 14 half-lives (7 days $\div$ 12 h), when IgG1 has just gone through one half-life. In another word, when an amount of IgG1 becomes 1/2, the same amount of chimeric IgE would have only 1/16 384 left, a difference of about 8000-fold. According to this simple analysis, the short half-life of chimeric IgE would seem a major reason for its low levels in H ϵ KI mice.

There are probably other reasons for the low levels of human IgE in H ϵ KI mice. Most notably, the human IgE-secreting plasma cells in the H ϵ KI mice as identified by

ELISPOT assays were much fewer than mouse IgG1-secreting plasma cells in the wild-type mice. This difference was also reflected by the fact that fewer human IgE-secreting hybridomas were obtained in a fusion experiment than would be obtainable in a fusion procedure to prepare IgG-secreting hybridomas. It remains to be analyzed whether the small numbers of human IgE-expressing B lymphoblasts and plasma cells in the H ϵ KI mice are due to a low rate of class-switching into human ϵ gene, or poor proliferation, growth, or survival of the human IgE-expressing B cells, or other mechanisms.

A knockin mouse strain, in which the gene segment encoding the CH1–CH2–CH3 of mouse γ 1 was replaced by the gene segment encoding CH1–CH2–CH3–CH4 of mouse ϵ immunoglobulin, was constructed by a German team for studying the roles of different immunoglobulin isotypes in basophils-mediated active systematic anaphylaxis (9). The total mouse IgE levels in this IgE knockin mice were increased about 10-fold over those in wild-type mice. Such a finding concerning the low IgE levels is coherent with our findings in the H ϵ KI mice and with our proposed explanations.

Although the human IgE levels in H ϵ KI mice are much lower than those of IgG1 in wild-type mice, the H ϵ KI mice are suitable for our purpose of preparing hybridomas secreting antigen-specific chimeric IgEs (with human ϵ and κ constant regions and mouse V_H and V_L regions). Upon immunization with a protein antigen, the chimeric IgE levels could be increased to 30 times the levels of mouse own IgE. In the present studies, we have illustrated the feasibility of preparing antigen-specific chimeric IgEs with papain and ovalbumin. We have also obtained monoclonal chimeric human IgEs specific for house dust mite antigen Der p2. In our experience, the limitation for preparing allergen-specific monoclonal chimeric IgEs is not the suitability of H ϵ KI mice, but purified allergens for immunizing the mice and screening the hybridoma clones.

Three allergen-specific IgE assays, which have been approved by FDA (USA) (2), are broadly used worldwide. The determination of allergen-specific IgE/total IgE ratios in a patient has been used in some clinical settings for predicting the patient's clinical response to allergen-specific immunotherapy (10), the efficacy of anti-IgE treatment (11), and the outcome of oral food challenges (3). Those assays for allergen-specific IgE quantification have been proven valuable for the physicians in designing and evaluating therapies for allergic patients. However, interassay variations have been a major problem of allergen-specific IgE assays (12–14). While such variations may be due to a number of factors, such as the variability of allergen-specific IgE in individual patients, different preparations of allergen extracts used (15) and

others, the lack of allergen-specific human IgEs as standards or calibrators could be a major cause. If the calibration system can adopt allergen-specific human IgEs, the results from various allergen-specific IgE assays may become comparable. However, to our knowledge, only one allergen-specific human IgE (an anti-Der p 2 chimeric IgE 2B12 from Indoor Biotechnologies Inc., Charlottesville, VA, USA) is available. The methodology developed in this study may thus fulfill this need by providing a feasible approach to prepare chimeric IgEs for various allergens of clinical importance.

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Author contributions

CSL and AFH designed and performed experiments and drafted the manuscript. CJL and CYT performed human C κ ES cell screening and generated knockin mice. JBC provided suggestions and experimental designs for ELISPOT. CC performed the real-time RT-PCR. YYS guided and improved the degranulation assays. TWC designed the project and wrote the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Material and methods.

Figure S1. Molecular cloning procedure of increasing homologous arms of ϵ gene fragment.

Figure S2. ELISPOT results.

Table S1. Primer sequences for BAC construction.

Table S2. Primer sequences for genotyping, southern blotting analyses and real time RT-PCR.

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Online supplement

Generating allergen-specific human IgEs for immunoassays by employing human ϵ gene knock-in mice

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Material and Methods

The Construction of C ϵ and C κ gene-targeting constructs

The bacterial clones, carrying BAC RP23-59O5, which contains gene segment encoding mouse C κ light chain, and BAC RP24-258E20, which contains gene segment encoding mouse C γ 1 heavy chain genes, were purchased from BACPAC Resources Center (Oakland, CA). Mouse C κ light chain and mouse C γ 1 heavy chain genes were replaced by human C κ light chain and C ϵ heavy chains following the manual of the Counter Selection BAC Modification Kit (Gene Bridges, Dresden, Germany).

Human C κ and C ϵ targeting constructs were generated by insertion of a neomycin selection cassette flanked by loxP sites into 500 bp after C κ and C ϵ in the modified BAC clone described above using the Quick and Easy BAC Modification Kit (Gene Bridges). Primer sequences used in this experiment were listed in Table S1 and additional methods were shown in Figure S1.

Generation of chimeric C ϵ and C κ knock-in mice

For generation of C κ and C ϵ knock-in mice, the constructs were linearized from the BAC backbone by NruI and NotI digestion and were electroporated into mouse embryonic stem (ES) cells (B6/J strain). ES cell clones with C κ and C ϵ were injected into B6/J-Tyr^{c-2J} blastocysts. The resulting male KI chimeric mice were bred with B6/J-Tyr^{c-2J} females, and the progeny were analyzed for germline transmission of the knock-in allele by PCR. Mice carrying the homozygous knock-in allele were mated with EIIa-Cre Tg mice to remove the loxP-flanked neomycin cassette. C κ and C ϵ knock-in mice were allowed to mate with each other to generate the H ϵ κ KI strain mice. Genotyping were performed by genomic PCR with primers p1, p2 and p3 for C ϵ knock-in mice and p4, p5 and p6 (as depicted in Fig.1A and 1B) for C κ knock-in mice. Genomic DNA of 3 genotypes of mice was digested with Bam HI and analyzed by Southern blotting using a probe (as depicted in Fig.1A) yielding a 1.2-kb band for the WT allele versus a 3.7-kb band for the ϵ knock-in allele. Primer sequences in this sentence were shown in Table S2 All experiments involving mice were approved by the Institutional Animal Care and Use Committee in Academia Sinica and conformed to the relevant regulations.

Real time RT-PCR for detecting human ϵ and mouse κ 1 mRNA

Total RNA of spleen cells was prepared by using a PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA). cDNA was prepared using a Superscript III reverse transcriptase (Invitrogen) , and real-time PCR (Applied Biosystems, Foster City, CA) was done with pairs of primers for the constant regions of the murine IgG1 and human IgE as well as with primers for beta-actin. Primer sequences used in the procedures of this section were shown in Table S2.

Determination of isotype-specific immunoglobulin-secreting plasma cell numbers by ELISPOT

Three mice, 7–8 weeks old, in each group of the 3 genotypes (wild type, heterozygous, and homozygous H ϵ KI) were immunized subcutaneously three times with 50 mg of papain (Sigma-Aldrich, St Louis, MO) emulsified with TiterMax[®] Gold (Sigma-Aldrich) at day 1, day 22 and day 36. The mice were sacrificed at days 50, 52 and 54 and spleens were taken. Two mice, 12-13 weeks old, in each group of the 3 genotypes (wild type, heterozygous, and homozygous H ϵ KI) were immunized subcutaneously two times with 50 μ g of papain (Sigma-Aldrich, St Louis, MO) emulsified with TiterMax[®] Gold (Sigma-Aldrich) at day 1 and day 22. The mice were sacrificed at days 28 and 30 and bone marrow cells were isolated from femur and tibia. MultiScreenHTS plates (Millipore, Billerica, MA) were coated with 1 μ g per well of goat anti-mouse IgG1 (Southern Biotech), or goat anti-mouse IgG-Fc, goat anti-mouse IgE, goat anti-human IgE (Bethyl Laboratories) in 100 μ l phosphate buffered saline (PBS) at 4°C overnight. The procedures were followed by the technical note from Millipore (1). 100 μ l of cell suspension containing 5×10^6 splenocytes were dispensed into the individual wells. HRP-conjugated goat anti-mouse IgG1 (Southern Biotech), or goat anti-mouse IgG-Fc, goat anti-mouse IgE, goat anti-human IgE (Bethyl Laboratories) were diluted 10,000 folds in 1% BSA/ PBS and 100 μ l of the diluted antibodies were dispensed into each well. The wells each were added 100 μ l of AEC solution (Life technologies) and incubated in the dark at room temperature for 30 minutes. The wells were scanned, and spots were counted with an AID iSpot FluoroSpot Reader System (AID Diagnostika GmbH, Germany).

Generation of antigen-specific chimeric human IgE mAbs

H κ KI mice 7–8 weeks old were immunized with 50 μ g of papain or ovalbumin (Sigma-Aldrich) emulsified with TiterMax[®] Gold (Sigma-Aldrich, St Louis, MO) injected subcutaneously twice at day 1 and day 29. A final boost was injected intraperitoneally with 100 μ g antigen in PBS 3 days before mice were sacrificed (day 43). The spleen cells from one immunized mouse were fused with FO myeloma cells by polyethylene glycol and the treated cells were dispensed in the micro culture wells of 20 plates (Corning Incorporated, NY), each with 96 wells. Culture supernatants of growing hybrid clones were screened for antibodies specific for the immunizing antigen by ELISA. Briefly, the wells of 96-well microtiter plates (Greiner Bio-One GmbH, Germany) were coated with 50 μ l of 1 μ g/ml antigen in coating buffer (50 mM sodium carbonate, pH 9.6) at 4°C overnight, followed by blocking with 1% bovine serum albumin in PBS buffer at room temperature for 1 h. The wells were then washed with PBS-T (0.05% Tween-20 in PBS) 5 times. The culture supernatants of the hybrid clones were applied to the wells and the plates were placed at room temperature for 1 h. After five washes as above, horseradish peroxidase (HRP)-conjugated goat anti-human IgE (1:10000 dilution, Bethyl Laboratories) was added to the wells and the plates were placed at room temperature for 1 h. After five washes as above, and further incubation with peroxidase substrate NeA-Blue (Clinical Science Products, Mansfield, MA) for 10 min, the reaction was stopped by adding 1 N HCl and optical density (OD) was measured at 450 nm with a Model 680 microplate reader (BioRad Laboratories, Hercules, CA).

Purification of human IgE and papain-specific chimeric human IgE mAbs

The IgE was purified from U266 and chimeric human IgE hybridoma cell culture supernatants by an immunoaffinity method, employing CNBr-activated Sepharose 4 Fast Flow resin (GE Healthcare, Fairfield, CT), which was coupled with omalizumab (a gift from Novartis, Basel, Switzerland) according to the manufacturer's instruction.

Reference

1. Elispot MultiScreen® HTS Filter Plates and Antibody Pairs. Available from:
http://www.emdmillipore.com/US/en/product/Elispot-MultiScreen%C2%AEHTS-Filter-Plates-and-Antibody-Pairs,MM_NF-C9039#documentation

Figure S1. Construction of the DNA segment with human C ϵ -encoding gene for recombination procedures and the human C ϵ -encoding gene knock-in BAC. Since it was futile to try to replace the C γ 1 encoding gene with the 4,000 bp long C ϵ -encoding gene using 50 bp homologous arms, the homologous arms were lengthened to 2,000 bp at both ends. Primers used in the procedure are listed in table S1.

Figure S2. ELISPOT analyses of isotype-specific immunoglobulin-secreting plasma cell numbers in the spleens from 3 genotypes of mice. **A**, representative fields in ELISPOT analysis of spleen cells. **B & C**, numbers of IgG and IgE-secreting cell spots in spleen cells shown as means $\pm$ SEM of three independent experiments. **D & E**, numbers of IgG and IgE-secreting cell spots in bone marrow cells shown as means $\pm$ SEM of two independent experiments.

Table S1. Primer sequences for bacterial artificial chromosome construction

Primers for cloning	Sequences (5' to 3')	PCR product lengths (bp)
For mouse ¥1 gene		
G1_CH1-rpsL-neo+ G1_M2-rpsL-neo-	gtcctgtcctgtcctgtctgacagtgtaatcacatatacttttctttaggcctggatgatggcgggatcg tattggacagggctcctcggggcctgggaaatgccattacaggaggtggctcagaagaactcgtaagaaggcg	4084
EcoR-mIGHG1-2kInt+ Cla-mIGHG1-CH1Int-	gaattcagacatcatgcagggccagt atcgattacaagaaaaagtatatgtg	2012
Cla-hIGHE-CH1Ex+ hIGHE_me2Int-	atcgatgcctccacacagagcccatc aaagctgggcctggtgga	4048
mIgG1Int+hIGHM2-Cla-del+ mIgG1Int+hIGHM2-Cla-del-	gcctccacacagagcccatc tacaagaaaaagtatatgtg	8797
Sac_mIGHG1m2-Int+ Xho_mIGHG1polyA-	ccgcgggccacctcctgtaatggc ctcgagctcagcacctgctcagat	2254
G1_M2_5h-neo+ G1_M2_5h-neo-	tgggtccaactgaattattctagcgaccttggcattgtcatgacctgccaggcggaattgggccgataa aggcattttccatatcccagggtttatcgaagtgttaaggaggaaggtcataactcaagctatcgagataa	1716
For mouse © chain		
mIGKC-rpsL-neo+ mIGKC-rpsL-neo-	atggagcccttcctgttacttcataccatcctctgtgcttccttcctcaggcctggatgatggcgggatcg agataggatggagctggggagctgggtggcgtctcaggacctttgtcttcagaagaactcgtaagaaggcg	1419
mIGKChm-hIGKC+ mIGKChm-hIGKC-	atggagcccttcctgttacttcataccatcctctgtgcttccttcctcaggaactgtggctgcacat agataggatggagctggggagctgggtggcgtctcaggacctttgtcttaacactctcccctgttg	424
mIGKCInt5hT7loxP+ mIGKCInt5hSP6loxP-	ttctcccctaagtcgtcaaaagtctgaagggggacagtctttacaagcagggcgaattgggccgataa ctttgcttcgccaagtttactgggttaggtgaatcagattacagaacatgtactcaagctatcgagataa	1716

Table S2. Primer sequences for genotyping, southern blotting analyses and real time RT-PCR

Primers for screening	Sequences (5' to 3')	PCR product lengths (bp)
p1	gaaggtttggtcctgtcctg	
p2	gctgctcagagtgtagaggta	257
p3	ggttttgtgtcgacccagt	362
p4	ggcagtgaacgacaaaatgg	207
p5	cctggccccattgttccta	300
p6	gtttggagcaccgcaaca	
Primers for southern blotting analyses		
probe+	gggataatcctggggaggta	937
probe-	tgtcaggatcaccagaccag	
Primers for real time RT-PCR		
RQ-Cγ1+	atggcaaggagttcaaatgc	264
RQ-Cγ1-	tttatccttggccatctgct	
RQ-Cε+	tctaccacgcaggagggt	125
RQ-Cε-	ctgtcctcaaagggtgtga	
RQ-BA+	gactacctatgaagatcct	500
RQ-BA-	ccacatctgctggaaggagg	



1A source
 S2B 7 ohm lamp
 10 ohm
 S2C 1 ohm lamp

1. Connect S, C, S, S and S
 to 10V



Test B and C in parallel
 connection



Circuit in parallel



Test C and S in parallel
 and light



S, S and S in parallel



