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Expression of Humanized Fab Fragments That Recognize the IgE-Binding Domain of Human FcεRIα in COS and CHO Cells

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Interfering with the binding of IgE to high-affinity IgE receptor α chain (FcεRIα) is a straightforward strategy for the specific prevention of the IgE-mediated allergic reaction specifically. A Fab fragment (Fab) of a humanized antibody against the membrane proximal IgE-binding domain of human FcεRIα inhibits the release of histamine from human basophils. We established an efficient expression system in which to produce directly the humanized anti-human FcεRIα Fabs without papain-digestion of the whole antibody. Four Fabs with different C-termini of CH1 were expressed directly in COS-7 cells transfected with expression vectors with or without the Fc gene downstream of a stop codon inserted within the hinge gene. The secretion of Fabs when transfected without the Fc gene was remarkably enhanced compared to that when transfected with the Fc gene. The ability of Fabs to inhibit IgE-FcεRIα binding when transfected without the Fc gene was equivalent to that of purified Fab prepared by papain-digestion of the whole antibody. No significant differences among the four Fabs were observed in secretion or activity. Clones of CHO-transfectant cells that secreted the Fabs constitutively were acclimatized to a serum-free medium. Analysis of the binding interface between the Fab and human FcεRIα will provide useful information for the design of therapeutic reagents for allergy and asthma.

Key words: CHO cells, COS cells, Fab fragments, high-affinity IgE receptor, humanized antibody.

The high-affinity receptor for IgE (FcεRI) is the key molecule in the triggering of IgE-mediated allergic reactions. Multivalent allergens bridge the receptor-bound IgE and induce the aggregation of FcεRI on the surface of mast cells and basophils, and trigger the subsequent release of mediators such as histamine that are responsible for allergic symptoms (*1*). FcεRI consists of non-covalently associated subunits: one α chain (FcεRIα), one β chain, and two disulfide-linked γ chains (*2, 3*). The β and γ chains are required for signal transduction (*4–6*). The binding site for the Fcε portion of IgE is located in the extracellular portion of FcεRIα (*7, 8*). The extracellular portion of FcεRIα is highly glycosylated and composed of two immunoglobulin-like domains, D1 and D2. The IgE-FcεRIα interaction has been vigorously investigated (*9, 10*). Recently, Garman *et al.* determined the crystal structures of the human FcεRIα and the human IgE/FcεRIα complex (*11, 12*).

Interfering with the binding of IgE to FcεRIα is considered to be a straightforward strategy for the specific prevention of the IgE-mediated allergic reaction (*13*). FcεRI-deficient mice generated by targeted disruption of the FcεRIα gene are reported to be resistant to anaphylaxis (*14*). Recombinant and synthetic peptides comprising structural elements of human IgE or human FcεRIα have been investigated as competitive inhibitors for the IgE-FcεRI interaction (*9, 10, 15–21*). Searches have been also made for monoclonal antibodies (mAbs) against IgE or FcεRIα (*22–29*). Two humanized anti-human IgE antibodies (Abs) have been reported (*26–28*), one of which is in phase III clinical trials (*29*).

Fab fragments (Fabs) of anti-human FcεRIα mAbs that prevent human IgE-binding to human FcεRI are also of potential therapeutic value. We cloned cDNAs for the V regions of mouse anti-human FcεRIα mAbs and by CDR grafting successfully humanized one of the mAbs, CRA2, which bound to the second immunoglobulin-like domain, D2, of FcεRIα as well as IgE, and inhibited IgE-binding to FcεRI-expressing cells (*30, 31*). Fab prepared by papain-digestion of the humanized CRA2 inhibited the release of histamine from human basophils (*31*). However, the enzymatic digestion and subsequent purification procedure is complex and not suitable for large scale production.

In this study, we established an efficient expression system for the direct production of Fabs of humanized CRA2

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Abbreviations: FcεRI, high affinity IgE receptor; FcεRIα, the α subunit of FcεRI; D1, the first domain; D2, the second domain; Ab, antibody; mAb, monoclonal Ab; V, variable; C, constant; L, light; H, heavy; Fab, Fab fragment; FabH, VH-CH1 chain of Fab; CHO, Chinese hamster ovary; CHO/αβγ, CHO transfectant expressing human FcεRI; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; CDR, complementarity determining region

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without papain-digestion of the whole antibody. Analysis of the binding interface between FcεRIα and the anti-FcεRIα Fab inhibitory for IgE-binding, including crystallization of the Fab/FcεRIα complex, would provide useful information for the design of therapeutic reagents for allergy and asthma.

MATERIALS AND METHODS

Construction of Expression Vectors of Humanized Fabs—pSRH, pSRL, and pMHgpt vectors were kindly provided by Dr. Hidetoshi Kanda, Eiken Chemical Co. Ltd. And Dr. Takeshi Watanabe, Kyushu University (*32*, *33*). A humanized κ-light (L) chain expression vector, pSRL-huCRA2, was previously constructed (Fig. 1A) (*31*). Eight humanized VH-CH1 (FabH) chain expression vectors for transfection of COS-7 cells were constructed from a humanized IgG1 heavy (H) chain expression vector, pSRH-huCRA2 (*31*), as follows (Fig. 2). Four sets of double-stranded oligonucleotides with *Pst*I cohesive ends corresponding to a part of the hinge coding sequence downstream of the *Pst*I site, to which a stop codon and a *Bam*HI site were added, were synthesized. The double-stranded oligonucleotides were inserted at the *Pst*I site within the hinge coding sequence of the pSRH-huCRA2 vector. The insertion sequences were checked with a 373A DNA sequencer (Applied Biosystems, Foster City, CA). The locations of the stop codon differed among the four vectors. The resultant vectors were designated pSR-huCRA2FabH, pSR-huCRA2FabH-D, pSR-huCRA2FabH-DK, and pSR-huCRA2FabH-DKTHT (Fig. 1B and 2). The four 5.9 kbps vectors were digested with *Bam*HI. The 1.0 kbp-fragment of the Fc gene was removed and the 4.9 kbp-fragment of each vector was self-ligated. The resultant vectors without the Fc gene were designated pSR-huCRA2FabHΔFc, pSR-huCRA2FabH-DΔFc, pSR-huCRA2FabH-DKΔFc, and pSR-huCRA2FabH-DKTHTΔFc (Fig. 1B and 2).

Fab expression vectors for the establishment of stable CHO transfectant cells (Fig. 2) were constructed as follows. pSR-huCRA2-NeoF vector (Takai *et al.*, manuscript in preparation), which contains genes of the L and H chains of humanized CRA2 Ab and the G418 resistant gene, was digested with *Bam*HI and *Hin*dIII, and a 7.8 kbp-fragment including the L chain gene and G418 resistant gene was recovered. Each of the four 4.9 kbp FabH chain expression vectors without the Fc gene was digested with *Bam*HI and *Hin*dIII and a 2.4 kbp-fragment containing the FabH chain gene was recovered. The 7.8 kbp-fragment and the 2.4 kbp-fragment were ligated. The resultant vectors, each of which had L and FabH chain genes for each humanized Fab and G418 resistant gene, were designated pSR-huCRA2FabΔFcNeo, pSR-huCRA2Fab-DΔFcNeo, pSR-huCRA2Fab-DKΔFcNeo, and pSR-huCRA2Fab-DKTHTΔ-FcNeo (Fig. 1C and 2).

Expression of Humanized Fabs in COS-7 Cells—Appropriate L and FabH chain expression vectors were co-transfected into COS-7 cells using LipofectAMINE reagent (Life Technologies, Grand Island, NY) as described previously (*30*, *31*). α-MEM (BRL, Bethesda, MD) supplemented with 10% heat-inactivated fetal calf serum was used as the medium. After culture for 5 to 15 days, the culture supernatants were harvested. The concentrations of the humanized Fabs in the culture supernatants were determined by sandwich ELISA using goat anti-human $(Fab')_2$ Ab (ICN, Aurora, OH) and horseradish peroxidase (HRP)-conjugated goat anti-human κ chain Ab (Binding Site, Birmingham, U.K.). Purified Fab prepared by papain-digestion of whole humanized CRA2 Ab (*31*) was used as the standard protein for quantification.

Flow Cytometry—The abilities of the secreted-humanized Fabs in the culture supernatants to bind to the CHO transfectant cells expressing human FcεRI (CHO/αβγ) (*30*, *31*) were confirmed by flow cytometry. CHO/αβγ cells (2×10^5) were incubated with culture supernatants diluted with FACS buffer (PBS, 0.1% bovine serum albumin, and 10 mM sodium azide, pH7.4) (200 μl) at 4°C for 30 min. The amount of culture supernatant added was adjusted by volume (15 μl) not by concentration, *i.e.*, the cells were incu-

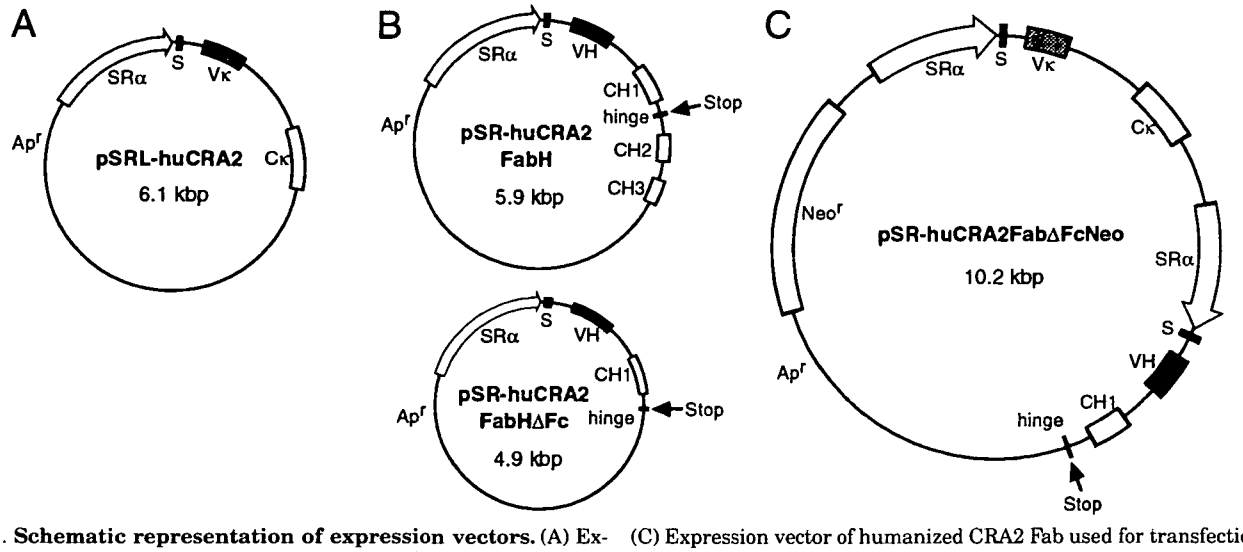


Fig. 1. **Schematic representation of expression vectors.** (A) Expression vector of the light chain of humanized CRA2 used for transfection of COS-7 cells. (B) Two types of expression vectors of the FabH chain of humanized CRA2 Fab used for transfection of COS-7 cells. (C) Expression vector of humanized CRA2 Fab used for transfection of CHO-K1 cells. SRα: SRα promoter. S: signal sequence. Ap^r: ampicillin resistant gene. Neo^r: G418 resistant gene. Stop: inserted stop codon.

bated in 200 μl that included 15 μl of culture supernatant. The cells were washed three times with FACS buffer and incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-human $(Fab')_2$ Ab (Cappel, Malvern, PA) at 4°C for 30 min. The cells were then washed and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

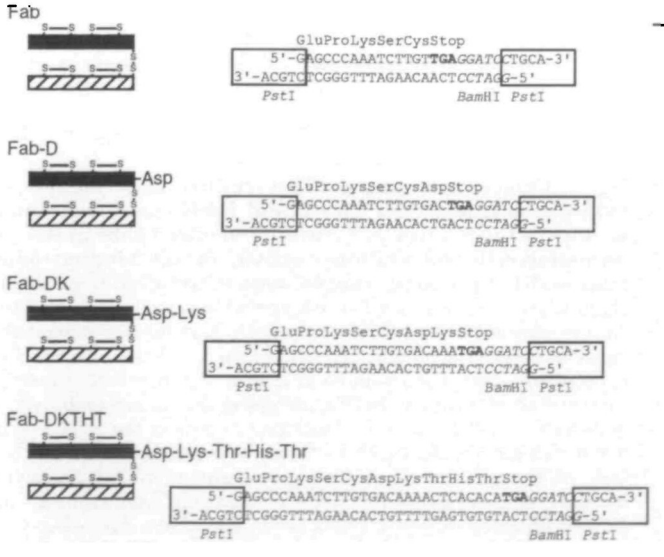
The abilities of humanized Fabs to inhibit the binding of FITC-conjugated human IgE to FcεRI were also confirmed. CHO/αβγ cells were incubated with diluted culture supernatants (200 μl) at 4°C for 30 min in the presence of FITC-conjugated IgE (1 μg/ml). As above, the culture supernatant was adjusted by volume (15 μl) not by concentration so that the cells were incubated in 200 μl including 15 μl of culture supernatant and 0.2 μg of FITC-conjugated IgE. The cells were washed and analyzed with a FACScan flow cytometer.

ELISA Using Immobilized Human FcεRIα—The inhibition of the binding of human IgE to immobilized human FcεRIα by the addition of culture supernatant including humanized Fabs was analyzed as described previously (*31*). Briefly, microtiter plates were coated with the recombinant extracellular portion of human FcεRIα (*16*) and blocked. Samples were tested in the presence of human myeloma IgE (10 ng/well). After incubation with a mixture of samples and IgE, the plates were washed and the plate-bound IgE was detected with an HRP-conjugated goat anti-human IgE Ab.

Immunoblotting—COS-7 cell supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4–20%, not boiled) under non-reducing or reducing conditions. After electrophoresis, the gels were used for immunoblotting as described previously with some modifications (*30*). Proteins transferred onto PVDF membranes (Immobilon; Millipore Co., Bedford, MA) were blocked with Block Ace (Snow Brand Products Co., Sapporo, Japan), incubated with a biotinylated anti-human IgG (H+L) Ab (Zymed Laboratories, San Francisco, CA), and detected by an avidin-conjugated peroxidase (Vector, Burlingame, CA) with diaminobentizine (Wako, Osaka, Japan) and H_2O_2. The medium, α-MEM (BRL) supplemented with 10% heat-inactivated fetal calf serum, was added to the buffer containing the biotinylated anti-human IgG (H+L) Ab to absorb fractions reactive to calf antibody (10% v/v, final concentration of the fetal calf serum: 1% v/v).

Establishment of Stable CHO Transfectant Cells Secreting Humanized Fabs into a Serum-Free Medium—The four vectors, each containing the gene for a humanized Fab and the G418 resistant gene, were transfected into CHO-K1 cells using LipofectAMINE reagent (Life Technologies). α-MEM (BRL) supplemented with 10% heat-inactivated fetal calf serum and 150–300 μg/ml of G418 (Geneticin Disulfate; Life Technologies) was used as the selection medium. Clones that stably secreted Fab at high concentration when cultured in the medium without G418 were acclimatized to serum-free medium without G418, CHO-S-SFMII (Life Technologies).

Purification of Humanized Fabs—The humanized Fabs secreted into the culture supernatant of CHO cells were purified using HiTrap Protein A (Pharmacia, Uppsala,



Vector	Fc gene	L gene	Neo^r	Transfectant cells
pSR-huCRA2FabH	+	−	−	COS-7
pSR-huCRA2FabHΔFc	−	−	−	COS-7
pSR-huCRA2FabHΔFcNeo	−	+	+	CHO-K1
pSR-huCRA2FabH-D	+	−	−	COS-7
pSR-huCRA2FabH-DΔFc	−	−	−	COS-7
pSR-huCRA2FabH-DΔFcNeo	−	+	+	CHO-K1
pSR-huCRA2FabH-DK	+	−	−	COS-7
pSR-huCRA2FabH-DKΔFc	−	−	−	COS-7
pSR-huCRA2FabH-DKΔFcNeo	−	+	+	CHO-K1
pSR-huCRA2FabH-DKTHT	+	−	−	COS-7
pSR-huCRA2FabH-DKTHTΔFc	−	−	−	COS-7
pSR-huCRA2FabH-DKTHTΔFcNeo	−	+	+	CHO-K1

Fig. 2. **Schematic representation of directly expressed humanized anti–human FcεRIα Fab fragments (Fabs).** Schematic view of four Fabs, four sets of oligonucleotides used for the construction of FabH expression vectors, and twelve vectors coding the FabH chain (eight for transfection of COS-7 cells and four for transfection of CHO cells) are shown. Stop codons are in bold, and the recognition sequences for *Pst*I and *Bam*HI are boxed and in italics, respectively. Amino acid residues are indicated above the corresponding codons in the sequence of the oligonucleotides.

Sweden) and eluted with 0.1 M glycine buffer (pH 2.7). The eluate was concentrated with Centricon 10 (Amicon, Beverly, MA) and fractionated by gel filtration (Sephadex75 HR10/30: Pharmacia).

RESULTS

FACS Analysis of Culture Supernatant of COS-7 Cells Transiently Expressing Humanized Anti-FcεRIα Fabs—A total of eight humanized FabH chain expression vectors were constructed for the transfection of COS-7 cells (Fig. 2). Four FabH chains with different C-terminal sequences are encoded by the eight vectors. Four vectors included the Fc gene downstream of a stop codon and the other four lacked the Fc gene. The L chain vector and FabH chain vector were co-transfected into COS-7 cells. The binding of the Fabs and the inhibition of FITC-conjugated human IgE-binding to CHO/αβγ cells expressing human FcεRI by the Fabs were confirmed by flow cytometry using equal volumes of supernatants (Fig. 3). All eight combinations exhibited obvious binding activity (Fig. 3A). Binding of the Fabs to the cells was enhanced when FabH chain vectors without the Fc gene were used (Fig. 3A, ΔFc). The four combinations without the Fc gene exhibited obvious inhibition (Fig. 3B, ΔFc), while the four with the gene exhibited only slight inhibition (Fig. 3B, +Fc).

Immunoblot Analysis of the Culture Supernatant of COS-7 Cells Transiently Expressing Humanized Anti-FcεRIα Fabs—The supernatants of COS-7 cells transfected with both the L chain expression vector and the FabH chain vector were analyzed by immunoblotting (Fig. 4A and B). A band of approximately 50 kDa for Fab under non-reducing conditions (Fig. 4A) and two bands of approximately 20–30 kDa for the L and FabH chains under reducing conditions (Fig. 4B) were detected in all eight combinations. When FabH chain vectors with the Fc gene were used, a few bands were detected that differed from those in the control lane of the medium under non-reducing conditions (Fig. 4A, +Fc gene, Medium).

Supernatants of COS-7 cells transfected with either FabH chain vector or L chain vector were also analyzed (Fig. 4C and D). The densities of bands detected as different from those in the control lane of the medium were remarkably lower than when both L and FabH chain vectors were transfected. When FabH chain vectors with the Fc gene were used, several bands were detected as different from those in the control lane under non-reducing conditions (Fig. 4C, +Fc gene, Medium). When FabH chain vectors without the Fc gene were used, a band of approximately 50 kDa under non-reducing conditions (Fig. 4C, ΔFc gene) and a band of approximately 20–30 kDa for the FabH chains under reducing conditions (Fig. 4D, ΔFc gene) were detected. When the L chain vector was used, no band was detected under non-reducing conditions (Fig. 4C, L) and two bands of approximately 20–30 kDa were detected under reducing conditions (Fig. 4D, L).

Quantification of Humanized Anti-FcεRIα Fabs Secreted into the Culture Supernatant of COS-7 Cells—The concentrations of humanized Fabs in the culture supernatants were determined by a sandwich ELISA using an anti-human $(Fab')_2$ Ab and an HRP-conjugated anti-human κ chain Ab. The concentrations of Fabs were remarkably higher when FabH chain vectors without the Fc gene were

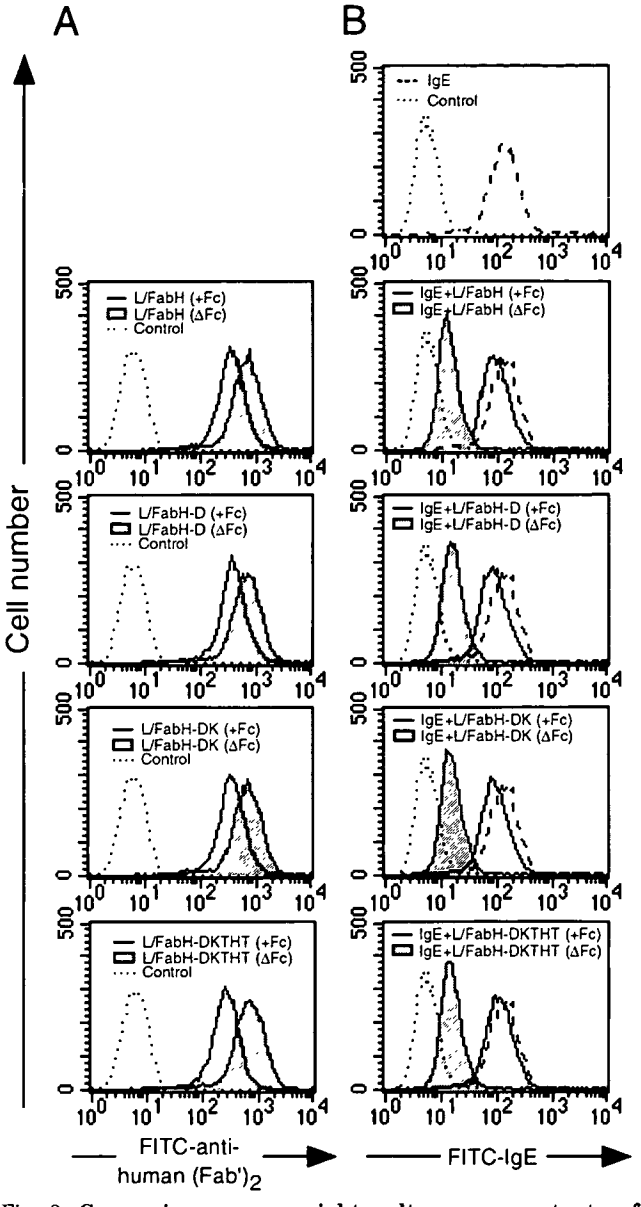


Fig. 3. **Comparison among eight culture supernatants of COS-7 cells transfected with L and FabH chain expression vectors in the binding activity of humanized Fabs in the supernatant cells and inhibitory activity for IgE-binding to human FcεRI expressing transfectants (CHO/αβγ).** L and FabH chain expression vectors were co-transfected into the cells and the culture supernatants were recovered. (A) Comparison among the supernatants in the binding activity of Fabs. CHO/αβγ cells were incubated with 200 μl of a dilution including 15 μl of culture supernatant, and stained with an FITC-conjugated anti-human $(Fab')_2$ Ab. A histogram for CHO/αβγ cells incubated only with the FITC-conjugated anti-human $(Fab')_2$ Ab is shown as a negative control (Control). (B) Comparison among the supernatants in inhibitory activity for IgE-binding to CHO/αβγ cells. CHO/αβγ cells were stained with FITC-conjugated human IgE (0.2 μg) in 200 μl of a dilution including 15 μl of culture supernatant. A histogram for CHO/αβγ cells incubated only with FITC-conjugated human IgE is shown as a positive control (IgE). A histogram for the nontreated CHO/αβγ cells is shown as a negative control (Control). L: Light chain. FabH, FabH-D, FabH-DK, and FabH-DKTH indicate four FabH chains whose C-terminal amino acid sequences were designed as Cys, Cys-Asp, Cys-Asp-Lys, and Cys-Asp-Lys-Thr-His-Thr, respectively (Fig. 2). (+Fc) and (ΔFc) indicate that FabH vector with the Fc gene and without the Fc gene, respectively, were used for transfection.

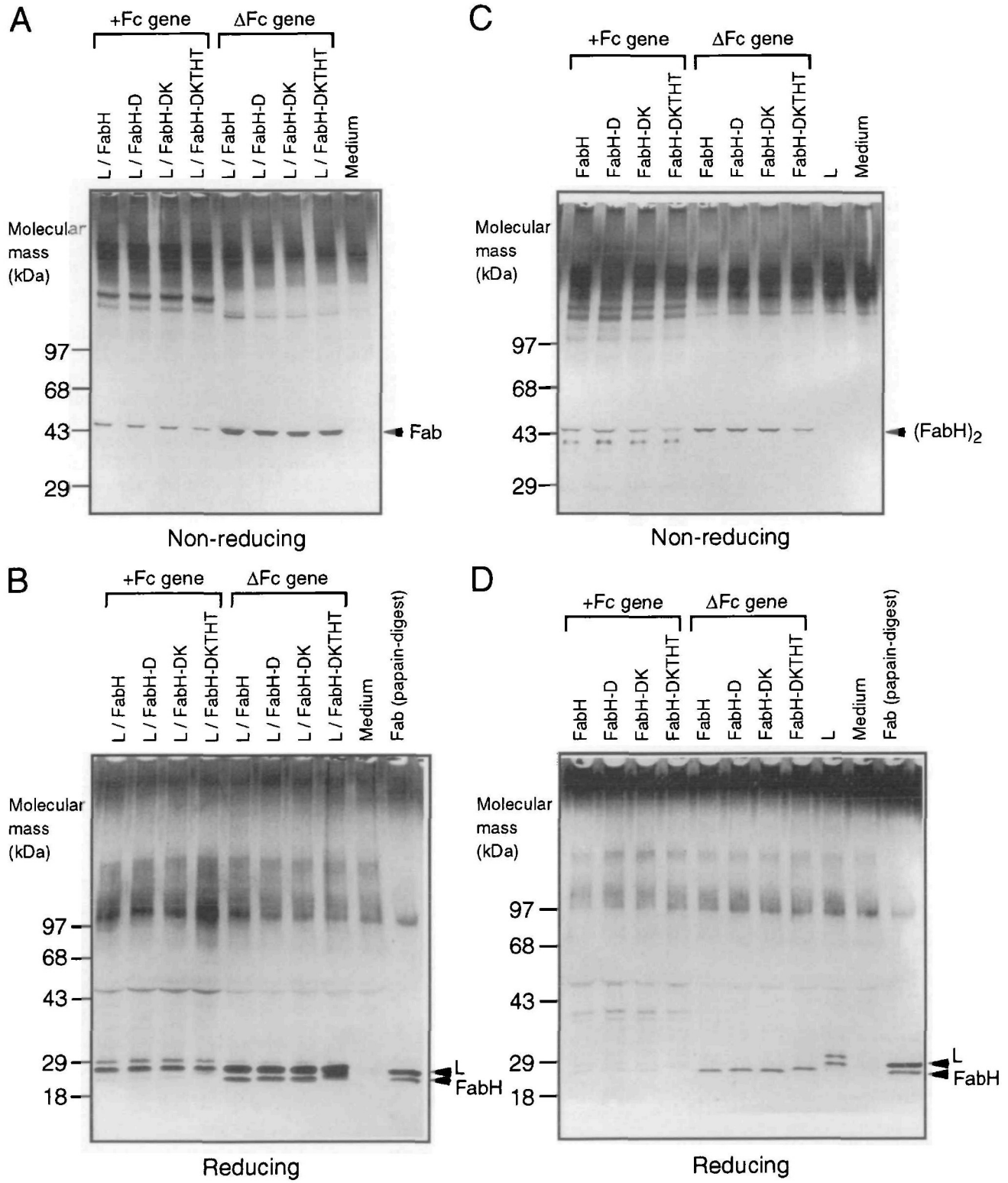


Fig. 4. **Immunoblot analysis of the culture supernatant of COS-7 cells transfected with L and/or FabH chain expression vectors.** L and FabH chain expression vectors were co-transfected into cells (A and B) or transfected alone (C and D). The culture supernatants were recovered, subjected to SDS-PAGE (4–20%) under non-reducing (A and C) or reducing conditions (B and D), and electroblotted onto PVDF membranes. The membranes were probed with combinations of a biotinylated anti-human IgG Ab and an avidin-conjugated peroxidase. L: Light chain. FabH, FabH-D, FabH-DK, and FabH-DKTH indicate four FabH chains whose C-terminal amino acid sequences were designed as Cys, Cys-Asp, Cys-Asp-Lys, and Cys-Asp-Lys-Thr-His-Thr, respectively (Fig. 2). "+Fc gene" and "ΔFc gene" indicate that the FabH vector with the Fc gene and without the Fc gene, respectively, were used for transfection. The bands for the humanized Fab, L chain, and FabH chain are indicated as Fab, L, and FabH, respectively (A, B, and D). $(FabH)_2$ is indicated as a possible homodimer of FabH (C). Lanes containing medium only are shown as negative controls (Medium). Lanes containing purified humanized Fab prepared by papain-digestion of whole humanized CRA2 (*31*) are shown as controls (B and D, Fab (papain-digest)).

used (Fig. 5).

Activity of Humanized Anti-FcεRIα Fabs to Inhibit IgE-FcεRIα Binding—The activities of Fabs, whose concentrations were determined by a sandwich ELISA, to inhibit IgE-binding to the plate-coated extracellular portion of human FcεRIα were analyzed (Fig. 6). Four Fabs in the supernatants of cells transfected with both L and FabH chain vectors without the Fc gene showed inhibition curves equivalent to purified Fab prepared by papain-digestion of whole humanized CRA2 Ab (Fig. 6, ΔFc gene). The other four Fabs in supernatants of cells transfected with vectors containing the Fc gene showed less inhibition (Fig. 6, +Fc gene).

Stable CHO-K1 Transfectant Cells Secreting Humanized Anti-FcεRIα Fabs into Serum-Free Medium—Highly productive stable clones of CHO-K1 transfectant cells were selected using the serum-containing medium and then acclimatized to serum-free medium (Fig. 7A). The cells were cultured as suspensions in serum-free medium. The concentrations of Fabs, the most productive clones secreted into the supernatant of confluent cultures in T75 flasks, were approximately 20 μg/ml. The Fabs were purified by Protein A affinity column chromatography and gel-filtration (Fig. 7B).

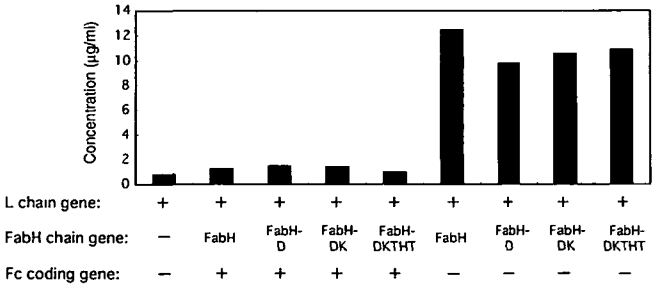


Fig. 5. **Quantification of humanized Fabs in the culture supernatant of COS-7 cells by sandwich ELISA using anti-human (Fab')$_2$ Ab and HRP-conjugated anti-human κ chain Ab.** FabH, FabH-D, FabH-DK, and FabH-DKTH indicate four FabH chains whose C-terminal amino acid sequences were designed as Cys, Cys-Asp, Cys-Asp-Lys, and Cys-Asp-Lys-Thr-His-Thr, respectively (Fig. 2). The value for the supernatant of cells transfected only with L chain expression vector is shown as a control because both the Abs used in the sandwich ELISA are reactive with the κ-light chain. Values are the averages of absorbances of duplicate wells.

DISCUSSION

Recently, we reported that Fab prepared by papain-digestion of humanized CRA2 against human FcεRIα inhibited the release of histamine from human basophils (*31*). However, the enzymatic digestion and subsequent purification procedure is complex and not suitable for large scale production. In this study, we established an expression system for the direct production of the humanized anti-FcεRIα Fab, which is potentially useful as a therapeutic ragent for allergy, in COS-7 and CHO-K1 cells. The humanized Fabs, which were transiently expressed in COS-7 cells and secreted into the supernatant, were able to inhibit the IgE-FcεRIα interaction to an extent equivalent to that of Fabs prepared by papain-digestion of whole humanized CRA2 (Fig.6, ΔFc gene). Clones of CHO transfectant cells, which stably produced the humanized Fabs in serum-free culture medium (approximately 20 μg/ml), were obtained (Fig. 7).

Fabs in the supernatants of COS-7 cells transfected with eight combinations of L and FabH chain expression vectors were able to bind FcεRIα and inhibit IgE binding to FcεRIα binding (Fig. 3 and Fig. 6). The concentration of the Fabs in the supernatants was remarkably higher when FabH vectors without the Fc gene were used for transfection (Fig. 3, Fig. 4AB and Fig. 5). Differences in the C-terminus of the FabH chain did not affect the secretion, assembly, or activity of Fab. The reason for the low level of Fab secretion when FabH vectors with the Fc gene were used for transfection is not clear. Interestingly, when FabH chain vectors with the Fc gene were used, a few or several bands reactive

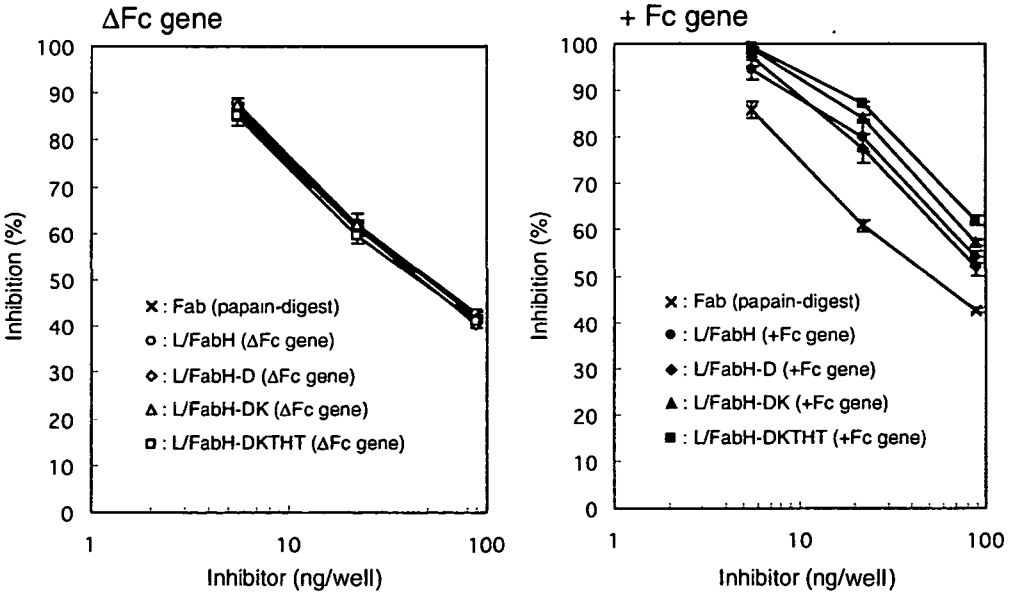


Fig. 6. **Competitive inhibition of human IgE-binding to the extracellular portion of human FcεRIα immobilized on plates by humanized Fabs.** Inhibition of human IgE-binding to the plates by Fabs in the culture supernatants of COS-7 cells was evaluated by ELISA. Fab concentrations were determined by a sandwich ELISA (Fig. 5). Fab (papain-digest): purified Fab prepared by papain digestion of whole humanized CRA2 Ab (*31*). L: L chain. FabH, FabH-D, FabH-DK, and FabH-DKTH indicate four FabH chains whose C-terminal amino acid sequences were designed as Cys, Cys-Asp, Cys-Asp-Lys, and Cys-Asp-Lys-Thr-His-Thr, respectively (Fig. 2). (ΔFc gene) and (+Fc gene) indicate that FabH vectors without and with the Fc gene, respectively, were used for transfection. Values are averages of duplicate experiments. The error bars show the range of the duplicate samples.

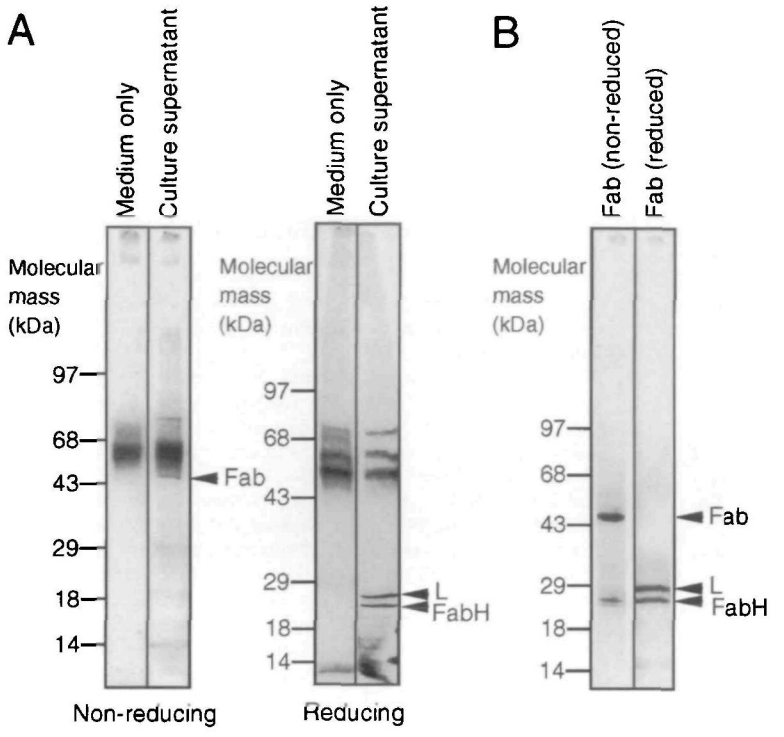


Fig. 7. **Stable expression of humanized Fabs in a serum-free culture of CHO transfectant cells.** SDS-PAGE (4–20%) was performed under non-reducing or reducing conditions, and the gels were stained with Coomassie Brilliant Blue. The results using one Fab whose C-terminal amino acid sequence is Cys (Fig. 2) are shown. The bands for the humanized Fab, Light chain, and FabH chain are indicated as Fab, L, and FabH, respectively. (A) Secretion of humanized Fab into the serum-free culture supernatant. A lane containing medium only is shown as a negative control (Medium only). (B) Purified humanized Fab. The Fab was purified by Protein A affinity column chromatography and gel-filtration.

with an anti-human IgG Ab were detected as different from those in the control lane of the medium under reducing conditions in immunoblot analysis (Fig. 4A, +Fc gene; and Fig. 4C, +Fc gene). Aggregation of the Fabs or FabH chains might have occurred. Northern blot analysis of the FabH chain mRNA and identification of the bands reactive with anti-human IgG Ab in the immunoblot analysis might provide information to explain the low level of secretion of the Fabs when FabH vectors with the Fc gene were used. The apparent lower inhibitory activities of IgE-FcεRIα binding when FabH chain vectors with the Fc gene were used (Fig. 6, +Fc gene) might be attributable to the ELISA for quantification of Fab in which L chain is detectable, because both antibodies used to capture and detect the Fabs are reactive to the κ-light chain (Fig. 5). The concentration of Fab could have been overestimated when determined by ELISA in the case where the concentration was low. The concentration of Fab in the supernatant when FabH chain vectors without the Fc gene were used was approximately 7–9 fold that when vectors with the Fc gene were used (Fig. 5), and the ability of Fab to inhibit IgE-FcεRIα binding was 2.5-4 times greater (Fig. 6). Therefore, the relative inhibitory activity of the supernatant is considered to be 18–36 times higher when vectors without the Fc gene are used for transfection than when vectors with the gene are used.

Immunoblot analysis of culture supernatants of COS-7 cells transfected with either FabH or L chain vectors (Fig. 4CD) revealed that the efficiency of secretion of the FabH chain and L chain when not assembled into Fab was remarkably low, that the FabH chain might form a homodimer or a complex with unknown component(s) detected as a band of approximately 50 kDa under non-reducing conditions, and that the L chain might aggregate or form complex(es) with other components because no band was detected under non-reducing conditions. Another band above the L chain band detected under reducing conditions might correspond to that of the precursor form of the L chain with the signal peptide (Fig. 4D, L). Assembly of the L and FabH chains facilitates the secretion into the supernatant of COS-7 cells (Fig. 4AB). Clones of stable CHO transfectant cells secreted assembled Fabs into serum-free medium as well as COS-7 cells (Fig. 7).

Recently, Garman *et al.* solved the crystal structure of the human IgE/FcεRIα complex (*12*). It was revealed that the binding of FcεRIα to the Fcε portion of IgE is asymmetrical and that FcεRIα interacts with Fcε at two distinct sites in the D2 domain, each of which involves one Cε3 domain of Fcε. In the literature, it is mentioned that inhibitors specific for either of the two binding sites might accelerate the dissociation of FcεRIα-bound IgE by capitalizing on the transient exposure of each site in the complex. The humanized CRA2 binds to D2 of human FcεRIα as well as IgE and effectively inhibits IgE-binding to FcεRIα (*30, 31*). We describe the direct expression of the Fab of the humanized anti-FcεRIα Ab in COS-7 and CHO-K1 cells in this study. Analysis of the binding interface between FcεRIα and the Fab, including crystallization of the Fab/FcεRIα complex, will provide useful information for the design of therapeutic reagents for IgE-mediated allergic diseases.

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