

Production of Humanized Antibody against Human High-affinity IgE Receptor in a Serum-free Culture of CHO Cells, and Purification of the Fab Fragments

Toshiro TAKAI,^{1,2,*} Kyoko TAKAHASHI,¹ Midori AKAGAWA-CHIHARA,¹ Minako FUKADA,¹ Toshihumi YUUKI,¹ Ichiro SHIBUYA,³ Ko OKUMURA,² Chisei RA,^{2,4} Toyokazu YOKOTA,¹ and Yasushi OKUMURA¹

¹Bioscience Research & Development Laboratory, Asahi Breweries, Ltd., 1-21 Midori 1-chome, Moriya-machi, Kitasoma-gun, Ibaraki 302-0106, Japan

²Department of Immunology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

³Institute for Production Research and Development, The Nikka Distilling Co., Ltd., 967, Matsuyama, Masuo, Kashiwa, Chiba 277-0033, Japan

⁴Division of Molecular Biology, Allergy Research Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

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We describe the preparation of Fab fragments of a humanized anti-human high-affinity IgE receptor (FcεRIα) antibody potentially useful for treatment of IgE-mediated allergic diseases. IgE-binding capacities of sixteen combinations of light and heavy chains of four recombinant anti-FcεRIα antibodies, chimeric CRA2, humanized CRA2, chimeric CRA4, and humanized CRA4, were compared. A combination in which both chains were of humanized CRA2 had the highest activity. Stable transfectant clones of four kinds of host cells expressing recombinant antibodies were established. CHO-K1 cells were the most productive. Serum-free media suitable for culture of the stable CHO-transfectant clones were screened. The concentration of the humanized CRA2, which the most productive clone secreted into the chosen serum-free medium, was approximately 100 μg/ml. A procedure for the purification of the antibody, papain-digestion, and purification of Fab fragments was established. The highly purified humanized Fab fragments are suitable for use to examine their *in vivo* activity and immunogenicity in primates.

Key words: high-affinity IgE receptor; humanized antibody; Fab fragments; serum-free culture; CHO cells

The high-affinity receptor for IgE (FcεRI) is the key molecule in the triggering of IgE-mediated aller-

gic reactions. Multivalent allergens bridge the receptor-bound IgE, induce the aggregation of FcεRI on the surface of mast cells and basophils, and trigger the subsequent release of mediators, such as histamine, responsible for allergic symptoms.¹⁾ The binding site for the Fcε portion of IgE is in the extracellular portion of the α chain of FcεRI.^{2,3)} FcεRI-deficient mice generated by targeted disruption of the FcεRIα gene were reported to be resistant to anaphylaxis.⁴⁾ Interfering with the binding of IgE to FcεRIα is considered a straightforward strategy for preventing the IgE-mediated allergic reaction specifically.⁵⁾

Recombinant and synthetic peptides comprising structural elements of human IgE or human FcεRIα have been investigated as competitive inhibitors for the IgE-FcεRI interaction.^{6–14)} Searches have also been done for monoclonal antibodies (mAbs) against IgE or FcεRIα.^{15–22)} The therapeutic potential of mAb has been limited by the immunogenicity of rodent mAbs in humans.^{23,24)} Winter and co-workers pioneered the humanization of antibody variable (V) regions by grafting the complementarity determining regions (CDRs), which are hypervariable loops involved in antigen binding, from rodent onto human V region frameworks.²⁵⁾ Two humanized anti-human IgE antibodies (Abs) have been reported^{19–21)} and one of them is in phase III clinical trials.²²⁾

Fab fragments of anti-human FcεRIα mAbs that prevent human IgE-binding to human FcεRI also

*¹ To whom correspondence should be addressed. Toshiro TAKAI, Present address: Allergy Research Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. Fax: +81-3-3813-5512

Abbreviations: FcεRI, high-affinity IgE receptor; FcεRIα the α subunit of FcεRI; Ab, antibody; mAb, monoclonal Ab; L, light; H, heavy; V, variable; CDR, complementarity determining region; CHO, Chinese hamster ovary; CHO/αβγ, CHO transfectant expressing human FcεRI; FCS, fetal calf serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunoassay; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase

would have potential therapeutic value. We cloned cDNA for V regions of mouse anti-human Fc ϵ RI α mAbs and successfully humanized one of the mAbs, CRA2, which bound to the second immunoglobulin-like domain of Fc ϵ RI α as well as did IgE and inhibited IgE-binding to Fc ϵ RI-expressing cells, by CDR-grafting.^{26,27} Fab fragments of the humanized CRA2 inhibited the release of histamine from human basophils.

The humanized CRA2 is specific to human Fc ϵ RI α and not reactive to rodent Fc ϵ RI α . Therefore, *in vivo* activity should be examined in primates or transgenic mice expressing human Fc ϵ RI α .^{28,29} Furthermore, reduction of the immunogenicity of the humanized CRA2 compared with that of the mouse CRA2 should be checked in primates.^{23,24} In this study, we established a procedure to prepare highly purified Fab fragments of the humanized CRA2. The procedure includes the use of a stable expression system in a serum-free culture of CHO-transfectant cells expressing the whole humanized Ab, purification of the Ab, papain digestion, and column chromatographies for purification of the Fab fragments.

Materials and Methods

Construction of expression vectors of recombinant anti-human high-affinity IgE receptor (Fc ϵ RI α) antibodies (Abs). Eight expression vectors of light (L) or heavy (H) chains of chimeric or humanized anti-human Fc ϵ RI α Abs were previously constructed.^{26,27} Three expression vectors of humanized CRA2, chimeric CRA2, and chimeric CRA4 for establishment of stable transfectant cells were constructed as follows. *Hin* d III-*Bam*H I fragments of three H chain expression vectors, pSRH-huCRA2, pSRH-chCRA2, and pSRH-chCRA4, containing an SR α promoter and the H chain gene were inserted into *Hin* d III/*Bam*H I sites downstream of an SR α promoter and the L chain gene of three L chain expression vectors, pSRL-huCRA2, pSRL-chCRA2, and pSRL-chCRA4, respectively. The resultant vectors, which had L and H chain genes of humanized CRA2, chimeric CRA2, or chimeric CRA4, were designated as pSR-huCRA2, pSR-chCRA2, and pSR-chCRA4. A *Hin* d III-*Xba* I fragment of the BCMGSneo vector³⁰ containing the G418 resistant gene was attached with *Cla* I linkers at both ends, digested with *Cla* I, and inserted to *Cla* I sites of pSR-huCRA2, pSR-chCRA2, and pSR-chCRA4. The resultant vectors with the G418 resistant gene inserted in the same direction as SR α promoters and Ab genes were designated as pSR-huCRA2neoF, pSR-chCRA2neoF, and pSR-chCRA4neoF. The resultant vectors with the G418 resistant gene inserted in reverse were designated as pSR-huCRA2neoR, pSR-chCRA2neoR, and pSR-chCRA4neoR.

Expression of recombinant anti-human Fc ϵ RI α Abs in COS7 cells. Appropriate L and H chain expression vectors were co-transfected into COS7 cells using LipofectAMINE reagent (Life Technologies, Grand Island, NY) as described previously.^{26,27} α -MEM (BRL, Bethesda, MD) with 10% heat-inactivated fetal calf serum (FCS) was used for the medium. After culture for 5 to 15 days, culture supernatants were harvested.

Measurement of recombinant anti-human Fc ϵ RI α Abs. The concentrations of fully assembled recombinant Abs in the culture supernatants were measured by a sandwich enzyme-linked immunoassay (ELISA) using a goat anti-human IgG Ab (Fc-specific) (Cappel, Malvern, PA) and a horse radish peroxidase (HRP)-conjugated goat anti-human κ chain Ab (Binding Site, Birmingham, U. K.).

Flow cytometry. The abilities of the secreted-recombinant Abs in the culture supernatants to bind to the CHO transfectant cells expressing human Fc ϵ RI (CHO/ $\alpha\beta\gamma$) were confirmed by flow cytometry as described previously.^{26,27} Briefly, CHO/ $\alpha\beta\gamma$ cells (2×10^5) were incubated with the culture supernatants diluted with buffer (200 μ l). Concentrations of the recombinant Abs were adjusted to 1 μ g/ml. The cells were washed and incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-human IgG Ab (Fc-specific). The cells were then washed and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

The abilities of recombinant Abs to inhibit the binding of FITC-conjugated human IgE to Fc ϵ RI were also confirmed as described previously.^{26,27} Briefly, CHO/ $\alpha\beta\gamma$ cells were incubated with diluted culture supernatants (200 μ l) in the presence of FITC-conjugated IgE. Concentrations of the recombinant Abs and FITC-conjugated human IgE were adjusted to 1 μ g/ml. The cells were washed and analyzed with a FACScan flow cytometer.

Establishment of stable transfectant cells secreting recombinant anti-human Fc ϵ RI α Abs. Four kinds of cells, CHO-K1 (Chinese hamster ovary), P3X63Ag8.563 (X653, mouse myeloma), SP2/0-Ag14 (SP2, mouse myeloma), and P815 (mouse mastocytoma), were used for establishment of stable transfectant cells secreting recombinant Abs. The vectors, which had the gene of a recombinant Ab and the G418 resistance gene, were transfected into CHO-K1 cells using LipofectAMINE reagent (Life Technologies) and into X653, SP2, and P815 cells using DOTAP reagent (Boehringer Mannheim Biochemica, Mannheim, Germany). α -MEM (BRL) with 10% heat-inactivated FCS and 2 mg/ml of G418 (Geneticin Disulfate; Life Technologies) was used as the selection medium for the CHO-K1 cells. RPMI-1640

(BRL) with 10% heat-inactivated FCS and 0.5 mg/ml of G418 was used for the selection medium for the X653, SP2, and P815 cells. Clones that stably secreted the recombinant Ab at a high concentration when cultured in the medium without G418 were selected from clones resistant to G418.

Serum-free culture of stable CHO-transfectant cells secreting humanized anti-human Fc ϵ RI α Ab. Clones which stably secreted humanized CRA2 Ab at high concentrations when cultured in the serum-containing medium without G418 were acclimatized to various serum-free media without G418. CHO-transfectant cells cultured in α -MEM (BRL) with 10% heat-inactivated FCS were stripped with trypsin-EDTA (Sigma, St. Louis, MO). The trypsin-digestion was stopped by addition of the α -MEM with 10% heat-inactivated FCS. The stripped cells were washed with PBS without calcium and magnesium ion. Serum-free media were prepared as described in the manufacturers' instructions. Cells suspended in PBS were allotted to the wells of flat-bottomed 24-well plates containing 500 μ l each of the serum-free media (100 μ l/well, approximately 5×10^4 cells/well) (Day 0). After three days, the culture supernatants were cultured with fresh media (1 ml) (Day 3). After a further four days, the culture supernatants were recovered (Day 7). The culture supernatants were put through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and ELISA. The serum-free media tested were SF-02 (Sanko Junyaku, Tokyo, Japan), MCD302 (Sigma), Nonserum P (International Reagents Co., Kobe, Japan), ASF104 (Ajinomoto, Tokyo, Japan), CHO-S-SFMII (Life Technologies), BIO-CHO1 (Biological Industry Co., Oshrat, Israel), KSF (Yagai Co., Yamagata, Japan), HYMEDIMUM 606 (Kohjin Bio, Saitama, Japan), E-RDF (Kyokuto Pharmaceutical Industrial Co. Ltd., Tokyo, Japan), SF-0 (Sanko Junyaku), and Hybridoma SFM (Life Technologies).

Serum-free production of humanized anti-human Fc ϵ RI α Ab. The MACCS-10 system (Shimazu, Kyoto, Japan) for perfusion culture was used for optimization of culture conditions. The culture volume was 900 ml. The total volume of the supernatant accumulated by the continuous culture was approximately 30 liters. The accumulated supernatant was concentrated by a CH₂ filtration system (Amicon, Beverly, MA). After the optimization of the culture conditions, large-scale production was committed to Toyobo (Osaka, Japan).

Purification of humanized anti-human Fc ϵ RI α Ab. The humanized CRA2 secreted into culture supernatant of CHO cells was purified using HiTrap Protein A (Pharmacia, Uppsala, Sweden) and eluted with 0.1 M glycine buffer (pH 2.7).

Papain-digestion of humanized anti-human Fc ϵ RI α Ab. Humanized CRA2 was digested with immobilized papain attached to beaded agarose (Sigma). The concentration of humanized CRA2 IgG was optimized to 2 mg/ml, the amount of immobilized papain to 0.05 U/mg-IgG (0.1 U/ml), the pH to 7.0, and the time for digestion to 15 h. During the digestion, the tubes containing the solution and the beaded agarose were agitated by rotation at 37°C in a Multishaker Oven HB (Titec, Saitama, Japan).

Purification of Fab fragments of humanized anti-human Fc ϵ RI α Ab. The solution of the papain digest was diluted and the pH was adjusted to pH 5.0. The solution was put onto HiTrap CM Sepharose (Pharmacia). The column was washed with 50 mM sodium acetate buffer (pH 5.0) and eluted with the same buffer containing 70 mM NaCl. The eluate was dialyzed with 20 mM Tris-HCl buffer (pH 8.0). The solution was put onto HiTrap Q Sepharose (Pharmacia). The column was washed with 20 mM Tris-HCl buffer (pH 8.0) and eluted with the same buffer containing 60 mM NaCl. The eluate was put through ultra filtration (100 kDa) with Centriplus 100 (Amicon). The fraction that passed through the membrane was concentrated by ultra filtration (10 kDa) with Centriplus 10 (Amicon). Protein concentration was measured by a BCA Protein Assay Kit (Pierce, Rockford, IL).

Measurement of endotoxin. Endotoxin was measured with Toxicolor (Seikagaku Corporation, Tokyo, Japan) or Endospecy (Seikagaku Corporation).

ELISA using immobilized human Fc ϵ RI α . Inhibition of human IgE from binding to immobilized human Fc ϵ RI α by addition of purified humanized Fab fragments was analyzed as described.²⁷⁾ Briefly, a microtiter plate was coated with the recombinant extracellular portion of human Fc ϵ RI α and blocked. Samples were tested in the presence of human myeloma IgE. After incubation with the mixture of samples and IgE, the plate was washed and the plate-bound IgE was detected with an HRP-conjugated goat anti-human IgE Ab.

Results and Discussion

FACS analysis of culture supernatant of COS7 cells transiently expressing recombinant anti-human Fc ϵ RI α Abs

Activities of sixteen combinations of the L and H chains of four recombinant anti-Fc ϵ RI α Abs, chimeric CRA2, humanized CRA2, chimeric CRA4, and humanized CRA4, to bind to CHO/ $\alpha\beta\gamma$ cells (Table 1) and to inhibit IgE-binding to the cells (Table 2) were compared. The results shown in Table 1 and Table 2 are summarized in Fig. 1.

Table 1. Binding of Sixteen Recombinant Anti-Human FcεRIα Abs to CHO/αβγ Cells^a

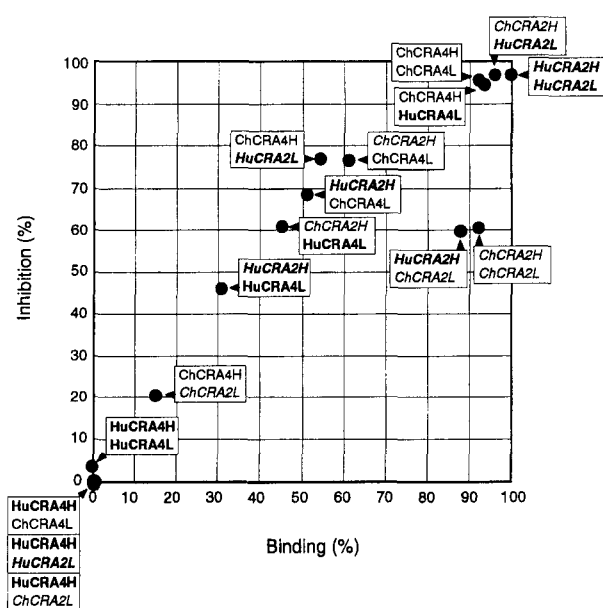
Light chain	Heavy chain			
	ChCRA2H	HuCRA2H	ChCRA4H	HuCRA4H
ChCRA2L	922	879	159	10.1
HuCRA2L	960	1000	534	17.1
ChCRA4L	617	516	923	11.8
HuCRA4L	458	314	936	12.1

^a Cell-bound Abs were detected with an FITC-conjugated anti-human IgG (Fc-specific) Ab. Mean fluorescences are shown. The value for cells incubated only with FITC-conjugated anti-human IgG Ab is 9.55 (background).

Table 2. Inhibition of Binding of FITC-Conjugated Human IgE to CHO/αβγ Cells by Sixteen Recombinant Anti-Human FcεRIα Abs^a

Light chain	Heavy chain			
	ChCRA2H	HuCRA2H	ChCRA4H	HuCRA4H
ChCRA2L	64.4	65.9	123	148
HuCRA2L	11.6	11.2	40.5	153
ChCRA4L	41.3	52.7	13.0	154
HuCRA4L	63.9	85.9	14.9	153

^a Mean fluorescences are shown. The value for cells incubated with FITC-conjugated human IgE without inhibitors is 153 (0% inhibition). The value for cells only is 6.49 (background).

**Fig. 1.** Activity of Sixteen Recombinant Anti-FcεRIα Abs to Bind to CHO/αβγ Cells and to Inhibit Human IgE-Binding to the Cells.

Results in Table 1 and Table 2 are summarized here. The fluorescences calculated by subtracting the background from the original mean fluorescences shown in Table 1 and Table 2 were used for calculation of the values for binding and inhibition. Binding of humanized CRA2 (HuCRA2H/HuCRA2L) is shown as 100% of binding. Combinations of heavy (H) and light (L) chains are shown in boxes. Ch: chimeric. Hu: humanized. H or L chains with CDRs of CRA2 are in italics. Humanized H or L chains are in bold.

Humanized CRA2 (HuCRA2H/HuCRA2L), a hemichimeric CRA2 the L chain of which was humanized (ChCRA2H/HuCRA2L), chimeric CRA4

(ChCRA4H/ChCRA4L), and a hemichimeric CRA4 the L chain of which was humanized (ChCRA4H/HuCRA4L) showed the highest activity. Substitution of ChCRA2L for HuCRA2L caused significant reduction of inhibitory activity in a hemichimeric CRA2 the H chain of which is humanized (HuCRA2H/ChCRA2L) and chimeric CRA2 (ChCRA2H/ChCRA2L). The reductions of the affinity are attributable to the chimeric VL sequence of the ChCRA2L, as we described recently.²⁷⁾

Four Abs which have the H chain of humanized CRA4 (HuCRA4H) had no binding capacity (HuCRA4H/HuCRA4L, HuCRA4H/ChCRA4L, HuCRA4H/HuCRA2L, and HuCRA4H/ChCRA2L). The loss of activity is attributable to the humanized VH sequence of the HuCRA4H chain, as we described recently.²⁷⁾

The other six Abs (ChCRA2H/ChCRA4L, ChCRA4H/HuCRA2L, HuCRA2H/ChCRA4L, ChCRA2H/HuCRA4L, HuCRA2H/HuCRA4L, and ChCRA4H/ChCRA2L), in which either one of the H or L chains had CDRs of CRA2 and the other CDRs of CRA4, showed significant activity. The epitopes for CRA2 and CRA4 are in the second immunoglobulin-like domain of FcεRIα but might not be completely uniform because they had independent VH and VL sequences.²⁶⁾ These six Abs were considered to recognize the epitope(s) on human FcεRIα for CRA2 or/and CRA4.

We considered that humanized CRA2 was suitable for further development as a candidate for a therapeutic inhibitor, because humanized CRA2 had the highest activity and was expected it to have low immunogenicity in humans.

Comparison of stable transfectant cells secreting recombinant anti-human Fc ϵ RI α Abs

The amount of recombinant Abs transiently expressed by COS7 is small and the procedure of transfection is complex. Therefore, a transient expression system is not suitable for large-scale production and a stable expression system is needed.

Four kinds of cells were transfected with expression vectors of chimeric CRA2 and/or chimeric CRA4. Secretion of recombinant Abs into serum-containing media without the drug for selection, G418, by the most productive stable transfectant clones in the confluent culture, is summarized in Table 3. The orientation of the G418 resistance gene in the expression vectors did not affect the productivity by the transfectant clones (data not shown). CHO-K1 was the most productive host cell. Transfectants of SP2, X653, and P815 could not survive in the medium containing G418 at 2 mg/ml and were selected in the medium containing G418 at lower concentration (0.5 mg/ml) than CHO-transfectants (2 mg/ml). The productivity might be dependent on the concentration of G418 used for the selection.

We chose CHO-K1 as the host for production of humanized CRA2. The stable CHO-transfectant clones of humanized CRA2 showed productivity as high as those of chimeric CRA2 and chimeric CRA4 (Table 3).

Screening of serum-free media suitable for culture of stable CHO-transfectant cells secreting humanized anti-human Fc ϵ RI α Abs

The procedure for purification of the recombinant Ab from serum-containing medium is considered complex. Therefore, a serum-containing medium is not suitable for large-scale production and a serum-free culture system is needed.

The CHO-transfectant clones secreting humanized CRA2 (Table 3) were acclimatized to various serum-free media. The concentrations of fully assembled Ab in the supernatants of eleven serum-free media were compared (Fig. 2A). Each of the concentration corresponds to total productivity of the batch and does not correspond to productivity of a single cell. Although serum-containing medium was changed to serum-free media in a single step, considerable proliferation of cells and production of Ab were observed in some serum-free media. In the two most

Table 3. Secretion (μ g/ml) of Recombinant Anti-Human Fc ϵ RI α Abs into Serum-Containing Media by Stable Transfectant Clones

Cells	Recombinant Anti-Human Fc ϵ RI α Ab		
	ChCRA2	ChCRA4	HuCRA2
CHO-K1	290	200	180
SP2	13	NT ^a	NT ^a
X653	5.3	3.2	NT ^a
P815	1.8	4.2	NT ^a

^a Not tested.

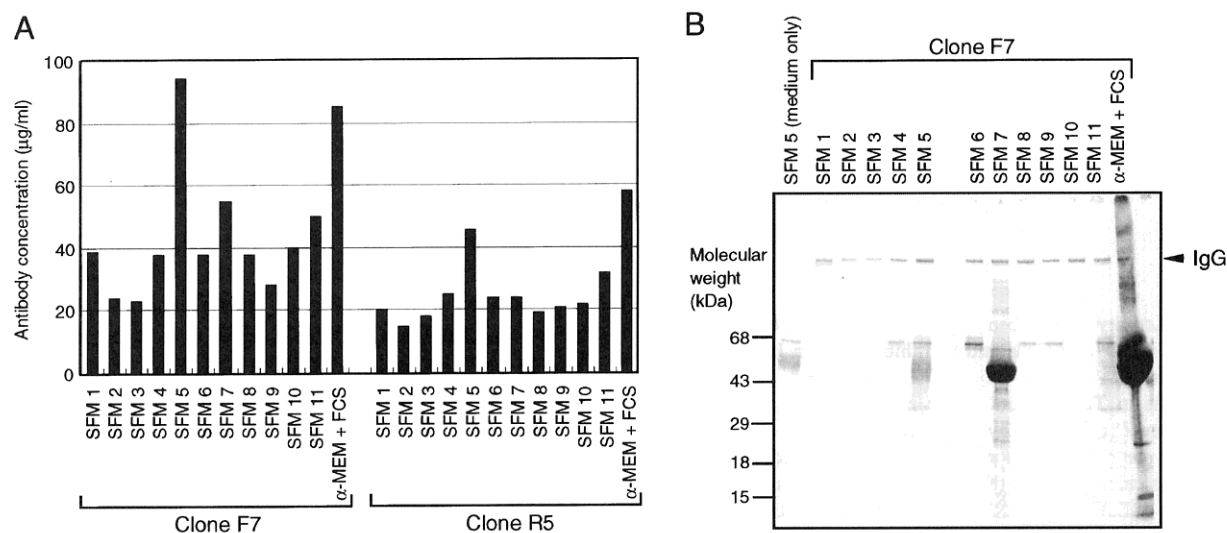


Fig. 2. Production of Humanized CRA2 by CHO-transfectant Cells in Eleven Serum-Free Media.

(A) Measurement of humanized CRA2 secreted to serum-free media. Concentrations of fully assembled humanized Ab secreted by two CHO-transfectant clones, Clone F7 and Clone R5, to eleven serum-free media (SFM1-11) and a serum-containing medium (α -MEM + FCS) were determined by sandwich ELISA. SFM1: SF-02 (Sanko Junyaku). SFM2: MCDB302 (Sigma). SFM3: Nonserum P (International Reagents Co.). SFM4: ASF104 (Ajinomoto). SFM5: CHO-S-SFMII (Life Technologies). SFM6: BIO-CHO1 (Biological Industry Co.). SFM7: KSF (Yagai Co.). SFM8: HYMEDIMUM 606 (Kohjin Bio). SFM9: E-RDF (Kyokuto Pharmaceutical Industrial Co. Ltd.). SFM10: SF-0 (Sanko Junyaku). SFM11: Hybridoma SFM (Life Technologies). α -MEM + FCS: α -MEM (BRL) supplemented with 10% heat-inactivated FCS. (B) SDS-PAGE (4–20%) of serum-free culture supernatants of a CHO-transfectant clone, Clone F7, under non-reducing conditions. The gel was stained with Coomassie Brilliant Blue. SFM5 (medium only): CHO-S-SFMII medium only.

productive clones secreting humanized CRA2, Clone F7 and Clone R5, a serum-free medium, CHO-S-SFMII, showed the highest Ab concentration (Fig. 2A, SFM5). On SDS-PAGE, a band for fully assembled humanized CRA2 Ab was observed in each lane for eleven serum-free culture supernatants (Fig. 2B, IgG).

We chose the serum-free medium, CHO-S-SFMII, for culture of Clone F7, the most productive CHO-transfectant clone secreting humanized CRA2. The cells were cultured as a suspension in the medium. The concentration of humanized CRA2 was approximately 100 μ g/ml.

Purification of papain-digested Fab fragments of humanized anti-human Fc ϵ RI α Ab

The procedure for the preparation of Fab fragments of humanized CRA2 is shown in Fig. 3A. Silver staining following SDS-PAGE of the samples after each step under reducing or non-reducing conditions is shown in Fig. 3B. Whole Ab was purified with Protein A affinity column chromatography (Fig. 3AB, IgG). The whole Ab was digested by immobilized papain attached to beaded agarose. The conditions for the digestion were optimized as described in Materials and Methods. The digest contained Fab, Fc, and various other peptide fragments from the humanized CRA2 IgG1 (Fig. 3B, Papain-digest). Various conditions for column chro-

matography were examined for removal of the Fc and other fragments from the Fab fragments. Optimized condition for purification of Fab fragments were as described in Materials and Methods. Purification of Fab fragments was accomplished by a combination of two ion-exchange column chromatographies (Fig. 3B, CM-70 mM NaCl, Q-60 mM NaCl). Purified Fab fragments of 50 kDa were passed through a membrane of 100 kDa for ultrafiltration and concentrated with a membrane of 10 kDa (Fig. 3B). Potential contamination of virus was prevented by ultrafiltration at 100 kDa.

Endotoxin was not detectable (lower than 0.125 EU/ml) in the concentrated sample (approximately 30 mg/ml). Recoveries of protein were 93% in the step including papain digestion and cation exchange column chromatography, 93% in the step of anion exchange column chromatography, and 78% in the step of two ultrafiltrations (100 kDa and 10 kDa). Therefore, total yield of the Fab fragments from whole antibody was approximately 67%. The apparent recoveries in the steps of papain digestion and cation exchange column chromatography are too high. The concentrations of Fab fragments were overestimated more than those of whole IgG when BCA Protein Assay Kit (Pierce) was used for measurement of the protein concentrations. Purity of the Fab fragments was estimated as more than 95% (Fig. 3B).

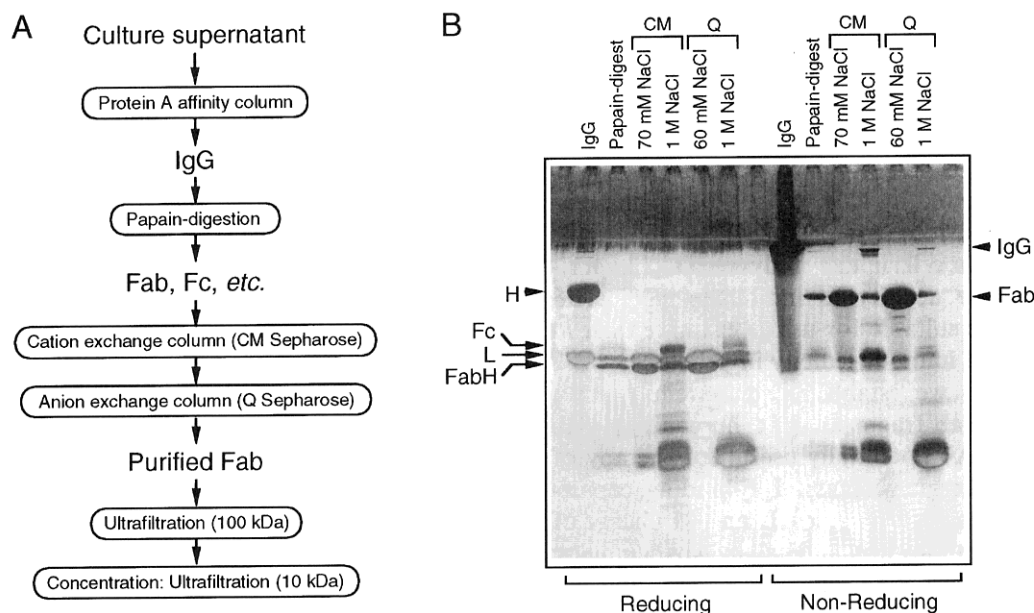


Fig. 3. Purification and Papain-Digestion of Humanized CRA2 and Purification of the Fab Fragments.

(A) Schematic of the procedure for preparation of Fab fragments of humanized CRA2. (B) Silver-staining after SDS-PAGE (20%) under reduced or non-reduced conditions. IgG: humanized CRA2 purified with Protein A affinity column chromatography. Papain-digest: papain-digest of purified humanized CRA2. CM-70 mM NaCl: eluate obtained with 50 mM sodium acetate buffer (pH 5.0) containing 70 mM NaCl from a CM Sepharose column (Pharmacia). CM-1 M NaCl: subsequent eluate obtained with the buffer containing 1 M NaCl. Q-60 mM: eluate obtained with 20 mM Tris-HCl buffer (pH 8.0) containing 60 mM NaCl from a Q Sepharose column (Pharmacia). Q-1 M NaCl: subsequent eluate obtained with the buffer containing 1 M NaCl. The bands for whole humanized Ab (IgG), Fab fragments (Fab), reduced CH2-CH3 chain (Fc), reduced heavy chain (H), reduced light chain (L), and reduced VH-CH1 chain (FabH) are indicated by arrows.

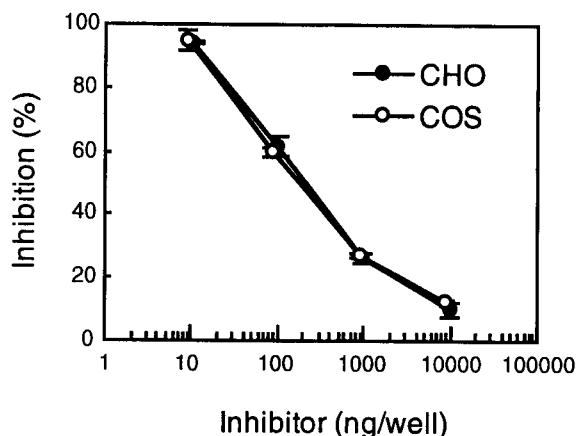


Fig. 4. Competitive Inhibition of Human IgE-binding to the Extracellular Portion of Human FcεRIα Immobilized on a Plate by Humanized Fab Fragments.

Inhibition of human IgE-binding to the plate by the Fab fragments prepared by papain-digestion of humanized CRA2 was evaluated by ELISA. Concentrations of the Fab fragments were measured by a BCA Protein Assay Kit (Pierce). CHO: purified Fab fragments prepared by digestion of humanized CRA2 produced by stable CHO-K1 transfectant cells (Clone F7). COS: purified Fab fragments prepared by digestion of humanized CRA2 transiently expressed by COS-7 cells.²⁷⁾ The values are averages of duplicated experiments. The error bars show the range for duplicate samples.

Activity of humanized anti-human FcεRIα Fab fragments to inhibit IgE-FcεRIα binding

Activity of Fab fragments to inhibit IgE-binding to plate-coated extracellular portion of human FcεRIα was analyzed (Fig. 4). Purified Fab fragments prepared by papain digestion of humanized CRA2 produced by stable CHO-transfectant cells showed an inhibition curve equivalent to that of Fab fragments prepared by digestion of humanized CRA2 transiently expressed by COS cells.²⁷⁾ The Fab fragments prepared from Ab produced by the stable CHO-transfectant cells in this study were highly purified, showed the activity to inhibit IgE-FcεRIα binding, and are suitable for use to examine their *in vivo* activity and immunogenicity in primates.

In this study, we established a system for the preparation of Fab fragments of a humanized anti-human FcεRIα Ab potentially useful for therapy of IgE-mediated allergic diseases. IgE-binding capacities of sixteen combinations of L and H chains of four recombinant anti-human FcεRIα Abs, chimeric CRA2, humanized CRA2, chimeric CRA4, and humanized CRA4, were compared. A combination in which both L and H chains were of humanized CRA2 showed the highest activity. CHO-K1 was the most productive host cell for stable secretion of the recombinant Abs. Serum-free media suitable for culture of the stable CHO-transfectant clones were screened and one was chosen for production of the humanized CRA2. Procedures for purification of the Ab, papain-digestion, and purification of the Fab frag-

ment were established.

The system has been scaled-up and the prepared Fab fragments have been used to examine their *in vivo* activity and immunogenicity in primates (Yasue *et al.*, manuscript in preparation). Furthermore, we recently reported other expression systems to produce the Fab fragments directly without papain digestion of whole antibody.^{31,32)}

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