

Inhibition of IgE-Dependent Histamine Release from Human Peripheral Blood Basophils by Humanized Fab Fragments That Recognize the Membrane Proximal Domain of the Human FcεRI α-Chain

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Key Words

Allergy · High-affinity IgE receptor · IgE · Degranulation · Humanized antibody · CDR grafting · Fab fragments · IgE network

Abstract

Background: Inhibition of the interaction between IgE and the α-chain of FcεRI (FcεRIα) is a straightforward strategy to develop therapeutic reagents for IgE-mediated allergic diseases. **Objective:** The purpose of this study is the humanization of CRA2 and/or CRA4, mouse anti-human FcεRIα monoclonal antibodies (mAbs) which recognize the IgE-binding membrane proximal immunoglobulin-like domain of FcεRIα. **Methods:** The two mAbs were humanized by CDR grafting onto human V region frameworks encoded by human germline V and J genes. The activities of the recombinant antibodies to bind FcεRIα and inhibit IgE binding to FcεRIα were analyzed by flow cytometry and ELISA. Human peripheral blood basophils were pretreated with the Fab fragments of the humanized CRA2 and stimulated with IgE and an anti-IgE

polyclonal antibody. The released histamine was measured. **Results:** The humanized CRA2 had almost the same activities of binding and inhibition of IgE binding to FcεRIα as the original mouse CRA2. Although the FcεRI-binding activity was maintained following humanization of the CRA4 light chain V region, it was lost by the humanization of the CRA4 heavy chain V region. Pretreatment of human peripheral blood basophils with the Fab fragments of the humanized CRA2 inhibited their subsequent degranulation activated by cross-linking of the FcεRI. **Conclusion:** In the humanized CRA2, all amino acid residues except CDR are replaced with the residues encoded by human germline genes. The humanization of CRA2 might be an important step in the development of immunotherapy to manipulate the IgE network in which mast cells, basophils, and various types of FcεRIα expressing cells are involved.

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Introduction

The high-affinity receptor for IgE (FcεRI), consisting of one α-chain (FcεRIα), one β-chain, and two disulfide-linked γ-chains [1, 2], is the key molecule in the triggering of the IgE-mediated allergic reaction. 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 responsible for allergic symptoms [3]. The binding site for IgE is located in the extracellular portion of FcεRIα [4, 5]. FcεRI-deficient mice generated by targeted disruption of the FcεRIα gene were reported to be resistant to anaphylaxis [6]. Interfering with the binding of IgE to FcεRIα is considered a straightforward strategy for preventing the IgE-mediated allergic reaction specifically [7].

Recombinant and synthetic peptides comprising structural elements of human IgE or human FcεRIα have been investigated as competitive inhibitors for the IgE-FcεRI interaction [8]. Searches have also been made for monoclonal antibodies (mAbs) against IgE or FcεRIα [9–12]. Two humanized anti-human IgE antibodies (Abs) have been reported [13, 14] and one of them is in phase III clinical trials [15]. In rat, the Fab fragments of an anti-rat FcεRIα mAb inhibited allergic reactions in vitro and in vivo [16]. Fab fragments of anti-human FcεRIα mAbs that prevent human IgE binding to human FcεRI also would have potential therapeutic value.

The therapeutic potential of mAbs has been limited by the immunogenicity of rodent mAbs in humans. A partial solution to this problem is to construct chimeric Abs by coupling the rodent antigen-binding variable (V) regions to human constant (C) regions [17, 18]. Winter et al. [19] pioneered the humanization of antibody V regions by grafting the complementarity-determining regions (CDRs), which are hypervariable loops involved in antigen binding, from rodent onto human V region frameworks (FRs).

Recently, we have reported cDNA cloning for V regions of mouse anti-human FcεRIα mAbs [20]. We generated two mouse anti-human FcεRIα mAbs, CRA2 and CRA4, which bind to the second immunoglobulin-like domain, D2, of FcεRIα as well as does IgE and inhibit IgE-binding to FcεRI expressing cells. In this paper, we document the humanization of the two anti-human FcεRIα mAbs, CRA2 and CRA4, by CDR-grafting onto human V region FRs.

Materials and Methods

Design of Humanized Abs

The amino acid sequences of V regions of CRA2 and CRA4 were deduced from their cDNA sequences [20]. The CDRs were identified by comparison of their Vκ and VH with the IgG compendium of Kabat et al. [21]. The corresponding sequences of FR1, FR2, and FR3 of CRA2 and CRA4 were then used to query a database of the amino acid sequences encoded by human germline V genes derived from the GenBank CDS database using the program GENETYX. The sequences with the highest degree of identity were then examined for conservation of buried residues [22] and canonical residues [23, 24] present within FR1, FR2, and FR3 of CRA2 and CRA4. Then FR4s of CRA2 and CRA4 were used to consult a database of the amino acid sequences encoded by human germline J genes. The sequences with the highest degree of identity were then examined for conservation of buried and canonical residues present within FR4s of CRA2 and CRA4.

Construction of Humanized Genes

Expression vectors for humanized Abs were constructed as described for mouse/human chimeric Abs with some modifications [20]. Briefly, the genes coding for humanized Vκ and VH of CRA2 and CRA4 were constructed by the CDR-grafting method utilizing long synthetic oligonucleotides and a PCR recombination method [25], and then connected to the PCR-amplified signal sequence encoding region of the heavy chain of the anti-cALLA mAb. The resulting DNA fragments were modified so as to contain *Xho*I sites at the 5' ends, and splice donor sequences and *Eco*RI sites at the 3' ends by a PCR method, treated with the restriction enzymes, and then inserted into pSRL and pSRH expression vectors. The resulting vectors were designated as pSRL-huCRA2 and pSRL-huCRA4 for the humanized κ light chain expression vectors and pSRH-huCRA2 and pSRH-huCRA4 for the humanized heavy chain expression vectors.

Expression and Purification of Recombinant Abs

Appropriate light (L) and heavy (H) chain expression vectors were co-transfected into COS7 cells using LipofectAMINE reagent (Life Technologies, Grand Island, N.Y., USA). α-MEM (BRL, Bethesda, Md., USA) supplemented with 10% heat-inactivated fetal calf serum was used for FACS analysis, and GIT medium (Nihon Pharmaceutical Co., Tokyo, Japan) without fractions of IgG and IgM was employed for purification of recombinant Abs. After culturing for 5–15 days, culture supernatants were harvested. The concentrations of fully assembled recombinant Abs in the culture supernatants were determined by a sandwich ELISA using a goat anti-human IgG Ab (Fc-specific) (Cappel, Malvern, Pa., USA) and a horseradish peroxidase (HRP)-conjugated goat anti-human κ-chain Ab (Binding Site, Birmingham, UK). Purification of recombinant Abs was performed as described [20].

Flow Cytometry

The abilities of the secreted-recombinant Abs in the culture supernatants to bind to the CHO transfectant cells expressing human FcεRI (CHO/αβγ) [20] were confirmed by flow cytometry. CHO/αβγ cells (2×10^5) were incubated with the culture supernatants diluted with FACS buffer (PBS, 0.1% bovine serum albumin, and 10 mM sodium azide, pH 7.4) (200 μl) at 4°C for 30 min. Concentrations of the recombinant Abs were adjusted to 1 μg/ml (determined by sandwich ELISA). The cells were washed three times with FACS buffer

and incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (Fc-specific) Ab (Caltag, San Francisco, Calif., USA) at 4°C for 30 min. The cells were then washed and analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif., USA).

The abilities of recombinant Abs 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. Concentrations of the recombinant Abs and FITC-conjugated human IgE were adjusted to 1 μg/ml. The cells were washed and analyzed with a FACScan flow cytometer.

ELISA

Microtiter plates (Immulon 2: Dynatech Laboratories, Inc., Chantilly, Va., USA) were coated with the recombinant extracellular portion of human FcεRIα (180 ng/well) [26] overnight at 4°C, washed three times with PBS containing 0.1% Tween 20, blocked with Block Ace (Snow Brand Product Co., Sapporo, Japan) for 2 h at 37°C and washed. Samples were serially diluted in PBS/10% Block Ace and added to the plates in duplicate. After a 3-hour incubation at 37°C, the plates were washed. Recombinant and mouse Abs bound to the plates were revealed with an HRP-conjugated goat anti-human IgG (Fc-specific) Ab (Cappel) and an HRP-conjugated goat anti-mouse immunoglobulin Ab (Amersham, Arlington Heights, Ill., USA), respectively. Inhibition of human IgE-binding was also analyzed. Samples were tested in the presence of human myeloma IgE (10 ng/well) (Yamasa Co., Choshi, Japan). After a 3-hour incubation at 37°C, plates were washed and the plate-bound IgE was revealed with an HRP-conjugated goat anti-human IgE Ab (ε-specific) (Cappel). After 1 h of incubation with the HRP-conjugated Abs at 37°C, the plates were washed and the substrate buffer (0.1 M citrate buffer pH 5.0, 0.5 mg/ml *o*-phenylenediamine dihydrochloride, 0.015% H₂O₂) was added. The reaction was stopped after 10–20 min by adding 0.3 M H₂SO₄, and the optical density of the samples was measured at a wavelength of 492 nm.

Preparation of Fab Fragments

The Fab fragments of mouse CRA2 were generated and purified with an ImmunoPure Fab Kit (Pierce, Rockford, Ill., USA). The Fab fragments of humanized CRA2 were generated with the same kit and purified using HiTrap Protein G (Pharmacia, Uppsala, Sweden) and eluted with 0.1 M citrate buffer (pH 3.5). The eluate was concentrated with Centricon 10 (Amicon, Beverly, Mass., USA) and fractionated by gel filtration (Sephadex75 HR10/30: Pharmacia). The purified Fab fragments were dialyzed against PBS. The concentrations of the purified Fab fragments were determined with a Protein Assay Kit (Bio-Rad).

Histamine Release Assay

The leukocytes used for histamine release assays were obtained from peripheral blood of volunteers who gave their informed consent. They were treated with acidic buffer to dissociate cell-bound immunoglobulins and then histamine release assays were performed as described by Pruzansky et al. [27]. The leukocytes were incubated with serially diluted humanized or mouse CRA2 Fab fragments at 37°C for 30 min, then human myeloma IgE (1 μg/ml) was added, and the cells were incubated for another 30 min at 37°C. After a wash, the cells were incubated with goat anti-human IgE Ab (3 μg/ml) (Cappel) for 30 min at 37°C. Cell-free supernatants and whole cells

solubilized with 0.1% Triton X-100 were collected and analyzed for histamine content using a Histamine ELISA (ICN, Costa Mesa, Calif., USA) or a Histamine Release Test Kit (Miles Inc., Spokane, Wash., USA) [28]. The percentage of released histamine was calculated as the amount of histamine in the supernatant relative to the total amount of supernatant and cellular histamine.

Results

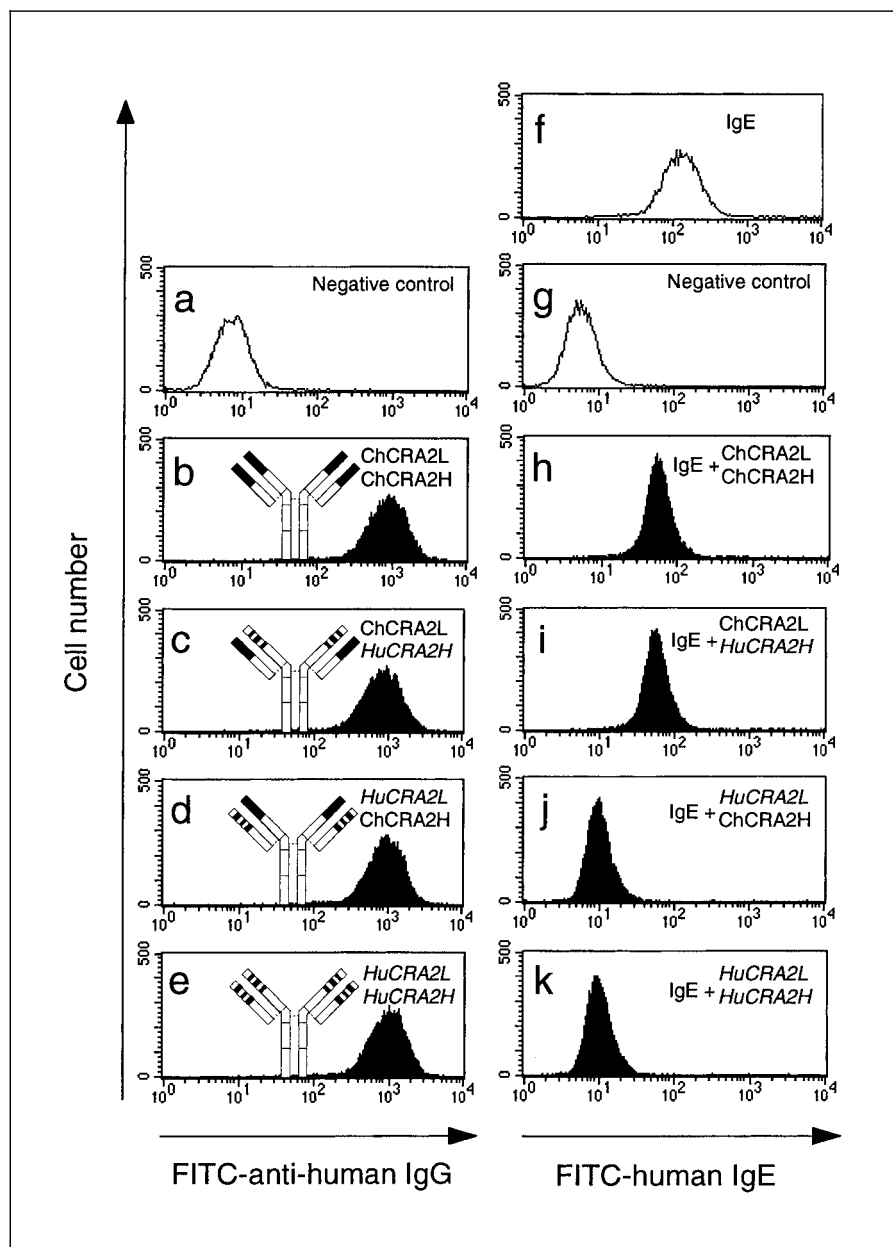
Design of Humanized Abs

For humanization we have chosen two mouse anti-human FcεRIα mAbs, CRA2 and CRA4. As described in Materials and Methods, the sequences of FRs of the V regions of the mAbs were used to consult a database of the amino acid sequences encoded by human germline V and J genes, respectively. The human germline V and J genes encoding the most highly homologous amino acid sequences to mouse FRs were selected. The amino acid sequences encoded by the human Vκ gene of subgroup IV [29, 30] and the human Jκ2 gene [21] were chosen for designing the humanized CRA2 VL region, and the human VH gene of DP54 [31] and the human JH6 gene [21] for the humanized CRA2 VH region. Those encoded by the human Vκ gene of L12a [32] and the human Jκ2 gene were selected for designing the humanized CRA4 VL region, and the human VH gene of DP14 [31] and the human JH6 gene for designing the humanized CRA4 VH region. The selected human germline genes were reported not to be pseudo-genes and to be expressed *in vivo*. Of the FRs of the human germline sequences present in the database GenBank CDS, those with the highest number of buried and canonical residues in common with the mouse FRs were chosen. The humanized V regions were designed for using the selected human germline FRs and the CDRs from the original mouse mAbs (fig. 1).

Expression of Chimeric and Humanized Abs against the Human FcεRI

The L and H chain expression vectors of chimeric or humanized Abs possessing human κ and IgG1 constant regions were cotransfected into COS7 cells. A total of eight recombinant Abs including chimeric, humanized, and 'hemichimeric' Abs, were transiently expressed and secreted into the supernatants of COS7 cells. The hemichimeric Ab is the Ab in which either the L or H chain is humanized and the other chain is chimeric [33]. The binding of the Abs and the inhibition of FITC-conjugated human IgE-binding to CHO/αβγ cells by the Abs were confirmed by flow cytometry using the culture supernatants (fig. 2). All four recombinant Abs that have CDRs of

Fig. 2. Comparison among recombinant anti-human FcεRIα Abs in binding activity and inhibitory activity for IgE-binding to human FcεRI expressing transfectants (CHO/αβγ). L and H chain expression vectors were cotransfected into COS7 cells and the culture supernatants were recovered. Ch = Chimeric; Hu = humanized. **A** Comparison among CRA2-related recombinant Abs.



Comparison of Mouse, Chimeric, and Humanized Abs against the Human FcεRI

Chimeric and humanized Abs that exhibited obvious activities were purified. The binding curves for the humanized CRA2 and the chimeric CRA4 assessed by ELISA using a recombinant extracellular portion of human FcεRIα immobilized on the plates were virtually identical to those of the original mouse CRA2 and CRA4, respectively. But the binding activity of the chimeric

CRA2 was reduced approximately to a tenth of the mouse CRA2 activity (fig. 3A). Similar results were obtained in competitive ELISA; the humanized CRA2 and the chimeric CRA4 were equal to the mouse CRA2 and the mouse CRA4, respectively, but the ability of chimeric CRA2 to inhibit IgE binding was approximately a tenth that of mouse CRA2 (fig. 3B). The humanized CRA4 did not exhibit any detectable activities of binding or inhibition (data not shown).

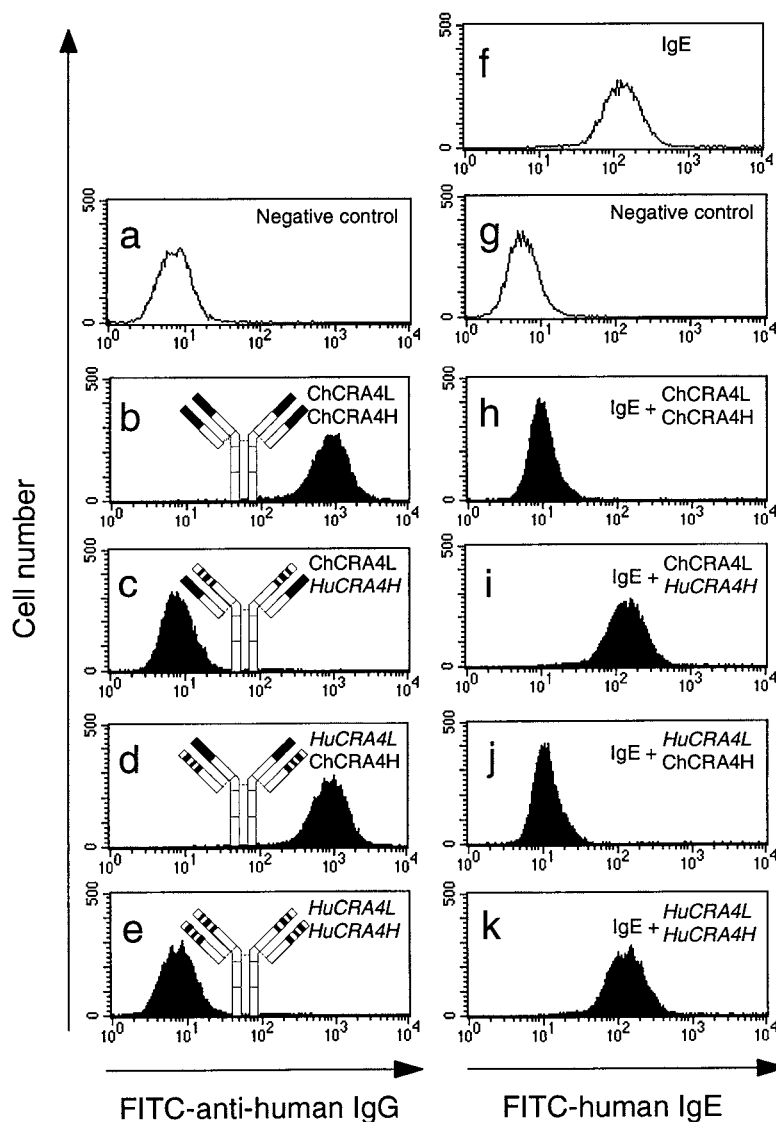


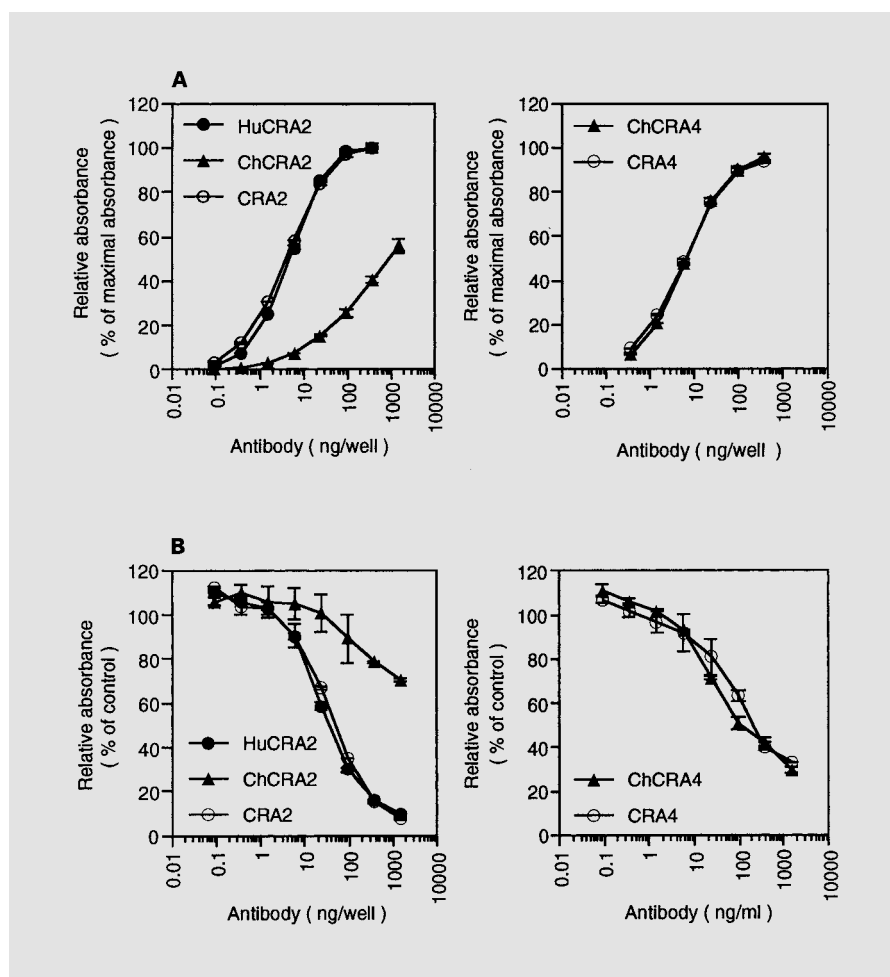
Fig. 2. B Comparison among CRA4-related recombinant Abs. The results of binding assay for the recombinant Abs are in the left column (**a–e**). CHO/ $\alpha\beta\gamma$ cells were incubated with the diluted culture supernatants including the recombinant Abs composed of the H and L chains shown in the histograms (**b–e**), followed by staining with an FITC-conjugated anti-human IgG (Fc-specific) Ab. Concentrations of the recombinant Abs were adjusted to 1 $\mu\text{g}/\text{ml}$ (measured by sandwich ELISA). A histogram for CHO/ $\alpha\beta\gamma$ cells incubated only with an FITC-conjugated anti-human IgG Ab is shown as a negative control (**a**). The results of competitive inhibition of IgE-binding to CHO/ $\alpha\beta\gamma$ cells with the recombinant Abs are displayed in the right column (**f–k**). CHO/ $\alpha\beta\gamma$ cells were stained with FITC-conjugated human IgE in the presence of the culture supernatants including the recombinant Abs composed of the H and L chains shown in the histograms (**h–k**). Concentrations of the recombinant Abs and FITC-conjugated human IgE were adjusted to 0.8 $\mu\text{g}/\text{ml}$. A histogram for CHO/ $\alpha\beta\gamma$ cells incubated only with FITC-conjugated human IgE is shown as a positive control (**f**). A histogram for the nontreated CHO/ $\alpha\beta\gamma$ cells is shown as a negative control (**g**).

Inhibition of Histamine Release by the Fab Fragments of the Humanized CRA2

The Fab fragments of the humanized and original mouse CRA2 were preincubated with human leukocytes, from which cell-bound immunoglobulins had been dissociated to assess their capacities to inhibit subsequent degranulation of human basophils activated by cross-linking of Fc ϵ RI with human myeloma IgE and a goat anti-human IgE Ab. The Fab fragments of the humanized

CRA2 were equal to those of the mouse CRA2 in their ability to inhibit histamine release from human peripheral blood basophils (fig. 4).

Fig. 3. Comparison of humanized (Hu), chimeric (Ch) and mouse anti-human FcεRIα Abs in binding activity and inhibitory activity for the IgE-binding to the extracellular portion of human FcεRIα immobilized on the plates. **A** Binding of humanized CRA2, chimeric CRA2, mouse CRA2, chimeric CRA4, and mouse CRA4 to the plates was measured by ELISA. **B** Competitive inhibition of IgE-binding with the Abs. Inhibition of human IgE-binding to the plates by humanized CRA2, chimeric CRA2, mouse CRA2, chimeric CRA4, and mouse CRA4 was evaluated by ELISA. Values are averages of duplicated experiments. The error bars show the range of duplicate samples.



Discussion

Reagents to inhibit the IgE binding to the FcεRI have been vigorously investigated in the last two decades. The inhibitors can be classified into two groups. Those of the first group, which bind to IgE, include the extracellular portion of human FcεRIα [26, 34, 35], the humanized anti-human IgE Ab [9, 13–15], and synthetic-oligonucleotide ligands [36]. Those of the second group, which bind to FcεRIα, include peptides derived from human IgE. A recombinant peptide fragment (76 amino acid residues) spanning the junction between Cε2 and Cε3 of human IgE was reported to inhibit the Prausnitz-Küstner reaction effectively [37]. Fab fragments of an anti-rat FcεRIα mAb were also reported to inhibit allergic reactions in vitro and in vivo in a rat system [10, 16]. Mouse mAbs against human FcεRIα were previously reported [11, 12]. But the

inhibitory effects of Fab fragments of anti-human FcεRI mAbs on allergic reactions have not been reported. Their therapeutic potential has been limited by the immunogenicity of rodent mAb in human. For the development of humanized Abs, we have chosen two mouse anti-human FcεRIα mAbs, CRA2 and CRA4, which bind the second immunoglobulin-like domain, D2, of FcεRIα and inhibit human IgE-binding to FcεRI [20].

A number of mouse mAbs have been humanized by CDR grafting to improve their therapeutic utility [19]. The transfer of mouse CDRs into human FRs is generally not sufficient to retain the binding affinity of the progenitor Ab. A transfer of several mouse FR residues, along with CDRs, has been necessary for retention of binding affinity in most cases. A variety of procedures, including an intricate computer-modeling technique, have been developed to select the most appropriate set of mouse FR

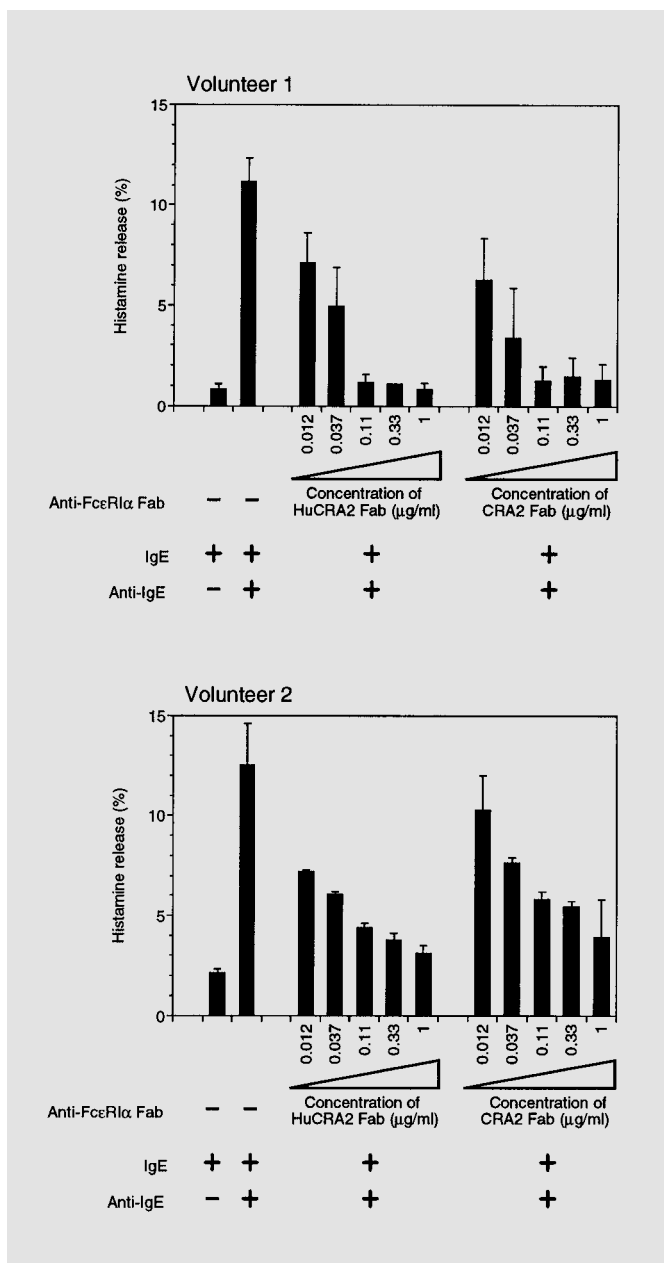


Fig. 4. Inhibition of histamine release from human peripheral blood basophils by the Fab fragment of the humanized (Hu) anti-human FcεRIα mAb. Leukocytes from human peripheral blood, from which cell-bound immunoglobulins had been dissociated, were preincubated with the Fab fragments of humanized CRA2 or mouse CRA2, then sensitized with human IgE. After washing, the cells were stimulated with an anti-human IgE Ab. Values are averages of duplicated experiments. The error bars show the range of duplicate samples. Results using leukocytes from two volunteers are shown.

residues required to maintain high binding affinity with a minimum mouse component in the final product. But these tend to be trial-and-error processes and the transferred mouse FR residues may have immunogenicity in humans. It is desirable that the first humanized version, whose FR residues were all human, retains the original binding affinity. Therefore, the use of the human FR sequences closest to the FRs of the progenitor mouse Ab to be humanized is a reasonable strategy [22, 33, 38, 39]. We adopted the sequences encoded by human germline V genes for FR1, FR2, and FR3, and those encoded by human germline J genes for FR4. Employing this strategy, the immunogenicity derived from FR residues into which somatic mutations were introduced in the affinity maturation process would be minimized [19] and a large repertoire of donors for CDR grafting would be made available by the combinations of FRs in a computer database. The humanized anti-human FcεRIα mAb, humanized CRA2, designed based on this strategy without the aid of computer-modeling techniques, retained the binding activity of the progenitor mouse CRA2 (fig. 2–4).

The reductions in the affinity of the chimeric CRA2 and the hemichimeric CRA2 whose L chain was chimeric (fig. 2A, h, i, 3) were attributable to the chimeric VL sequence. In the chimeric VL sequence, mutations were introduced into the amino acids in the N-terminal region of the mouse VL sequence (fig. 1B). This was the result of the use of genomic DNA encoding not only the H chain signal sequence but also the N-terminal region of the mouse anti-human cALLA mAb VH region. The loss of the activities of the humanized CRA4 and the hemichimeric CRA4 whose H chain was humanized (fig. 2B, c, e, i, k) is attributable to the humanized VH sequence. The degree of identity of FR residues of humanized V regions with the corresponding mouse FR residues was compared. The scores of the humanized CRA4 VH in the total identity of FR residues and more conspicuously in the identity of buried residues (71%) were lower than those of the successfully humanized ones, humanized CRA2 VH (95%), humanized CRA2 VL (91%), and humanized CRA4 VL (85%) (table 1). While the amino acids in the immediate neighborhood of the CDRs were all conserved in the successfully humanized V regions, two residues downstream of CDR2 (numbered 66) and upstream of CDR3 (numbered 94) were not conserved in the humanized CRA4 VH. The residue numbered 94 is an even more important residue because it not only neighbors CDR3, but is a buried and at the same time a canonical residue that affects the H1 loop [23, 24] (fig. 1C). The change in the charge of this residue that results when leu-

Table 1. Comparison of humanized FRs with their progenitor murine FRs

Humanized FRs	Identity with their progenitor mAbs, %		Number of different residues from their progenitor mAbs	
	all FR residues	buried residues in FRs	contacting residues in FRs	canonical residues in FRs
HuCRA2VH	85	95	1	0
HuCRA2Vk	78	91	1	1
HuCRA4VH	75	71	1	1
HuCRA4Vk	77	85	1	2

FR = Framework.

cine is replaced by arginine may cause some change in the conformation of the antigen binding loops and/or the VH domain. Therefore we consider that the failure of the humanization of CRA4 VH probably resulted from alterations in the buried residues or/and the residues in the immediate neighborhood of the CDRs.

The pretreatment of human peripheral blood basophils with the monovalent Fab fragments of the humanized CRA2 inhibited the subsequent degranulation of basophils activated by cross-linking of FcεRI with IgE and an anti-IgE polyclonal Ab as well as the pretreatment with the mouse CRA2 Fab (fig. 4). The monovalent Fab fragments of the humanized CRA2 themselves did not induce histamine release at high concentration (10 µg/ml) (data not shown). All amino acid residues except CDR in the humanized CRA2 are human residues encoded by human germline genes. For further development of the humanized CRA2 Fab as a therapeutic reagent, the activities and the immunogenic properties in vivo should be estimated in primate or in human FcεRIα gene transgenic mice [40, 41].

Critical roles for mast cells in promoting the allergic reaction through the IgE-FcεRI-mast cells axis have been suggested [42]. Inhibitors of IgE-FcεRI interaction on mast cells and basophils, such as the Fab fragments of the humanized CRA2, have therapeutic potential [7]. Furthermore, the expression of FcεRI in various other types of cells in humans has been reported [43]. Langerhans cells from patients with atopic dermatitis also express FcεRI and can be activated by its cross-linking [44]. Monocytes also express FcεRI, and monocyte-enriched PBMCs effectively present allergens to T cells via FcεRI and allergen-specific IgE [45, 46]. Eosinophils [47] and platelets [48] were also documented to express FcεRI, although there are three recent reports that have demonstrated a lack of functional FcεRI on eosinophils from

atopic donors [49–51]. Since these cells may be clinically involved in the development of allergic diseases, the anti-FcεRI Fab or Fv fragments should be able to target various cells expressing FcεRI, and to deliver drugs to these cells. As compared to IgE-derived peptides [37], the fragments of the humanized FcεRI-specific mAb have the possible benefits of binding specifically to FcεRI-expressing cells but not to low-affinity IgE receptor (FcεRII)-expressing cells, modifications such as bispecific antibodies for drug delivery to FcεRI-expressing cells, and a potential for further maturation of the affinity [52]. The successful humanization of CRA2 is thus an important step in the development of immunotherapy to manipulate the IgE network in which mast cells and various other types of cells are involved [53].

The recombinant Abs constructed in this study are also applicable to the analysis of the interaction between IgE and FcεRI because their epitopes on D2 of FcεRIα may overlap with the IgE-binding site on FcεRI [54, 55]. The structure of the antigen-binding sites of these Abs would provide useful information with which to rationally design inhibitors with low-molecular weight for IgE binding [56].

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