J. Biochem. **133**, 43–49 (2003)
DOI: 10.1093/jb/mvg001

Human FcεRIα–Specific Human Single-Chain Fv (scFv) Antibody with Antagonistic Activity toward IgE/FcεRIα-Binding

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Received August 8, 2002; accepted September 19, 2002

The α-chain of FcεRI (FcεRIα) plays a critical role in the binding of IgE to FcεRI. A fully human antibody interfering with this interaction may be useful for the prevention of IgE-mediated allergic diseases. Here, we describe the successful isolation of a human single-chain Fv antibody specific to human FcεRIα using human antibody phage display libraries. Using the non-immune phage antibody libraries constructed from peripheral blood lymphocyte cDNA from 20 healthy subjects, we isolated three phage clones (designated as FcRε27, FcRε51, and FcRε70) through two rounds of biopanning selection. The purified soluble scFv, FcRε51, inhibited the binding of IgE to recombinant FcεRIα, although both FcRε27 and FcRε70 showed fine binding specificity to FcεRIα. Since FcRε51 was determined to be a monomer by HPLC, BIAcore analysis was performed. The dissociation constant of FcRε51 to FcεRIα was estimated to be 20 nM, *i.e.*, fortyfold lower than that of IgE binding to FcεRIα (K_d = 0.5 nM). With these characteristics, FcRε51 exhibited inhibitory activity on the release of histamine from passively sensitized human peripheral blood mononuclear cells.

Key words: allergy, Fc epsilon RI, human, phage antibody library, single-chain variable fragment, scFv.

Abbreviations: AP, alkaline-phosphatase; BSA, bovine serum albumin; HAS, human serum albumin; HRP, horseradish peroxidase; IgE, immunoglobulin E; IL-6, interleukin-6; IPTG, isopropyl-thio-β-D-galactopyranoside; MCP-1, monocyte chemoattractant protein-1; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PEG, poly(ethylene glycol); ScFv, single chain variable fragment; TNF-R1, tumor necrosis factor receptor 1; TU, transforming unit.

Higher animals are endowed with antibodies as the sole professional defense molecules that, like bullets, target an unlimited number of molecules. Since J. Koller and C. Milstein reported the production of a murine monoclonal antibody by means of cell fusion technology (*1*), a tremendous number of studies has been carried out in a search for therapeutic antibodies. However, few attempts have been successful regarding human therapy. The reason is the immunogenicity of murine or rat monoclonal antibodies in humans (*2–4*). Nevertheless, no technology has been available for generating human antibodies directed to human self molecules which is principally forbidden in the human immune system. There has been, however, a recent breakthrough as a result of two cutting-edge technologies involving a human-immunoglobulin-transgenic mouse and a human antibody display phage library. In this study, we attempted to create a therapeutic human antibody for immunoglobulin E (IgE)-mediated allergic diseases. The interaction between IgE and its high affinity receptor, FcεRI (*5*, *6*) on mast cells and basophiles, is central to allergic diseases (*7*, *8*). When receptor-bound IgE is cross-linked by a multivalent allergen, an activation signal causes the release of inflammatory mediators, resulting in a type I immediate hypersensitivity reaction. Interference with the binding of IgE to FcεRIα is considered to be a strategy for specific prevention of an IgE-mediated allergic reaction (*9*). In the case of IgE as a target molecule, two murine mAbs have been reported to have been humanized to avoid the problems of antigenicity (*2*, *10*). One of them, omalizumab, is under phase III clinical trials (*11–13*). Its counterpart, FcεRI, consists of one α-chain, one β-chain, and two disulfide-linked γ-chains. The binding site for IgE is located in the extracellular portion of FcεRIα, indicating that FcεRIα is an attractive target for the inhibition of IgE-mediated allergic diseases. Targeting this molecule, the murine anti-human FcεRIα mAb was humanized and characterized by Takai *et al*. (*14*, *15*).

In this study, we attempted to produce a human single-chain antibody fragment (scFv) specific to human FcεRIα using large-scale phage libraries displaying scFv derived from non-immune human peripheral blood mononuclear cells (PBMCs). As human FcεRIα-specific human scFvs were successfully isolated, we report here their characterization. One of the clones, FcRε51, may be a leading scFv antibody for the therapeutic treatment of IgE-mediated allergic diseases.

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MATERIALS AND METHODS

Recombinant FcεRIα (rFcεRIα)—First-strand cDNA was prepared from human peripheral blood mononuclear cells (PBMCs) (*16*). A DNA fragment coding the extracytoplasmic portion of rFcεRIα flanked by initiation and alanine codons at the 5′end was generated by polymerase chain reaction (PCR) with synthetic sense primer KEB-59 (5′-TATACATATGGCAGTCCCTCAGAAACCTAAGG; incorporating a unique *Nde*I site) and anti-sense primer KEB-60 (TGGTCCCATGGGCCTCGAAAATAATGTCATTACAACTCC; incorporating a unique *Acc65*I site). The resulting PCR product was cleaved with *Nde*I and *Acc65*I, and then ligated with the pET-29b plasmid (pFcεRIα), in which six successive histidine residues were inserted immediately upstream of the stop codon. The whole sequence was confirmed by sequencing analysis. pFcεRIα was transfected into *E. coli* strain BL21-Star. Gene expression was induced with 1 mM isopropyl-thio-β-D-galactopyranoside (IPTG). rFcεRIα was purified from a cell lysate by affinity chromatography on nickel-nitrilotriacetic acid resin (Amersham Pharmacia Biotech) following the supplier's instructions. The function of rFcεRIα was determined on the binding activity to anti-human FcεRIα mAb (CRA1, CosmoBio) and human IgE.

Construction of a scFv Library—Fifty milliliters of heparinized venous blood was collected from each of 20 healthy volunteers, and PBMCs were isolated by density gradient centrifugation using Ficoll. Total RNA from the purified PBMCs was extracted with ISOGEN (Nippon Gene, Toyama). The first-strand cDNA was synthesized with a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Backinghamshire) using the DNA primer for the Ig constant region for the γ, μ, κ, or λ chain. The V genes for the γ, μ, κ, or λ chain was separately amplified by PCR using appropriate V gene-family-based back and forward primers (*17–19*). The PCR products of VH and VL gene segments were separately purified and assembled by PCR with a linker DNA that encoded a $(Gly_4Ser)_3$ peptide to construct a single-chain Fv (scFv) gene. After adding the flanking oligonucleotides containing the appended restriction sites to the reaction mixture, the assembled scFv genes were reamplified by PCR and gel-purified. The purified scFv gene repertoires were digested with *Not*I and *Sfi*I restriction enzymes, gel-purified, and then ligated to phagemid pCANTAB5E (Amersham Pharmacia Biotech). The ligation products were purified by extraction with phenol/chloroform and precipitation with ethanol. The purified DNA was then electroporated into TG1 electrocompetent cells (Stratagene, La Jolla, CA) with a Gene Pulser (Bio-Rad, Richmond, CA). The diversity of each library was defined as the number of kinds of recombinant single ampicillin-resistant clones obtained on consecutive ligation and transformation of the plasmid into competent cells prior to any amplification of the genes. Four phagemid libraries were constructed, named Vγ-Vκ, Vγ-Vλ, Vμ-Vκ, and Vμ-Vλ; they contained 1.1×10^8, 2.1×10^8, 8.4×10^7, and 5.3×10^7 members, respectively. The VH and VL genes from arbitrarily chosen clones in the four phagemid libraries were sequenced, and the germ-line gene was assigned based on homology to a database (VBASE) of germ-line V-genes compiled by Tomlinson *et al*. (*20*).

Preparation of an scFv Display Phage—M13KO7 helper phage (final, *ca.* 10^9 plaque–forming units/ml) was added to *E. coli* TG1 cells harboring the constructed phagemids grown to an OD_{600} of 0.5–0.7 at 30°C in a 2 YT medium containing 2% glucose and 100 μg/ml ampicillin. After being gently shaken at 30°C for 1 h, the cells were collected by centrifugation, suspended in a 2 YT medium containing 100 μg/ml ampicillin and 50 μg/ml kanamycin, and incubated with shaking overnight at 30°C. After the cells had been removed by centrifugation, phage particles in the supernatant were purified and concentrated by two poly(ethylene glycol) (PEG) precipitations by adding a 1/5 volume of 20% PEG in 2.5M NaCl (*21*). The concentrated phage was resuspended in TE (10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA) to a concentration of about 10^{13} transducing units/ml (ampicillin-resistant clones).

Phage Antibody Selection—Biopanning was performed as described by Kaji *et al.* (*22*). Briefly, four μg of rFcεRIα in 0.1 M $NaHCO_3$, pH 8.6, was incubated at 4°C overnight in a 35 mm petri dish (Iwaki Glass, Tokyo). The rFcεRIα-coated plate was washed once with phosphate-buffered saline (PBS) and then blocked with either 0.5% gelatin for the 1st selection or 5% skim milk for the 2nd selection. The mixture of Vγ-Vκ and Vγ-Vλ phage libraries (5×10^{11} transforming units [TU] each) or Vμ-Vκ and Vμ-Vλ phage libraries (5×10^{11} TU each) was added to the rFcεRIα-plastic plate at room temperature. Two hours later, the plate was washed 10 times with phosphate-buffered saline (PBS) containing 0.1% Tween20. The bound phages were eluted for 5 min with 400 μl of 0.1 M glycine-HCl (pH 2.2), and then immediately neutralized with 1 M-Tris HCl (pH 9.1) and amplified by infection with log phase *E. coli* strain TG1 cells, as described (*17*). One milliliter of these phages (containing approximately 10^{12} TU) was used for the next round of selection.

Soluble scFv—The soluble scFvs of the selected clones after the final selection were prepared by infecting phage clones with *E. coli* HB2151 according to the manufacturer's instructions (Amersham Pharmacia Biotech). The scFv fragments were then affinity-purified using an anti-Etag monoclonal antibody (Amersham Pharmacia Biotech). The eluted fractions were dialyzed against PBS and then analyzed by SDS-PAGE. Protein concentrations were determined with a protein assay kit (Bio-rad) based on the Bradford method. The purified scFvs were analyzed by HPLC using HiLoad 16/60 Superdex (Amersham Pharmacia Biotech).

ELISA—ELISA was performed as described (*22*, *23*). Briefly, all antigens (80 ng /40 μl) were adsorbed to a microtiter plate (Nunc, Denmark). Phage clones (40 μl of PEG-precipitated phage [typically 10^{12} TU]) were added to wells, followed by incubation with biotinylated anti-M13 mAb at a dilution of 1:1,000 (Pharmacia, CA), followed by detection with alkaline-phosphatase (AP)–conjugated streptavidin (1:1,000: Vector Laboratories, Burlingame). For the detection of scFv, anti-Etag mAb (Pharmacia) was used at a dilution of 1:1,000 as a primary antibody, followed by AP-conjugated anti–mouse IgG (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:2,000 as a secondary antibody. Absorbance was measured at 405 nm by the use of a microplate reader (NJ-2300; Nunc, Tokyo).

Immunoblotting Analysis—12% SDS-PAGE and blotting were carried out as described (*22*), using a semidry electroblotter (Sartorius, Tokyo). The transferred PVDF membrane (Applied Biosystems) was incubated with horseradish peroxidase (HRP)–conjugated anti–E-tag mAb. Development was performed by ECL (Amersham Pharmacia Biotech) according to the manufacturer's instructions using a luminoimage analyzer (LAS-1000, Fuji Film, Tokyo). An NEB prestained protein marker (NE Biolabs) was used as a protein marker.

DNA Sequencing—The nucleotide sequences of the scFv genes were determined with a Dye Terminator Cycle Sequencing FS Ready Reaction kit (PE Applied Biosystems, Foster City, CA) with primer S7 (pUC19 reverse in the pUC backbone: 5′-AGCGGATAACAATTTCACACAGG-3′) and S8 (in the *fd* gene 3: 5′-GTCGTCTTTCCAGACGTTAGT-3′). The amino acid residues of each variable domain were according to Kabat *et al*. (*24*, *25*).

Surface Plasmon Resonance Analysis—The binding kinetics of scFvs as to immobilized rFcεRIα were measured by means of surface plasmon resonance (SPR) using a Pharmacia BIAcore 2000 optical biosenser (Biacore Uppsala, Sweden) according to the manufacturer's instructions. The rFcεRIα was immobilized on a CM5 sensor chip in 10 mM sodium acetate, pH 4.0, using the amine coupling kit supplied by the manufacturer. Unreacted moieties on the surface were blocked with ethanolamine. A rFcεRIα concentration of 50 μg/ml and a contact time of 5 min at a flow rate of 5 μl/min gave approximately 240 resonance units (RU). All measurements were conducted in HBS buffer (10 mM HEPES, pH 7.4, containing 0.15 M NaCl, 3 mM EDTA, and 0.005% Tween20) at a flow rate of 5 μl/min at 20°C. After each measurement, the chip surface was regenerated with 10 μl of 200 mM glycine-HCl buffer containing 200 mM NaCl (pH 2.2). Human IgE (Chemicon, Temecula, CA) and scFv antibodies were assessed over the concentration range of 5–0 nM. As unrelated scFvs showed no binding activity on these sensorgrams, response curves were prepared by subtracting the signal generated from control flow cell from the flow cell containing rFcεRIα. Sensorgram curves were evaluated with BIAevalution 2.1 to determine the rate constants k_{on} and k_{off}. The dissociation constant (K_d) was calculated with the equation $K_d = k_{off}/k_{on}$.

Histamine Release Assay—PBMCs were treated with 0.01 M lactic acid (pH 3.9) containing 0.13 M NaCl–0.005 M KCl to dissociate cell-bound immunoglobulins, and then the histamine release assays were performed as described by Pruzansky *et al*. (*26*). The PBMCs were sensitized with the human myeloma IgE (2.5 μg/ml) for 10 min at 4°C. Before the sensitization, the cells were treated with the indicated concentration of scFv at 4°C for 20 min. Sensitized cells were stimulated with plate-coated goat anti–human IgE Ab (Sigma) for 45 min at 37°C. The histamine in the supernatant and cell fractions was measured with an RIA kit (Immunotech, Westbrook, ME). 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.

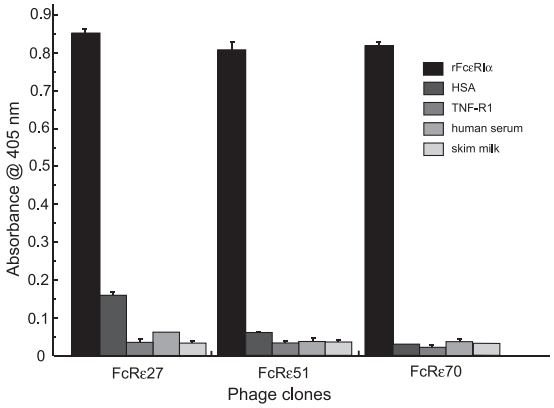

Fig. 1. **Phage clones selected with rFcεRIα.** ELISA plates were coated with 80 ng/well of HSA, human serum, TNF-R1, gelatin, or skim milk for 12 h. After incubation of the wells with the FcRε27, FcRε51, or FcRε70 phage clone (1×10^{11} TU/40 μl/well) for 1 h, the binding of phage clones was detected with biotinylated anti-M13 mAb in combination with AP-conjugated streptavidin, as described under "MATERIALS AND METHODS."

RESULTS

Selection of FcεRIα-Specific scFv Display Phage Clones—FcεRIα-specific scFv phage clones were selected from the mixture of the Vγ-Vκ and Vγ-Vλ phage libraries. In total, two rounds of biopanning were performed on immobilized rFcεRIα, with varying protein concentrations. After two rounds of biopanning selection, 144 clones were randomly selected and examined by ELISA screening for binding to rFcεRIα, and then four scFv phages out of the 144 tested clones were analysed. The DNA sequencing data confirmed that 3 of the clones were unique, their variable heavy and light chains having different nucleotide and amino acid sequences; these clones were designated as FcRε27, FcRε51, and FcRε70, respectively. As shown in Fig. 1, these phage clones specifically bound to rFcεRIα, but not to gelatin, skim milk, BSA, human serum, recombinant human tumor necrosis factor receptor 1 (TNF-R1), or human serum albumin (HSA). We were unable to isolate the specific clones from the pool of Vμ-Vκ and Vμ-Vλ (data not shown).

Binding Activity of the Soluble FcεRIα-Specific scFvs—The three rFcεRIα-specific clones, FcRε27, FcRε51, and FcRε70, were expressed in shaker flasks using nonsuppressor host *E. coli* HB2151 cells. Soluble scFv proteins were purified from the culture supernatants or periplasmic extracts of *E. coli* on an anti–E tag mAb column. SDS-PAGE analysis of each purified scFv preparation gave a single protein band corresponding to the expected molecular size of 29–30 kDa (Fig. 2). The soluble scFvs were examined as to their binding activity toward FcεRIα. As shown in Fig. 3, all three scFvs bound to rFcεRIα. No cross-reaction was seen with HSA, TNF-R1, human serum, or 5% skim milk.

VH/VL Sequences of scFvs—The VH and VL domains of anti-FcεRIα scFvs were sequenced and compared to elucidate the contributions of the variable heavy (VH)- and variable light (VL)-chains to the antigen specificity. The gene usage and deduced amino acid sequences of the scFv clones are shown in Fig. 4. The VH genes of the

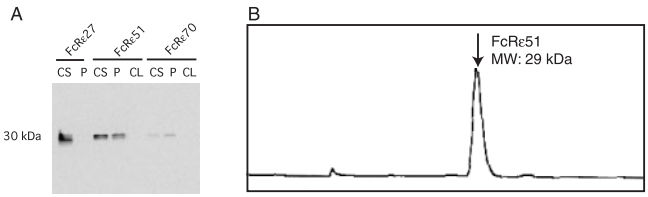

Fig. 2. **Immunoblotting analysis of scFvs derived from an *E. coli* extract.** (A) The supernatant (CS), periplasmic extract (P), and whole cell extract (CL) of a HB2151 cell culture infected with the indicated phage clone were recovered and subjected to 12% SDS-PAGE under non-reducing conditions. After electroblotting, the PVDF membranes were incubated with HRP-conjugated anti–E tag mAb. (B) Size-exclusion HPLC (HiLoad 16/60 Superdex, Pharmacia) of scFv preparations.

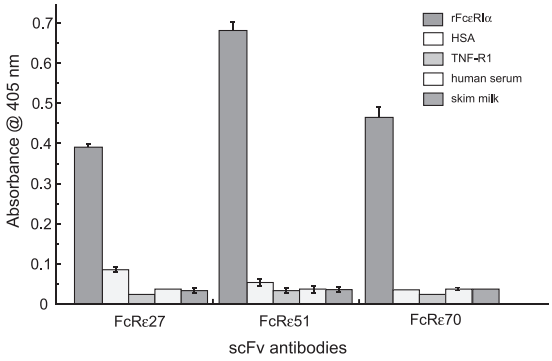

Fig. 3. **Specific binding of the purified soluble scFvs to rFcεRIα.** ELISA was performed as described in Fig. 1 except that FcRε27, FcRε51, or FcRε70 (1 μg/40 μl/well) was added to wells in place of a phage clone.

FcRε27, FcRε51, and FcRε70 scFvs belonged to V_H1, V_H3, and V_H5 family, respectively. The light chains of FcRε27 and FcRε70 belonged to the Vλ1 family, whereas FcRε51 belonged to the Vλ3 family.

Inhibitory Activity of FcRε51 scFv on the Binding of IgE to FcεRIα—We examined the inhibitory activity of soluble scFvs on the interaction of FcεRI with IgE. After biotinylated FcεRIα had been preincubated with the scFv phage for 30 min, the mixture was added to IgE-coated plates. The binding of FcεRIα to IgE was detected with AP-conjugated streptavidin. As shown in Fig. 5A, the FcRε51 phage showed significant inhibitory activity, while the FcRε27 and FcRε70 phages did not exhibit any detectable inhibition. To confirm the inhibitory activity of FcRε51 scFv, varying concentrations of the purified scFv were used for this experiment. As shown in Fig. 5B, FcRε51 scFv inhibited the binding of IgE to its receptor at 10 μg/ml, and 90% inhibition was observed at 100 μg/ml, while an irrelevant scFv did not exhibit any inhibitory action. The addition of FcRε51 scFv after the incubation with IgE had no effect on the interaction of FcεRIα with IgE (data not shown).

Binding Affinity of FcRε51 scFv—As size-exclusion HPLC analysis showed that FcRε51 was in the monomer form (Fig. 2), affinity analysis was performed by means of surface plasmon resonance. rFcεRIα was coupled to the sensor chip, CM5 of BIAcore, and various concentrations of scFv were run over the chip. The binding of IgE to the immobilized rFcεRIα was also examined under identical conditions (Fig. 6). In the case of the k_{on} value, the binding of rFcεRIα/FcRε51 scFv and rFcεRIα/IgE was almost identical, *i.e.*, 3.6×10^5 and 3.3×10^5 s^{-1} M^{-1}, respectively. In contrast, the k_{off} value was 7.2×10^{-3} s^{-1} in the case of FcRε51 *vs*. rFcεRIα, but 1.6×10^{-4} s^{-1} in the case of IgE *vs*. rFcεRIα, resulting in 40-fold lower affinity of FcRε51 to rFcεRIα in comparison with that of IgE, *i.e.*, K_d = 20 nM *vs.* 0.5 nM in the case of IgE.

Inhibition of Histamine Release from IgE-Triggered PBMCs by FcRε51 scFv—FcRε51 scFv was examined as to its ability to inhibit the passive sensitization of human PBMCs with IgE. The histamine concentration of culture supernatants was measured by means of an RIA assay. As shown in Fig. 7, FcRε51 exhibited approximately 50 % inhibition at 50 μg/ml and 30% inhibition at 10 μg/ml of the histamine release from PBMCs. On the other hand, an unrelated scFv showed no inhibition. It is of note that the PBMCs treated with FcRε51 alone did not show induction of degranulation of histamine, indicating that FcRε51 has no agonistic activity.

A. Amino acid sequences of the V_H domains

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                    FR1                 CDR1      FR2              CDR2                  FR3
FcRε27   QVQLQQSGAEVKKPGASVKVSCKASGYNFT NFDLN WVRQAAGQRPEWMG WMNP--KSGNSGYAQSFRG RLTMTRNISITTAYMELSNLMSEDTAVYYCTR
FcRε51   QVQLQEWGGGLVQPGGSLRLSCAASGFTVS SNYMS WVRQAPGKGLEWVS VIYRGGSGDNTYYAGSVKG RFTISRDNSKNTVYLQMNSLRAEDTAVYYCVR
FcRε70   QVQLQQSGPEVKKPGESLTISCQGDGYNFN RYWVG WVRQRPGQGLEWMG VIYP--GDSDTRYSPSFRG QVTISADKSVNTAYLQWVSLKASDTAIYYCVR

              CDR3          FR4
FcRε27   GVGGSS-------FN WGHGTLVTVSS
FcRε51   SSDVGYGILRGYMDV WGKGTTVTVSS
FcRε70   HFFEGD---EDAFDI WGQGTMVTVSS
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B. Amino acid sequences of the V_L domains

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                  FR1                CDR1          FR2          CDR2              FR3
FcRε27   QSVLTQPPSVSAAPGQRVTISC SGSISNIGHNSVS WYQHLPGTAPRLLIY RNNQRPS GVPDRFSGSKSGTSASLAISGLRSEDEADYYC
FcRε51   HVILTQPPSVSVSPGQTARIPC SGQDLTNK--YVS WYQLQSGQSPALVIY EDSKRPS GIPERFSGSNSGDTATLTITGTQAADEADFFC
FcRε70    SVVTQPPSVSATPGQKVTISC SGNNSNIGNNYVS WYQQLPGTAPKLLIY DNNKRPS GIPDRFSGSKSGTSATLGVTGLQTGDEADYYC

             CDR3        FR4
FcRε27   AAWDDSLSDYV FGTGTQLTVLG
FcRε51   QAYDTNG--WV FGAGTKLTVLG
FcRε70   GCWDSSLSAVV FGGGTKLTVLG
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Fig. 4. **Amino acid sequences of the VH and VL domains of FcRε27, FcRε51, and FcRε70.** (A) Amino acid sequences of the VH domains. The complementary-determining regions (CDR1–CDR3) and the flanking regions (FR1-4) were deduced according to Kabat *et al*. (Refs. *24* and *25*). (B) Amino acid sequences of the VL domains. CDR regions are indicated in bold.

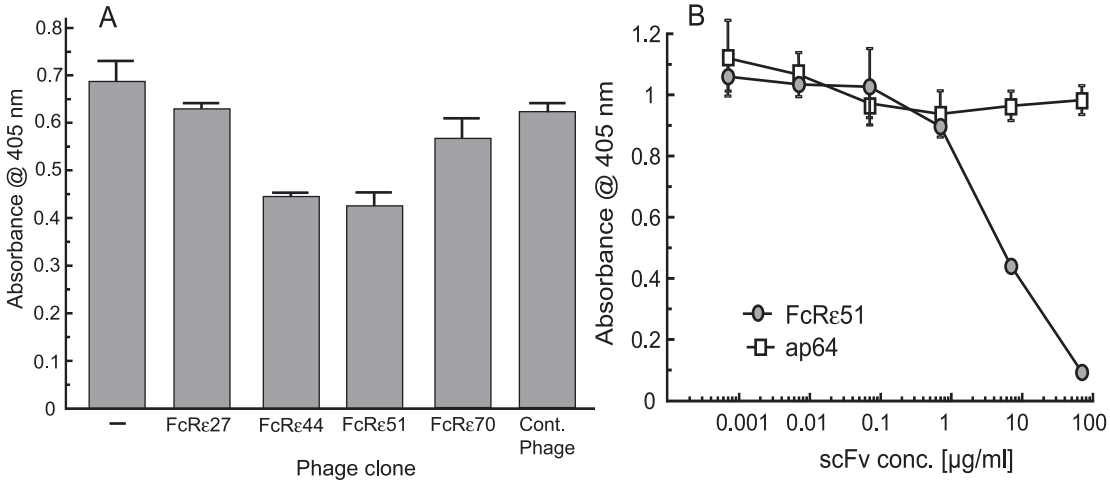
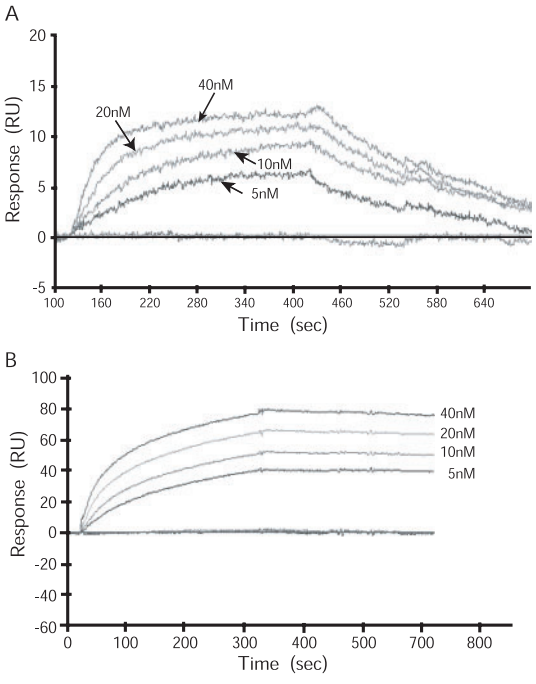


Fig. 5. **FcRε51 inhibits the binding of FcεRIα to human IgE.** ELISA plates were coated with human IgE (80 ng/40 μl/well). Twelve hours later, biotinylated rFcεRIα preincubated with or without a phage clone (A) or the indicated concentration of FcRε51 (B) for 0.5 h was added to IgE-coated wells. The binding of rFcεRIα was detected with AP-conjugated streptavidin. As judged on sequencing analysis of the V genes, FcRε44 and FcRε51 were identical. Therefore, FcRε44 was omitted in the analysis in (B).



C

Protein	k_{on} ($sec^{-1}M^{-1}$)	k_{off} (sec^{-1})	K_d (k_{off}/k_{on}) (nM)
FcRε51	3.6×10^5	7.2×10^{-3}	20
human IgE	3.3×10^5	1.6×10^{-4}	0.50

Fig. 6. **BIAcore analysis of FcRε51.** BIAcore sensorgrams recording the association and dissociation of FcRε51(A) and human IgE (B). rFcεRIα (50 μg/ml) was immobilized on CM5 sensor chips as described under "MATERIALS AND METHODS." The rate constants, k_{on} and k_{off}, and the dissociation constant (K_d) are shown in (C). No binding to an unrelated protein (human IgE) was observed.

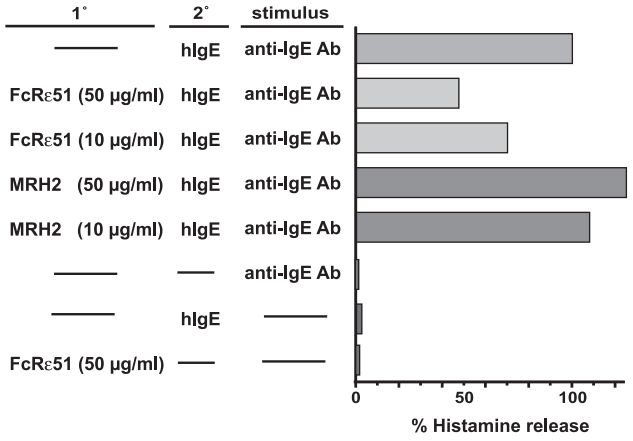


Fig. 7. **Inhibition of histamine release from PBMCs by FcRε51.** PBMCs were treated with an acidic buffer to dissociate the cell-bound immunoglobulins. These cells were preincubated with the indicated concentrations of FcRε51 or an unrelated scFv (MRH2) for 20 min, followed by sensitization with 2.5 μg/ml of human IgE. After washing, the sensitized cells were stimulated on anti–human IgE-coated plates (3 μg/ml /well) at 37 °C for 45 min. The histamine content of the supernatant was measured using an RIA kit (Immunotech, Westbrook, ME). 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.

DISCUSSION

For this study, human scFv display phage libraries were carefully constructed according to the method reported by Marks *et al*. (*17*). Our libraries have several superior features. First, four libraries were constructed, which were designated as Vγ-Vκ, Vγ-Vλ, Vμ-Vκ, and Vμ-Vλ, according to the assembly pattern. Secondly, these libraries were prepared on a large scale by using peripheral blood lymphocytes obtained from 20 healthy volunteers, which reduced the bias of V-gene usage among individu-

als to as little as possible. Arbitrarily chosen clones in the four phagemid libraries were examined for their V-gene DNA sequences. All the selected clones had scFv DNA, and the VH-gene usage in these libraries was quite similar to that of PBMCs reported by Brezinschek *et al.* (*27*) (data not shown). Thirdly, the V gene fragments were derived from the cDNA of PBMCs, which underwent immunological selection *in vivo*. This feature may reduce the degree of possible antigenicity of V domains to as little as possible. Fourthly, they were non-immune libraries. Probably, owing to these features, human FcεRIα-specific scFv antibodies were successfully isolated from the non-immune human-scFv displaying phage libraries. After two rounds of biopanning selection using rFcεRIα-coated plates, three specific scFv antibodies were isolated from a pool of two libraries, Vγ-Vκ and Vγ-Vλ. On the other hand, we were unable to isolate such antibodies from the pool of Vμ-Vκ and Vμ-Vλ (data not shown). Initially, we speculated that an anti-self antibody could be derived from the Vμ-library because Vμ is quite similar to the germ-line V gene, which has not undergone immunological *in vivo* selection. However, our results suggested the importance of V gene-maturation *in vivo* with regard to self-specificity. Using our libraries, we have isolated scFv antibodies specific to either human interleukin-6 (IL-6) or human monocyte chemoattractant protein-1 (MCP-1) (manuscript in preparation). The isolation of scFv antibodies against EGFR from the non-immune library has also been reported (*28*, *29*). Thus, these data suggest that the non-immune library may be practically used to isolate the self-recognizing antibodies.

IgE antibodies bind to specific high-affinity receptors (FcεRI) on mast cells, leading to mast cell degranulation and the release of mediators, such as histamine, which produce symptoms associated with allergy. Recent studies have shown that humanized monoclonal antibodies specific to IgE or FcεRIα are of potential therapeutic value in the treatment of allergies. In this study, we have isolated fully human scFv antibodies specific to human FcεRIα. BIAcore analysis demonstrated that the FcεRIα-specific scFv antibody, FcRε51, bound to FcεRIα with an affinity of K_d = 20 nM, which is relatively higher than the average affinity of an scFv antibody. It has been reported with the use of a smaller phage library of 1×10^7 to 2×10^8 clones, the isolation of scFv antibodies with moderate binding affinities was attained. The pretreatment of PBMCs with FcRε51 significantly inhibited the subsequent degranulation by cross-linking of FcεRI with IgE and an anti-IgE polyclonal Ab (Fig. 6). A 10-fold excess of scFv compared to IgE was required to inhibit 50% of the response. This may be due to the monovalency of FcRε51 and the much faster rate of dissociation than that of IgE (Fig. 5C). Antibody engineering may improve FcRε51 to attain higher avidity and effective antagonistic activity. It has been reported that a bispecific scFv dimer, composed of two scFvs directed against different epitopes on VEGF, showed up to 20-fold higher avidity than either of the individual parent scFvs (*29*). FcRε51 alone did not induce histamine release, indicating that FcRε51 has no agonistic activity. In relation to this issue, it has been reported that exposure to monomeric IgE can enhance mast cell survival, cytokine production, and FcεRI expression without histamine release (*30–32*). It remains to be determined whether or not monovalent FcRε51 has an effect on mast cell activation, including FcεRI-expression or cell survival.

The scFv antibody, FcRε51, is a human antibody that is highly superior to a humanized antibody in terms of immunogenicity in humans. This class of agents should be of potential therapeutic value in the treatment of allergies.

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan.

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