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Trimolecular Complex Formation of IgE, FcεRI, and a Recombinant Nonanaphylactic Single-Chain Antibody Fragment with High Affinity for IgE[1]

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IgE is a central molecule in allergic disease. We have isolated cDNAs coding for the heavy and light chains of a murine mAb specific to human IgE and expressed a recombinant single-chain variable fragment (ScFv) derived thereof in *Escherichia coli*. The purified recombinant ScFv has a molecular mass of 28 kDa as measured by mass spectrometry and shows a β-sheet fold as determined by circular dichroism. In biosensor-based studies it was demonstrated that the ScFv rapidly and stably binds to human IgE with an affinity of K_D of 1.52×10^{-10} M, which is almost as high as the affinity of IgE for FcεRI, and that the ScFv is able to recognize FcεRI-bound IgE and to prevent IgE binding to FcεRI. The ScFv reacts specifically with IgE but not with other isotypes, allows the measurement of allergen-specific IgE in serum samples, and specifically targets cells that contain FcεRI- or FcεRII-bound IgE or that secrete IgE. Using negative-stain electron microscopy we demonstrated the formation of bimolecular complexes consisting of two ScFv molecules and one IgE and trimolecular complexes consisting of IgE, FcεRI, and ScFv in which only one ScFv is able to bind to IgE. Accordingly, we found that the ScFv does not cross-link basophil-bound IgE and hence does not induce histamine release or activation of basophils as demonstrated by FACS analysis of CD203c expression and by histamine release experiments. In vivo skin testing confirmed the lack of allergenic activity of the ScFv. The recombinant ScFv may represent a universal tool for the IgE-targeted treatment of allergies. *The Journal of Immunology,* **2009, 182: 4817–4829.**

IgE-mediated allergies represent a global health problem due to the continuously increasing prevalence of the disease and the fact that untreated allergies progress from mild (e.g., rhinitis) to severe manifestations, such as allergic asthma (1–3).

IgE is the least abundant Ig but it has a central role in allergic diseases (4, 5). IgE binds to various immune cells via the high-affinity receptor FcεRI and via the low-affinity receptor FcεRII (CD23) and, upon allergen recognition, can trigger various inflammatory responses (6–8). Allergen-induced cross-linking of FcεRI-bound IgE is a key event in triggering the activation and degranulation of mast cells and basophils, which leads to the fast release of inflammatory mediators (e.g., histamine, leukotrienes), proteases, and proinflammatory cytokines (9, 10). The binding of IgE to FcεRI is characterized by several interesting features. IgE binds with extremely high affinity to FcεRI and remains attached to FcεRI-expressing effector cells for much longer periods than its half-life in serum or body fluids. The IgE-FcεRI interaction represents a 1:1 interaction due to structural constraints of the IgE molecule (11, 12). It has been reported that monomeric IgE binding is crucial for the survival of effector cells, whereas cross-linking by allergens or other ligands represents the signal for cell activation (9, 13). Interestingly, high levels of IgE up-regulate the expression of FcεRI on effector cells, whereas low levels of IgE cause a down-regulation of the specific receptors (14).

Both FcεRI and FcεRII are also present on APCs, and it has been demonstrated that IgE can facilitate the presentation of allergens to T cells, leading to stronger activation of T cells when the allergen is presented via this pathway compared with non-IgE-mediated Ag presentation (15–18).

Also, IgE production seems to be strongly regulated by IgE-dependent mechanisms. There is evidence for local IgE production in the target organs of allergy, for example, in the respiratory mucosa (19), and it has been demonstrated that allergen contact via the respiratory mucosa, presumably by stimulating allergen-specific IgE-producing cells, strongly enhances IgE secretion, leading to increases of allergen-specific serum IgE levels and sensitivity in the target organs of allergy (20, 21).

Because of the central role of IgE in allergic disease, many therapeutic strategies for targeting the IgE molecule have been suggested, of which one compound, a monoclonal anti-human IgE Ab termed omalizumab, is available for the treatment of certain forms of allergy (22, 23).

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Herein we report the expression and molecular and immunological characterization of a recombinant single-chain variable fragment (ScFv12)[3] with specificity to IgE. Recombinant ScFv was expressed and purified to homogeneity and its fold was studied by circular dichroism analysis. The kinetics of the binding of ScFv12 to IgE were assessed by surface plasmon resonance (SPR) experiments, and it was demonstrated that the ScFv efficiently binds to IgE in solution, prevents IgE binding to FcεRI, and targets FcεRI- and FcεRII-bound IgE, which also was confirmed in FACS assays using IgE-bearing cells as well as IgE-producing cells. This unusual behavior of preventing IgE binding to the receptor and recognizing receptor-bound IgE, which distinguishes ScFv12 from omalizumab, is due to a bilateral recognition of IgE, which overlaps with the binding sites for FcεRI. This feature was visualized by negative stain electron microscopy of bi- and trimolecular complexes consisting of IgE and ScFv12, as well as of IgE, ScFv12, and FcεRI. The lack of allergenic activity of ScFv12 was studied by basophil activation assays in vitro and by skin testing in vivo in man to demonstrate its safety and therapeutic potential.

Materials and Methods

Antibodies, recombinant FcεRI α-chain

mAb12 was generated by immunization of BALB/c mice with purified IgE (ND) Fc (24). Hybridoma cells were grown in DMEM containing 10% (v/v) FBS and 50 μg/ml gentamicin (all substrates by Invitrogen) at 37°C, 5% CO_2, and 95% humidity.

Chimeric Bip1, a humanized monoclonal IgE Ab specific to the major birch pollen allergen, Bet v 1, has been described (24). Recombinant FcεRI α-chain–human serum albumin (HSA)–FcεRI α-chain (α-HSA-α), consisting of two molecules of the extracellular portion of FcεRI α-chain that were fused to the C and N termini, respectively, of HSA was prepared at Novartis.

Construction and expression of a recombinant ScFv

mAb12-producing hybridoma cells were harvested by centrifugation (800 × *g*, 10 min, room temperature) and resuspended in GIT buffer (4 M guanidine-isothiocyanate, 3 M sodium acetate (pH 6), 0.83% (v/v) 2-ME). Messenger RNA was isolated from hybridoma cell homogenate by CsCl gradient centrifugation (25).

Double-stranded cDNAs of the L chain and of the variable domain and the first constant domain of the H chain (V_H-Cγ_1) were obtained using a cDNA synthesis system (Roche). The first strand of the cDNA was synthesized by reverse transcription using primers (MWG Biotech) specifically binding to the 3′ end of the mRNA encoding the constant domain of the L chain (5′-TCC TTC TAG ATT ACT AAC ACT CTC CCC TGT TGA A-3′) and to the 3′ end of the sequence of the mRNA encoding the first constant domain of the IgG H chain (Cγ_1) of mAb12 (5′-AGG CTT ACT AGT ACA ATC CCT GGG CAC AAT-3′), respectively. The second strand was constructed by replacement synthesis according to the manufacturer's instructions. After ligating *Eco*RI linkers to the blunt-ended termini (Boehringer Mannheim) and digestion with *Eco*RI, the cDNAs were ligated to *Eco*RI-digested λgt11 vector arms (Stratagene). Phages were assembled using Gigapack III Gold-11 packaging extracts (Stratagene). Positive clones were identified by hybridization with ^{32}P-labeled oligonucleotides specific to the cDNA encoding C_L (5′-AAT GGG TGA AGT TGA TGT CTT GTG AGT GGC-3′) and Cγ_1 (5′-TCT TGT CCA CCT TGG TGC TGC TGG CCG GGT-3′), respectively. After transferring the inserts of two positive clones into the plasmid pUC19, the authentic cDNA sequences could be determined (MWG Biotech) and aligned with mouse variable domain germline sequences of the ImMunoGeneTics (IMGT) database using V-QUEST alignment software (26). The molecular masses of V_H and V_L were calculated using PeptideMass (27) (ExPASy(Expert Protein Analysis System) Proteomics Tools, Swiss Institute of Bioinformatics, Geneva, Switzerland).

The cDNAs encoding the variable domains of the H chain (V_H) and the L chain (V_L) were amplified by PCR using phage DNA as templates and specific primers binding to the 5′ and to the 3′ ends of murine variable domain sequences of the H chain and of the L chain (heavy primers 1 and 2 and light primer mix, recombinant phage Ab system/mouse ScFv module; Amersham Biosciences), respectively. The PCR products were assembled by a second PCR step using linker primers (linker primer mix; Amersham Biosciences) connecting the 3′ end of V_H with the 5′ end of V_L by a linker of 45-bp length (see Fig. 2). A third PCR step introduced an *Sfi*I restriction site at the 5′ end and a *Not*I restriction site at the 3′ end of the ScFv12 DNA (RS primer mix; Amersham Biosciences). The purified ScFv12 PCR product was ligated into pSTblue-1 AccepTor Vector (Novagen) via single deoxyadenosine overhangs introduced at the 3′ ends by *Taq*DNA polymerase. The plasmid could be amplified and sequenced using a T7 promoter primer (5′-TAA TAC GAC TCA CTA TAG GG-3′) and an SP6 promoter primer (5′-CAT TTA GGT GAC ACT ATA G-3′) (MWG Biotech).

Positive clones were digested with *Sfi*I and *Not*I, and the cut ScFv12 DNA was purified from an agarose gel and ligated into the pre-cut expression vector pCANTAB 5 E (Amersham Biosciences). *E. coli* strain HB2151 (RPAS expression module; Amersham Biosciences) was transformed with the ligation product (28) and plated onto Luria-Bertani agarose plates containing 100 μg/ml ampicillin. To identify clones expressing functional ScFv12, nitrocellulose filters (Schleicher & Schuell BioScience), presoaked with 5 mM isopropyl β-D-thiogalactoside, were placed onto the plates and incubated at 37°C for 8 h. Subsequently, the membranes were exposed to liquid nitrogen for 30 s to disrupt the bacterial cells. After blocking the membranes with BSA (43 mM Na_2HPO_4, 7 mM NaH_2PO_4, 0.5% (v/v) Tween 20, 0.5% (w/v) BSA, 0.05% (w/v) NaN_3), the filters were incubated overnight at 4°C with culture supernatants of a hybridoma cell line expressing human IgE (24). Bound IgE was detected with ^{125}I-labeled anti-human IgE (RAST RIA; Sweden Diagnostics) which had been diluted 1/10, and colonies expressing functional recombinant ScFv12 were identified by autoradiography.

IgE-reactive clones were picked from the plate and grown overnight in 5 ml of 2× YT-AG medium (1 liter contains 17 g of bacto-tryptone, 10 g of bacto-yeast extract, 5 g of NaCl (pH 7.4), 100 μg/ml ampicillin, 2% (w/v) glucose) at 30°C. Four milliliters of the overnight cultures was transferred to 50 ml of fresh 2× YT-AG medium, incubated at 30°C for 1 h, and centrifuged (1000 × *g*, 10 min, room temperature). The bacteria were resuspended in 50 ml of 2× YT-AI medium (2× YT medium containing 100 μg/ml ampicillin and 1 mM isopropyl β-D-thiogalactoside) and incubated at 30°C overnight. Cells and debris were removed from the culture by centrifugation and filtration, and proteins from the culture supernatants were separated by SDS-polyacrylamide gel (12.5%) electrophoresis under nonreducing conditions (29) and blotted onto nitrocellulose (Schleicher & Schuell BioScience) (30). After blocking the membranes with BSA, ScFv12 was identified with a HRP-conjugated mouse mAb specific to the C-terminal E tag of ScFv12 (HRP/anti-E tag conjugate; Amersham Biosciences). Bound anti-E tag Ab was visualized by incubating the blot in 50 mM Tris (pH 7.6) containing 0.6 mg/ml diaminobenzidine and 0.1% (v/v) H_2O_2.

Expression of soluble ScFv12 and purification by affinity chromatography

A 4 ml overnight culture of an *E. coli* HB2151 clone expressing functional ScFv12 was used to inoculate 50 ml of fresh 2× YT-AG medium and, after incubation at 30°C for 1 h, transferred to four flasks containing 250 ml of 2× YT-AI medium each and cultured overnight at 30°C. The overnight cultures were centrifuged, filtered through Steritop/Express Plus membrane (pore size 22 μm; Millipore), concentrated using Amicon Ultra centrifugal filter tubes (Millipore) to a final volume of ~100 ml, and filtered again. Recombinant ScFv12 was purified from the supernatant by affinity chromatography using a 5-ml HiTrap anti-E tag affinity column (RPAS purification module; Amersham Biosciences). The eluate was immediately dialyzed against PBS (pH 7.5) and concentrated using Amicon Ultra centrifugal filter tubes (Millipore). Purity of the sample was shown by SDS-PAGE, and the protein concentration was determined using a Micro BCA protein assay kit (Pierce).

MALDI-TOF mass spectrometry of ScFv12

Purified ScFv12 was desalted and further purified via reversed phase-HPLC (HP 1050 binary gradient; column, Nucleosil-100 RP-18 150 × 4 mm, 5 μm; solvent A, water, 0.1% TFA; solvent B, acetonitrile, 0.1% TFA; gradient, 10% B to 90% B in 15 min; flow, 1 ml/min; detection, UV at 215 nm; injected volume, 10 μl). Mass spectra (piCHEM) were acquired from the purified sample by MALDI-TOF on a Kratos PC Axima CFR V2.2.1 (Kratos Analytical).

[3] Abbreviations used in this paper: DC, dendritic cell; HSA, human serum albumin; MFI, mean fluorescence intensity; RU, resonance unit; ScFv, single-chain variable fragment; SPR, surface plasmon resonance.

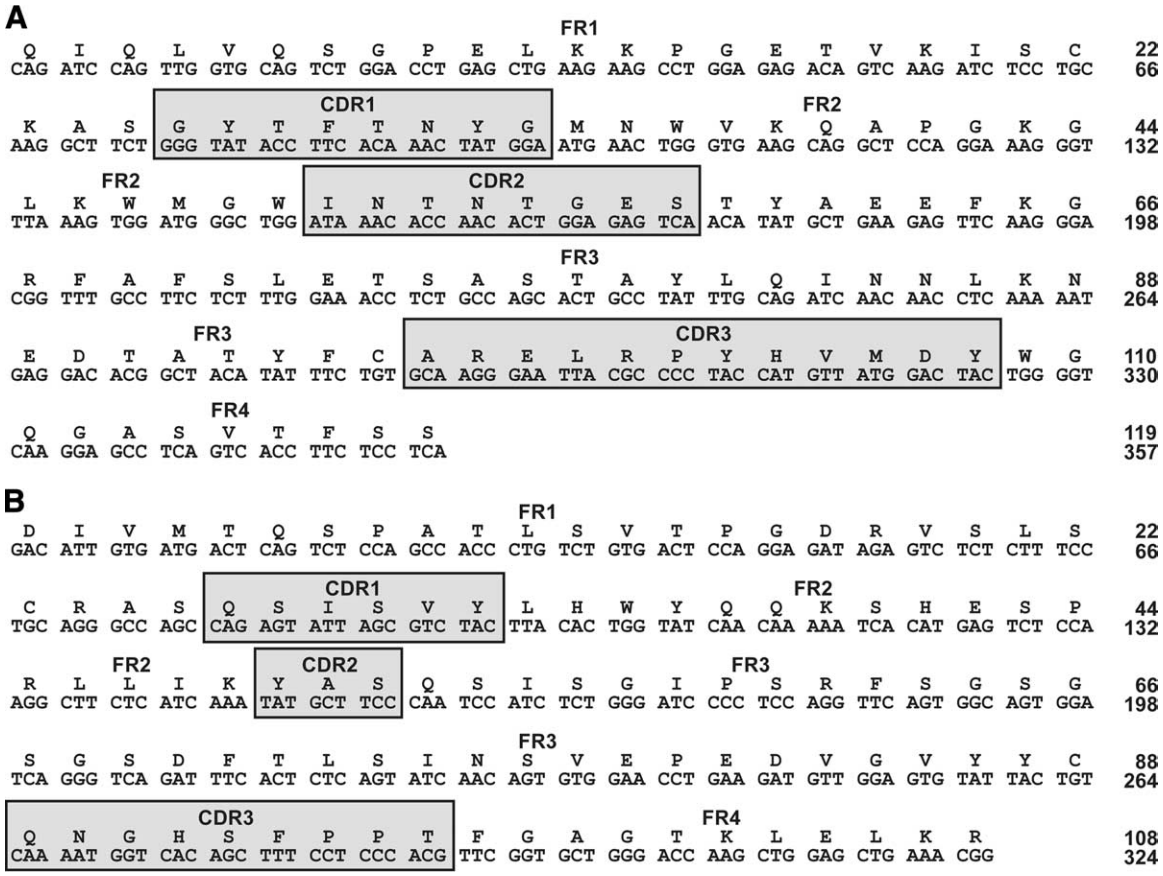


FIGURE 1. cDNA and deduced amino acid sequences of the variable regions of the H (*A*) and the L chain (*B*) of mAb12 are displayed. Nucleotides and amino acids are numbered on the right margin. Framework regions (FRs) and complementarity determining regions (CDRs, gray boxes) are shown.

Circular dichroism analysis of ScFv12

CD spectroscopy was performed on a Jasco J-810 spectropolarimeter. The spectra were recorded using a 1-mm pathlength cell (equilibrated at room temperature) in a wavelength range from 185 to 260 nm with 1-nm resolution at 20 nm/min scanning speed and 1-s response time.

Assessing isotype specificity of ScFv12 by ELISA

ELISA plates (Costar; Corning) were coated (coating buffer, 0.1 M $NaHCO_3$ (pH 9.6)) overnight at 4°C with 300 ng of human IgM, IgG1, IgG2, IgG3, and IgG4 (Sigma-Aldrich) and IgE purified from serum of an allergic individual by affinity chromatography (24). After washing with TBST (Tris-buffered saline (pH 7.5) with 0.05% (v/v) Tween 20) and blocking with TBST/1% (w/v) BSA for 2.5 h at 37°C, 100 μl of purified ScFv12, diluted in TBST/0.5% (w/v) BSA to a concentration of 0.2 μg/ml, was applied and incubated overnight at 4°C. After washing with PBST (PBS, 0.5% (v/v) Tween 20), HRP-conjugated monoclonal mouse IgG anti-E tag Ab (Amersham Biosciences), diluted 1/8000 in PBST/0.5% (w/v) BSA, was applied, and after incubation at 37°C for 1 h and at 4°C for 1 h, unbound detection Ab was removed by washing with PBST. Detection of bound ScFv12 was performed using 50 mM citric acid (pH 4), containing 0.2 mg/ml ABTS and 0.16% (v/v) H_2O_2, in a Dynatech MR7000 ELISA reader.

The same assay was performed using mAb12 (13 μg/ml) instead of ScFv12. For all steps TBST was used as washing buffer. Bound mAb12 was detected with alkaline phosphatase-conjugated rat anti-mouse IgG1 mAb (BD Pharmingen), diluted 1/1000 in TBST/0.5% (w/v) BSA, with 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich), 5 mg dissolved in 5 ml of 11.5% (v/v) diethanolamine (pH 9.8) containing 0.02% (w/v) sodium azide. All samples were measured in duplicates, and results show mean values with a variation of $<2\%$.

Detection of allergen-specific IgE with ScFv12

ELISA plates were coated overnight at 4°C with 300 ng/well purified recombinant allergens (rBet v 1, rBet v 2, rPhl p 1, rPhl p 5, and rDer p 2; Biomay). After washing and blocking with TBST/1% (w/v) BSA, sera from five patients with well-defined sensitization profiles and serum from a nonallergic individual, diluted 1/4 in TBST/0.5% (w/v) BSA, were applied and incubated overnight at 4°C. After washing with TBST, 100 μl of purified ScFv12 (0.2 μg/ml) diluted in TBST/0.5% (w/v) BSA was added and incubated overnight at 4°C. Bound ScFv12 was detected with HRP-conjugated anti-E tag Ab, as described above. The same assay was performed using mAb12 instead of ScFv12, as described above. All samples were measured in duplicates, and results represent means with a variation of $<15\%$.

In parallel, 1-μg aliquots of each allergen were dotted onto nitrocellulose membranes. The membranes were washed and blocked in buffer A (40 mM Na_2HPO_4, 0.6 mM NaH_2PO_4, 0.5% (v/v) Tween 20, 0.5% (w/v) BSA, 0.05% (w/v) NaN_3) for 50 min at room temperature and incubated overnight at 4°C with sera of the same individuals mentioned above, diluted 1/6 in buffer A. After washing, ^{125}I-labeled anti-human IgE (IBL International), diluted 1/10 in buffer A, was applied for 2 h at room temperature. The membranes were washed and air dried. Bound IgE was visualized by autoradiography.

Determination of the affinity and kinetics of the interaction between ScFv12 and IgE

The surface of a C1 sensor chip (GE Healthcare Biacore) was activated by injection of a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) at a flow rate of 5 μl/min for 7 min. As capturing Ab, monoclonal mouse IgG anti-E tag Ab (Amersham Biosciences), which had been diluted in 10 mM sodium acetate (pH 5) to a concentration of 15 μg/ml, was injected at 5 μl/min into flow cell 2. Then, the surface of the sensor chip was deactivated by injection of 1 M ethanolamine-HCl (pH 8.5) at a flow rate of 5 μl/min for 7 min. Using the same protocol, an isotype-matched Ab without specificity to IgE was immobilized in flow cell 1, which served as a reference cell. The final immobilization levels were 543 resonance unites (RU) (anti-E tag Ab, flow cell 2) and 411 RU (control Ab, flow cell 1), respectively.

The capturing level of ScFv12 required to obtain a maximal response of 100 RU was calculated to be 20 RU. ScFv12 (concentration of 64 nM,

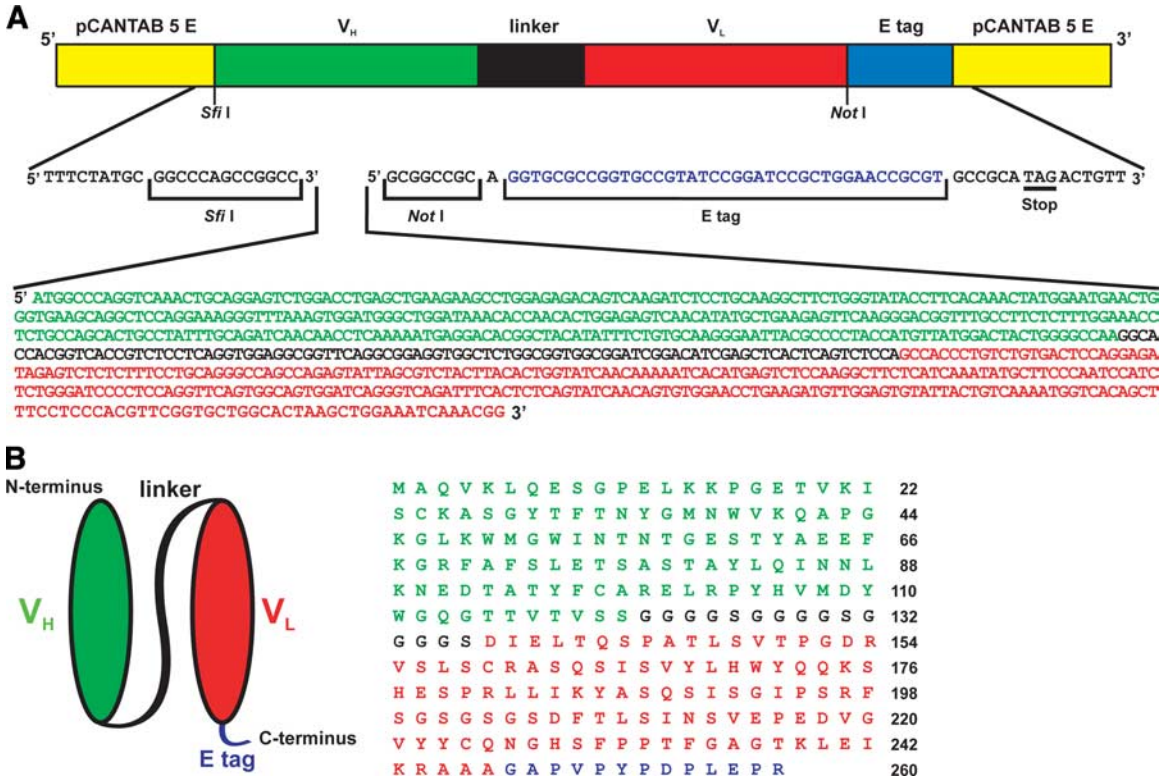


FIGURE 2. The ScFv12 DNA consisting of cDNAs coding for V_H, linker and V_L was ligated via the *Sfi*I and the *Not*I restriction sites into the expression vector pCANTAB 5 E. The sequence encoding the E tag, located downstream of the *Not*I restriction site of the vector, was attached to the 3′ end of the V_L cDNA (*A*). Deduced amino acid sequence and schematic structure of ScFv12 are shown (*B*). V_H is displayed in green, V_L in red, the linker peptide is shown in black and the E tag in blue, and portions belonging to the expression vector pCANTAB 5 E are shown in yellow.

diluted in HBS-EP (0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20 (pH 7.4)) was injected into flow cell 2 (5 μl/min) for 3 min and, after a stabilization time of 10 min with injection of running buffer, the final capturing level of 20 RU was obtained. Interaction of ScFv12 with chimeric Bip 1, a purified monoclonal human IgE specific to the major birch pollen allergen Bet v 1 (24), was assessed by injection of chimeric Bip 1, diluted in HBS-EP running buffer, at 2-fold increasing concentrations (starting concentration, 0.5 nM; top concentration, 64 nM) into the flow cells at a flow rate of 30 μl/min for 3 min. Dissociation of IgE was investigated by injection of HBS-EP at 30 μl/min for 30 min. Regeneration of the sensor chip surface was performed by injection of 10 mM glycine-HCl (pH 2.5) (20 μl/min, 30 s). Dissociation constant, on-rate, and off-rate were calculated with BIAevaluation 3.2 (GE Healthcare Biacore) using a 1:1 interaction model.

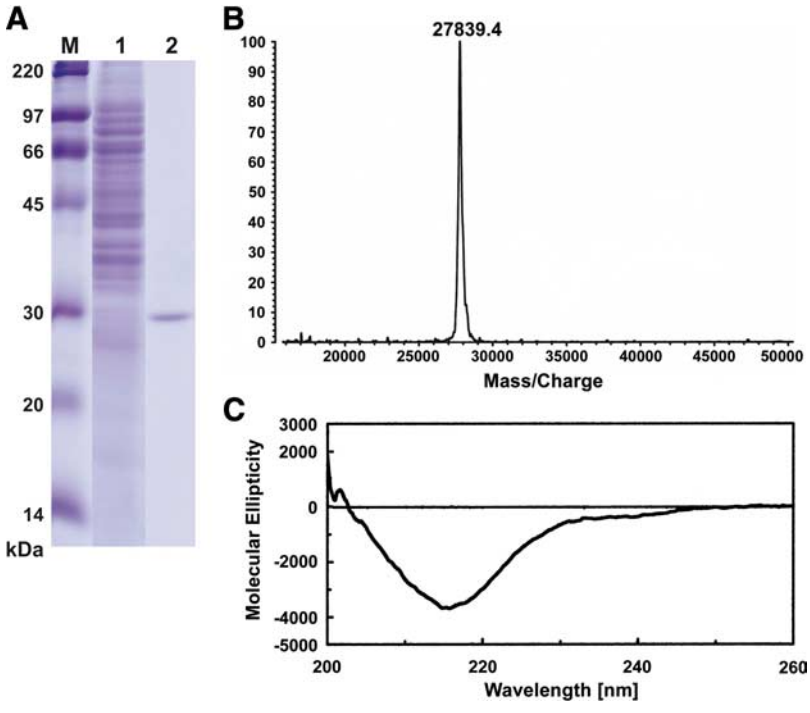


FIGURE 3. *A*, Coomassie blue-stained SDS PAGE containing a protein molecular mass marker (*lane M*), the supernatant of an *E. coli* HB2151 culture expressing ScFv12 (*lane 1*), and purified ScFv12 (*lane 2*). *B*, Purified ScFv12 was analyzed by mass spectrometry: The *x*-axis displays the mass/charge ratio, and the signal intensity is shown on the *y*-axis as the percentage of the most intense signal obtained in the investigated mass range. *C*, The circular dichroism spectrum of ScFv12 was recorded in a wavelength range from 185 to 260 nm (*x*-axis) and is expressed as mean residue ellipticity (*y*-axis).

On a second C1 sensor chip, FcεRI (α-HSA-α), which had been diluted in 10 mM sodium acetate (pH 5) to a concentration of 13 μg/ml, was immobilized in flow cell 2 using the same protocol as described above. The immobilization level was 118 RU. Flow cell 1 was activated and blocked without coupling of protein to serve as reference cell. Binding of ScFv12 to α-chain-bound IgE was investigated by injection of purified monoclonal IgE (concentration of 32 nM in HBS-EP, 5 μl/min, 3 min) into the flow cell containing immobilized α-chain and, following a stabilization time of 10 min, by subsequent injection of ScFv12 (concentration of 64 nM in HBS-EP, 20 μl/min, 3 min) or of an isotype control (concentration of 64 nM in HBS-EP, 20 μl/min, 3 min), respectively. To assess dissociation of α-chain-bound material (ScFv12, IgE), HBS-EP was injected at a flow rate of 20 μl/min for 30 min. The surface was regenerated by injection of 10 mM glycine-HCl (pH 2) (20 μl/min, 30 s).

Inhibition of the interaction between IgE and α-chain by ScFv12 was studied by preincubation of chimeric Bip 1 (concentration of 30 nM) with a 40-fold excess of ScFv12 (concentration of 1200 nM) or an isotype control (concentration of 1200 nM) at room temperature for 3 h and subsequent injection (20 μl/min, 3 min) into the flow cell containing immobilized α-chain.

All measurements were performed at 25°C.

Negative stain electron microscopy

Negative stain electron microscopy was performed as previously described (31). Briefly, purified soluble molecules were diluted to 3–8 μg/ml in borate saline buffer (BSB) (0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl (pH 8.3)) and affixed to carbon support membrane for preliminary analysis of appropriate concentrations (for viewing) and molecular homogeneity. The membrane was stained with 1% uranyl formate and mounted on 600-mesh copper grids for analysis. Electron micrographs were recorded at ×100,000 magnification at 100 kV on a JEOL JEM-1200 electron microscope. To generate molecular complexes, the reactants were mixed at 60–160 μg/ml in BSB for ~30 min and were diluted 20-fold in BSB just before attachment to carbon membranes. Following electron microscopy analysis, the ratios of the reactants were sometimes adjusted to maximize the ratio of complexes to unreacted molecules. For molecular complexes composed of IgE, α-HSA-α, ScFv12, and IgG anti-E tag, the IgE and α-HSA-α (in molar excess) were incubated first for 30 min followed by the addition of ScFv12 and anti-E tag and a second 30-min incubation. The reactants were then diluted 20-fold for application to the carbon membrane. The distinguishing molecular morphologies of the reactants were initially confirmed by the analysis of each component independently and then by creating and analyzing pairwise and subsequently three- and then four-component complexes.

Targeting of basophils by flow cytometric analysis

Basophils were obtained from peripheral blood of a healthy donor by enrichment of mononuclear cells using Ficoll. Mononuclear cells were preincubated with ScFv12 (10 μg/ml) at 4°C for 30 min, washed in PBS at 4°C, and then incubated with anti-E tag Ab (Amersham Biosciences) for 30 min. For control purposes, mononuclear cells were incubated with control buffer (PBS) instead of ScFv12, washed in PBS at 4°C, and then incubated with anti-IgE mAb E-124.2.8 (Dε2) (Immunotech) or with an isotype-matched IgG1 control Ab (BD Biosciences) under the same conditions as described above. The cells were washed and stained with a FITC-labeled goat anti-mouse IgG1 Ab (Caltag Laboratories). For detection of basophils, cells were stained with the PE-conjugated CD203c-specific mAb 97A6 (Immunotech) and with allophycocyanin-labeled CD123-specific mAb AC145 (Miltenyi Biotec). Cells were analyzed by multicolor flow cytometry on a FACSCalibur (BD Biosciences). Ab reactivity was expressed as mean fluorescence intensity (MFI).

Targeting of dendritic cells (DCs) and inhibition of IgE-binding to DCs

Normal oral mucosa from the vestibular region was obtained from patients undergoing intraoral surgery at the Department of Oral and Maxillofacial Surgery (University of Bonn Medical Center). Specimens were obtained after informed consent from patients was given according to the approval of the local ethics committee.

Crude epithelial cell suspensions were prepared by trypsinization in a 0.5% trypsin buffer without Ca^{2+} for 2 h at 37°C as described elsewhere (32). IgE was removed from the cell surface by using a pH 2.7 stripping buffer containing 10 mM lactic acid, 0.13 M NaCl, and 5 mM KCl. Cells were incubated in stripping buffer for 5 min at room temperature followed by washing steps. Afterward, cells were incubated in RPMI 1640 cell culture medium for 30 min before immunolabeling (32).

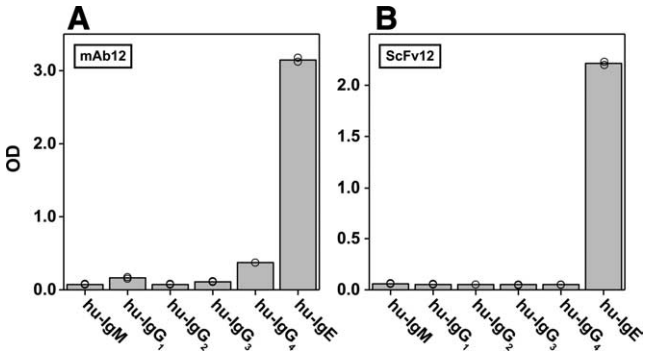


FIGURE 4. MAb12 and ScFv12 are specific to IgE Abs. Purified human IgM, IgG1–4, and IgE were coated to ELISA plates and probed with mAb12 (*A*) and ScFv12 (*B*), respectively. OD values correspond to the amount of bound mAb12 and ScFv12. Results of duplicate determinations are shown as circles; bars display mean values.

An indirect triple staining for 0.5–2 × 10^5 unfixed, vital cells was performed as described (32). Finally, the cells were washed twice and analyzed by flow cytometry.

Cells were analyzed on a FACSCanto (BD Biosciences) (32). For quantitative evaluation, dead cells were excluded by 7-aminoactinomycin D (Sigma-Aldrich) staining, and DCs were detected by a PE-labeled mAb specific to CD1a (T6RD1, IgG1; Beckman Coulter) whose expression in the periphery (i.e., outside primary lymphoid organs) is confined to DCs (33, 34). The high-affinity receptor for IgE, FcεRI, was detected either by mAb 22E7 (IgG1; generous gift of Dr. J. Kochan from Hoffmann-La Roche), which is directed against the α-chain of FcεRI but does not interfere with the binding site for IgE, or mAb 15A5 (IgG1; generous gift of Dr. J. Kochan), which interferes with the binding site for IgE (data not shown). Binding of IgE was detected by E124.2.8 (IgG1; Beckman Coulter), which reacts with the Dε2 constant region of human IgE. Acquired data were analyzed by FlowJo software (Tree Star).

Targeting of CD23-bearing cells and inhibition of binding of IgE to CD23 by ScFv12

Serum IgE from a birch pollen allergic patient was preincubated with ScFv12, anti-IgE mAb (BD Pharmingen; see Fig. 10*B*, anti-IgE #2), isotype controls, or FACS buffer (PBS containing 0.1% BSA and 0.1% NaN_3) for 1 h at room temperature. Loading of IgE on CD23-expressing EBV-B cells was performed as described (35). The cells were washed with FACS buffer and incubated with monoclonal mouse anti-IgE biotin (SouthernBiotech) for 30 min at 4°C. After washing, the cells were stained with streptavidin-PE (BD Biosciences) for 20 min at 4°C, washed, and analyzed in a FACSCanto flow cytometer (BD Biosciences). For the targeting experiments, cells were loaded with serum IgE (35), washed, and incubated with ScFv12, anti-IgE (SouthernBiotech; see Fig. 10*A*, anti-IgE #1), anti-E tag mAb (Amersham Biosciences), isotype controls, or FACS buffer for 1 h at 4°C. The washed cells were then incubated with goat polyclonal anti-mouse Ig FITC (BD Biosciences) for 30 min at 4°C for detection of anti-IgE (SouthernBiotech) and anti-E tag mAb (Amersham Biosciences) or, for detection of bound ScFv12, with anti-E tag mAb followed by polyclonal anti-mouse Ig FITC. Following a final wash, the cells were analyzed by FACS. Each experiment was performed in duplicates and 10,000 cells were analyzed for each sample.

Targeting of IgE-secreting cells by flow cytometric analysis and effects of ScFv12 on IgE secretion in an in vitro system

To assess the binding of ScFv12 to the IgE-secreting cell line U266 (36) (American Type Culture Collection), the cells were exposed to ScFv12 (5 μg/ml and 10 μg/ml). Bound ScFv12 was detected with mouse monoclonal anti-E tag Ab (10 μg/ml) (Amersham Biosciences) followed by detection of the anti-E tag Ab with a FITC-labeled goat anti-mouse antiserum (BD Biosciences). Washing steps followed each incubation step (1 h at 4°C). For control purposes each of the reagents was omitted once.

All cell preparations were stained with 50 μl of 7-aminoactinomycin D (25 μg/ml; Sigma-Aldrich) to determine viability. FACScanning (BD Biosciences) was done using 10,000 cells per condition. Data were analyzed using CellQuest software (BD Biosciences).

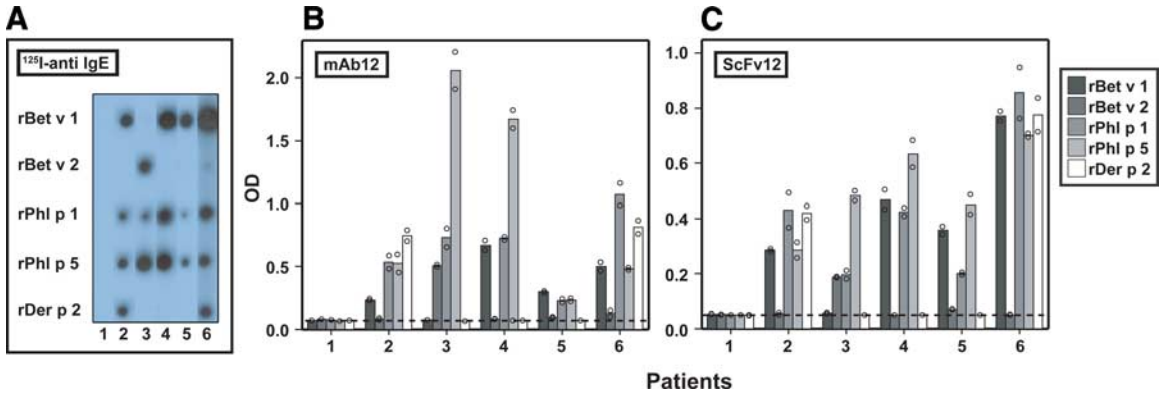


FIGURE 5. Allergen-bound IgE was detected with mAb12 and ScFv12. *A*, Recombinant allergens were dotted onto nitrocellulose membranes and incubated with sera from a nonallergic individual (patient 1) and from five allergic individuals (patients 2–6). IgE was detected using ^{125}I-labeled anti-human IgE Abs. *B* and *C*, The same panel of allergens was coated to ELISA plates and incubated with sera from the same individuals. IgE reactivities of the sera to the different allergens (OD values, *y*-axes) were detected using mAb12 (*B*) or ScFv12 (*C*). The cut-off levels for specific reactivities are indicated (dashed lines). Results of duplicate determinations are shown as circles; bars display mean values.

When ScFv12 was added to PBMC cultures from allergic patients (37, 38), no effects on allergen-specific IgE production was observed (data not shown).

In vivo half-life of ScFv12

To approximate the in vivo half-life of ScFv12, BALB/c mice ($n = 2$) (Charles River Laboratories) were administered each 40 μg of ScFv12 in 300 μl of sterile PBS i.p. Blood was taken before injection of ScFv12, 15 min, 45 min, 1 h 15 min, 2 h 15 min, and 3 h 15 min after injection.

ScFv12 concentrations in the sera were determined by quantitative ELISA. Three hundred nanograms of purified human IgE was coated to ELISA plates per well overnight (Nunc MaxiSorp). After blocking with BSA, a dilution series of purified ScFv12 with known concentrations, ranging from 0.5 to 32 ng/ml (six replicates per dilution), and the sera, diluted

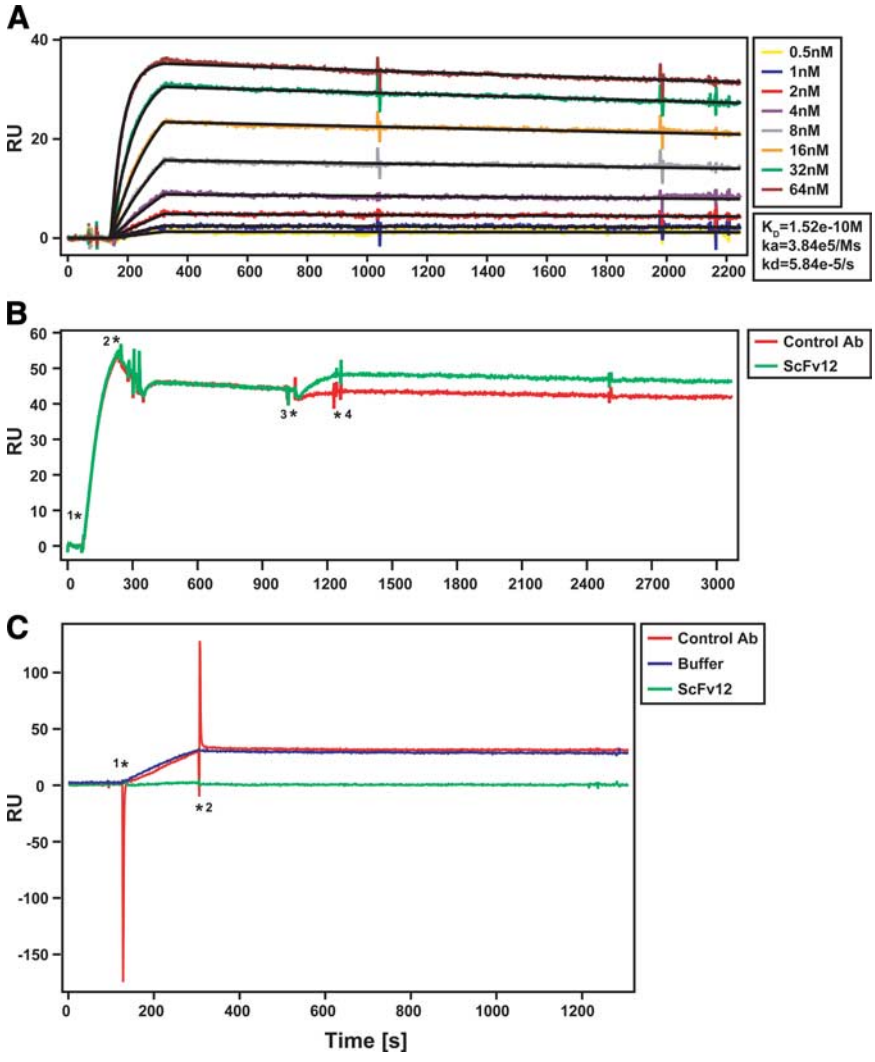


FIGURE 6. *A*, To assess affinity and kinetic constants of the interaction between human IgE and ScFv12, IgE was injected at 2-fold increasing concentrations from 0.5 to 64 nM (curves bottom to top) into the flow cell containing immobilized ScFv12. Recorded curves (colored) and calculated curves (black) were superimposed. *B*, Formation of a trimolecular complex consisting of chip-coupled FcεRI α-chain, IgE, and ScFv12. IgE was injected and captured by immobilized α-chain (asterisks 1 and 2), followed by a stabilization phase (asterisks 2 and 3). Thereafter, ScFv12 (green curve) or the control Ab (red curve) were injected and their association (asterisks 3 and 4) and potential dissociation (after asterisk 4) were monitored. *C*, Binding of IgE to FcεRI α-chain is inhibited by ScFv12. IgE was preincubated with ScFv12 (green curve), an isotype control (red curve), or running buffer (blue curve), respectively, and then exposed to chip-bound α-chain. Association (asterisks 1 and 2) and potential dissociation (after asterisk 2) were monitored; *x*-axes, time (seconds); *y*-axes, signal intensities (RU).

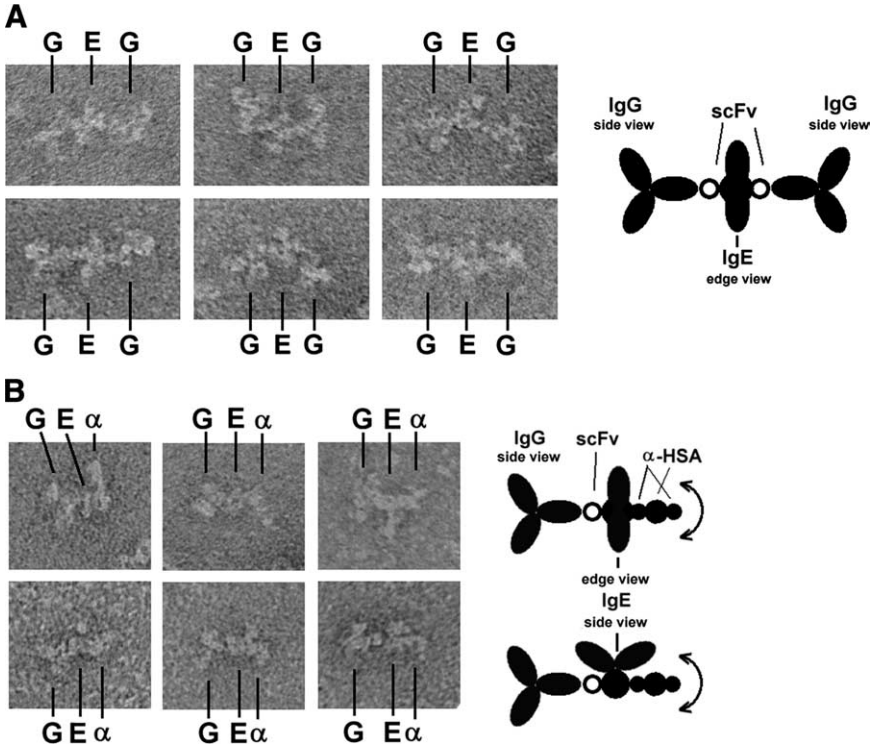


FIGURE 7. Complexes consisting of IgE/ScFv12/anti-E tag IgG and of α-HSA-α/IgE/ScFv12/anti-E tag IgG were directly visualized by negative stain electron microscopy. *A*, A panel of negative stain electron micrographs (*left*) and an interpretive diagram (*right*) showing complexes of IgE (E) with ScFv12 (scFv) and IgG anti-E tag (G). *B*, A panel of negative stain electron micrographs (*left*) and interpretive diagrams (*right*) showing complexes of IgE (E), α-HSA-α (α), ScFv12 (scFv), and IgG anti-E tag (G).

1/100 (three replicates per serum), were applied and incubated overnight. Bound ScFv12 was detected using monoclonal mouse IgG1 anti-E tag Ab (Amersham Biosciences), diluted to 1 μg/ml, and, after a washing step, using alkaline phosphatase-conjugated rat anti-mouse IgG1 mAb (BD Pharmingen). Sera, standards, and detection Abs were diluted in TBST. Concentrations of ScFv12 in the serum samples and the serum half-life of ScFv12 were calculated using Microsoft Excel.

Animal experiments were performed according to the local guidelines with permission from the Austrian Committee for Animal Welfare.

Evaluation of basophil activation by flow cytometry and histamine release assays

Peripheral blood was obtained from a healthy donor after informed consent was given. Blood was collected in heparinized tubes. Blood aliquots (100 μl) were incubated with serial dilutions of mAb12 (0.001–10 nM), ScFv12 (0.002–20 nM), with anti-IgE mAb E124.2.8 (Dε2) (10 nM), monoclonal anti-E tag Ab (1 μg/ml; Amersham Biosciences), or control buffer (PBS) for 15 min at 37°C, and then washed in PBS containing 20 mM EDTA. After this washing step, one set of cells that had been preincubated with a dilution series of ScFv12 (0.002–20 nM) additionally was incubated with monoclonal anti-E tag Ab (Amersham Biosciences) at 1 μg/ml for 5 min at 37°C to induce cross-linking of ScFv12 attached to receptor-bound IgE. Thereafter, cells were incubated with 10 μl of PE-conjugated CD203c mAb 97A6 for 15 min at room temperature. After erythrocyte lysis using FACS lysing solution (BD Biosciences), cells were washed, resuspended in PBS, and analyzed by two-color flow cytometry on a FACScan (BD Biosciences) using FlowJo Software (Tree Star). Up-regulation of CD203c was calculated from MFIs obtained with stimulated (MFI_{stim}) and unstimulated ($MFI_{control}$) cells, and is expressed as stimulation index (MFI_{stim}: $MFI_{control}$) (39). The PE-conjugated mAb 97A6 (CD203c) and anti-IgE mAb E-124.2.8 were purchased from Immunotech.

Histamine release experiments were performed as described (40).

Skin-prick test experiments

A nonallergic individual was skin prick tested with 20-μl aliquots of ScFv12 and mAb12, respectively, and for control purposes with PBS and histamine on the forearm. The test solutions comprised purified ScFv12 and mAb12 in 2-fold increasing concentrations (ScFv12, 0.04, 0.07, 0.14, 0.29, 0.57, 1.14, 2.29 μM; mAb12, 0.02, 0.04, 0.07, 0.14, 0.29, 0.57, 1.14 μM). Concentrations were chosen to reflect a molar ratio of ScFv12/mAb12 of 2:1. Results were recorded 15 min after testing by transferring the ballpoint pen-marked wheal area with a tape to paper and by photodocumentation. The mean diameters of the wheals were calculated by $(D + d)/2$, where D indicates the largest diameter and d indicates the smallest diameter of the wheal.

Results

Isolation and characterization of the cDNAs encoding the variable domains of the H and the L chain of mAb12

To avoid PCR errors, the cDNAs of V_H and V_L of mAb12 were isolated from a cDNA library constructed from the hybridoma line by oligonucleotide screening. cDNA and deduced amino acid sequences of V_H (357 nt, 119 aa, calculated molecular mass (average mass) of 13,357 Da) and V_L (324 nt, 108 aa, calculated molecular mass (average mass) of 11,729.1 Da) were determined (Fig. 1). The sequences of V_H and V_L of mAb12 have been deposited in GenBank (www.ncbi.nlm.nih.gov/Genbank/) under the following

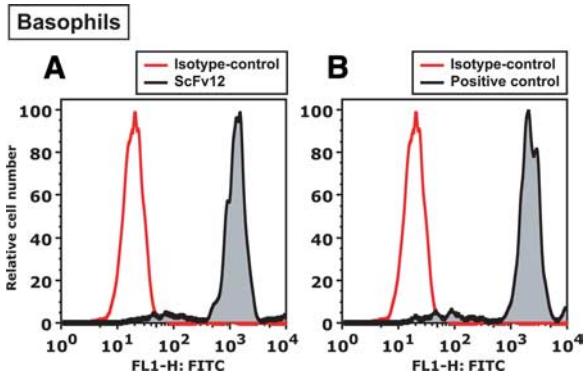


FIGURE 8. Human basophils were targeted with ScFv12. PBMCs were isolated and gated on basophils with anti-CD123 and anti-CD203c Abs. Basophils were detected with ScFv12 (*A*, black curve) and, for control purposes, with anti-IgE mAb E-124.2.8 (*B*, black curve). The background signal was assessed by incubation with an isotype-matched control Ab without reactivity to human IgE (*A* and *B*, red curves); *x*-axes, signal intensities; *y*-axes, relative cell numbers.

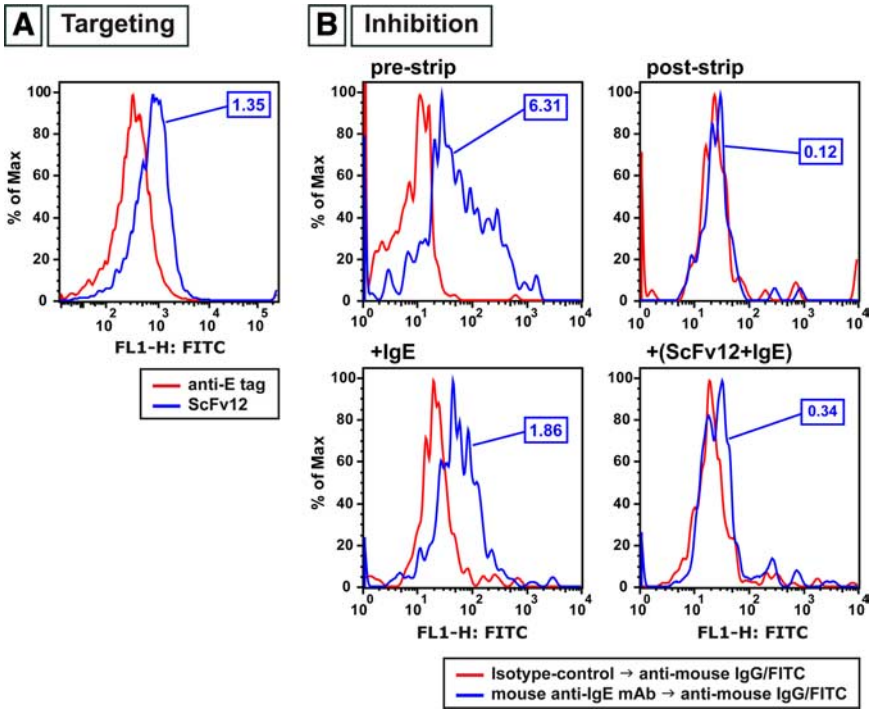


FIGURE 9. *A*, IgE-bearing DCs were specifically targeted with ScFv12. Human mucosal DCs were incubated with ScFv12 followed by incubation with monoclonal anti-E tag Ab (blue curve) or with monoclonal anti-E tag Ab alone (red curve). Detection was performed with FITC-labeled goat anti-mouse IgG. *B*, IgE binding to DCs was inhibited by ScFv12. IgE bound to the surface of DCs was detected before stripping the cells (pre-strip), after stripping the cells (poststrip), after incubation of the stripped cells with IgE (+IgE), and after incubation of the stripped cells with IgE that had been preincubated with ScFv12 (+(ScFv12+IgE)). Cells were stained with a mouse anti-IgE mAb (blue curve) or with an isotype control (red curve) followed by FITC-conjugated anti-mouse IgG; *x*-axes, signal intensities; *y*-axes, relative cell numbers.

accession numbers: EU048270 (mAb12 V_H-Cγ_1) and EU048271 (mAb12 V_L-Cκ).

Mouse mAb12 belongs to the IgG1 isotype; the L chain is a κ-chain. Alignment of the sequences encoding the variable domains of the H and the L chain of mAb12 with the closest related germline sequences of the IMGT database showed an unexpectedly low rate of somatic mutations (<1%) in the V regions of the H and L chain excluding D and J regions (V_H, 99.65% identity; V_L, 99.64% identity).

Construction, expression, and characterization of a recombinant ScFv derived from mAb12

The cDNAs of V_H and V_L were assembled using a linker of 45-bp length by PCR and the resulting ScFv12 DNA was ligated into the *Sfi*I/*Not*I site of the expression vector pCANTAB 5 E upstream of a sequence encoding a 13-aa peptide (E tag) (Fig. 2). Recombinant ScFv12 could be purified from supernatants of overnight cultures to homogeneity as a soluble protein by affinity chromatography using an anti-E tag Ab (Fig. 3*A*). Approximately 1.1 mg of ScFv12 was obtained from 1 liter of cultured *E. coli*.

The molecular mass of ScFv12, as determined by mass spectrometry (Fig. 3*B*), is 27,839.4 Da, corresponding to the molecular mass of 27,842.99 Da (average mass), which was calculated according to the deduced amino acid sequence (PeptideMass, ExPASy Proteomics Tools, Swiss Institute of Bioinformatics, Geneva, Switzerland). Analysis of the secondary structure of the purified protein by circular dichroism spectroscopy (Fig. 3*C*) showed that ScFv12 represents a folded protein that mainly consists of β-sheets. The obtained pattern corresponds to the typical secondary structure of functional ScFvs (41).

Recombinant ScFv12 specifically reacts with human IgE and detects allergen-bound IgE

As determined by ELISA, ScFv12 as well as mAb12 strongly bind to human IgE and lack any relevant reactivity with purified human IgM and IgG subclasses (Fig. 4).

Sera from five allergic patients with known IgE reactivity profiles to five different recombinant allergens (birch pollen, rBet v 1, rBet v 2; timothy grass pollen, rPhl p 1, rPhl p 5; house dust mite, rDer p 2) were tested for IgE reactivity with the very same allergens using ScFv12 and mAb12 as detection reagents by ELISA

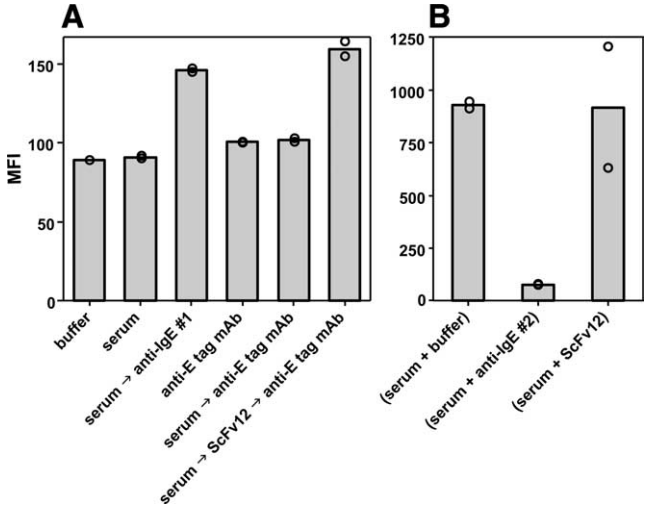


FIGURE 10. *A*, CD23-bound IgE on human B cells was targeted with ScFv12. IgE from an allergic patient's serum was bound to CD23 on EBV-infected B cells. IgE-bearing cells were then incubated with a monoclonal anti-IgE Ab (serum → anti-IgE #1), monoclonal anti-E tag Ab (serum → anti-E tag mAb), or with ScFv12 followed by incubation with anti-E tag mAb (serum → ScFv12 → anti-E tag mAb). Detection of bound anti-IgE and anti-E tag mAb was performed with a FITC-labeled detection Ab. A possible interaction of anti-E tag mAb with the cells was assessed by incubation of the cells devoid of IgE with anti-E tag mAb (anti-E tag mAb); the background of the FITC-labeled detection Ab was determined by incubation with IgE-lacking (buffer) or IgE-bearing cells (serum). *B*, Serum from an allergic patient was preincubated with ScFv12 (serum+ScFv12), a monoclonal anti-IgE Ab (serum+anti-IgE #2), or FACS buffer (serum+buffer) before addition to the B cells. Bound IgE was detected using a biotinylated monoclonal anti-IgE Ab and stained with streptavidin-PE. The MFIs of duplicates (○) and their mean values (bars) are shown.

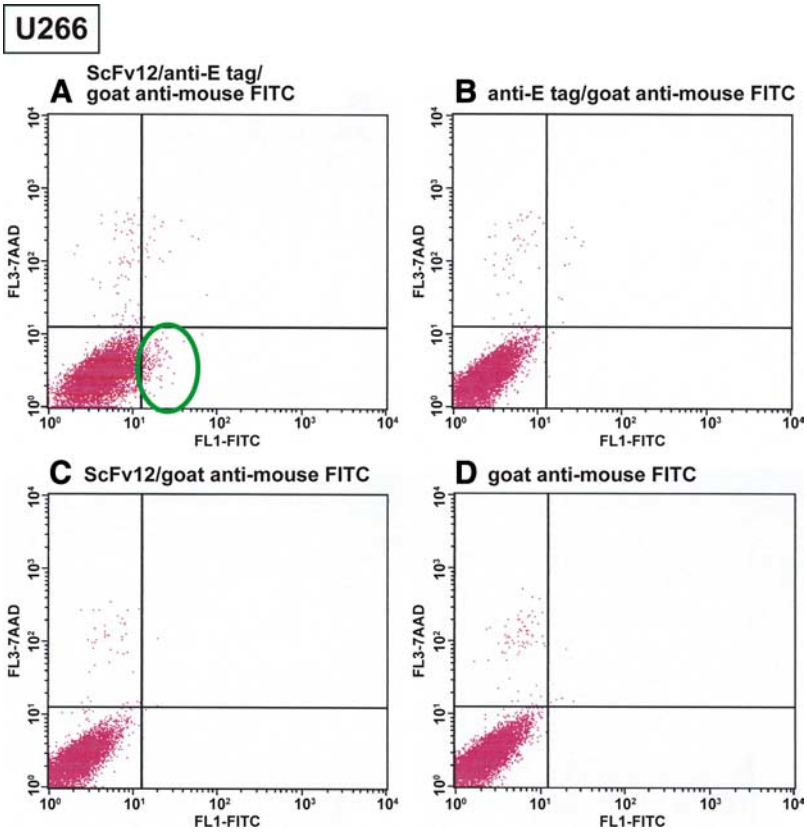


FIGURE 11. IgE-secreting plasma cells were specifically targeted using ScFv12. *A*, Scatter plot of U266 cells exposed to ScFv12, anti-E tag, and FITC-labeled goat anti-mouse Ab. Positive cells are encircled. Control stains of U266 without ScFv12 (*B*), without anti-E tag (*C*), and without ScFv12 and anti-E tag (*D*) are shown; *x*-axes, signal intensities (FITC); *y*-axes: signal intensities (7-aminoactinomycin D).

(Fig. 5, *B* and *C*). The IgE reactivity profiles with the purified allergens determined with ScFv12 as well as with mAb12 were in accordance with those obtained with established IgE detection systems (Fig. 5*A*).

ScFv12 binds with high affinity to isolated and FcεRI-bound human IgE

Interaction of ScFv12 with IgE and FcεRI α-chain-bound IgE was assessed in SPR-based assays. ScFv12 binds to IgE with high affinity ($K_D = 1.52 \times 10^{-10}$ M), exhibiting a high association rate constant ($K_a = 3.84 \times 10^5$/Ms) and slow dissociation ($K_d = 5.84 \times 10^{-5}$/s) (Fig. 6*A*). Notably, affinity and kinetics of the binding of ScFv12 to human IgE are in the same range as those measured with the same technology for the interaction between IgE and FcεRI α-chain and between IgE and complete mAb12 (42), respectively. Next, we studied whether ScFv12 can bind to α-chain-bound IgE and prevent binding of IgE to the α-chain. Fig. 6*B* shows that ScFv12 reacts with α-chain-bound IgE and shows a very slow dissociation rate. Preincubation of IgE with ScFv12 completely prevented IgE from binding to the α-chain (Fig. 6*C*).

Visualization of the binding of ScFv12 to IgE and to complexes consisting of IgE and FcεRI α-chain by negative stain electron microscopy

Each of the reactants (IgE, α-HSA-α, ScFv12, and IgG anti-E tag) proved homogeneous and displayed a morphology constant with their known structural properties (general shape and dimensions, data not shown). α-HSA-α was observed to cross-link two IgE molecules, but it did not form higher order complexes, consistent with the ability of IgE Fc to react with only a single FcεRI α-chain (data not shown). In contrast, two ScFvs could bind to a single IgE, one on either side of the Fc region. Although the small ScFv molecules were difficult to see when bound to IgE, their presence was indicated by the subsequent reaction of IgG anti-E tag mAbs to either side of the IgE molecules (Fig. 7*A*). Note that the IgE molecules in the complexes were typically oriented so as to provide an "on edge" view rather than the more typical side (broad profile) view observed for Ig molecules when attached to carbon membranes. We previously reported a similar IgE on edge configuration when mAb12 $F(ab')_2$ was complexed with IgE (24).

To verify the stoichiometry of α-HSA-α constructs with the Fc of IgE, the molecules were reacted at varying molar ratios. The largest complexes formed had configurations consistent with one α-HSA-α in complex with two IgE molecules (data not shown), as would be expected for IgE molecules with a functional valency of one for binding Fc to the FcεRI α-chain. To visualize the reaction of IgE molecules in complex with both the FcεRI α-chain and ScFv12, we first reacted IgE with α-HSA-α in molar excess and,

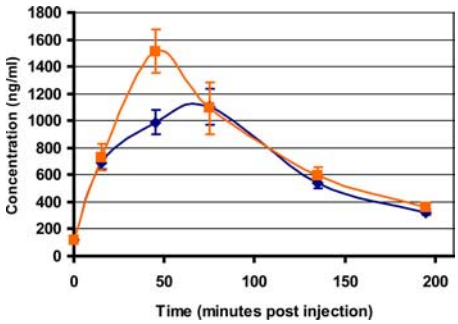
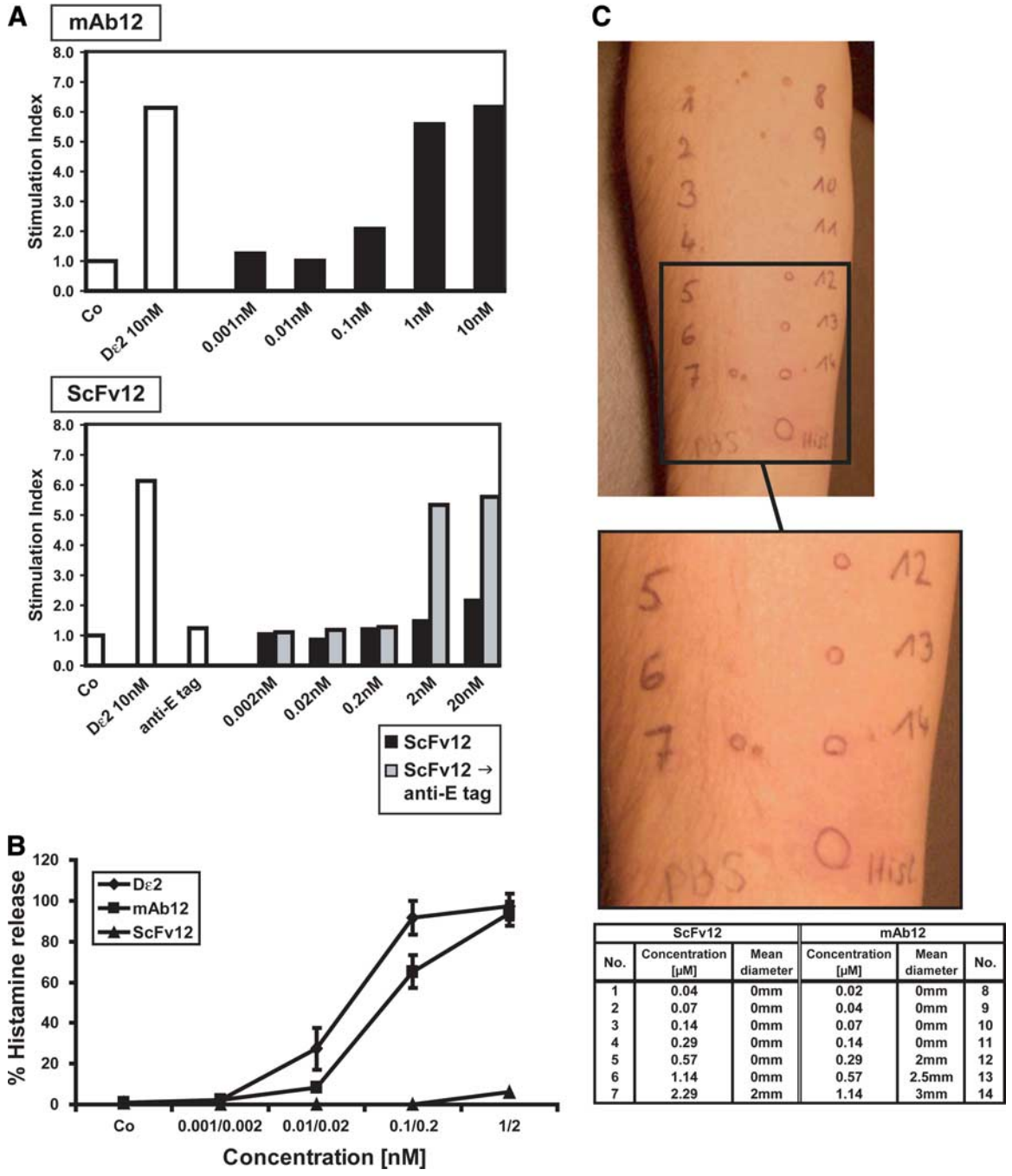


FIGURE 12. Serum concentrations of ScFv12 (*y*-axes, ng/ml) after i.p. injection into BALB/c mice as determined by quantitative ELISA are shown over time (*x*-axes, minutes after injection of ScFv12). Mean values ± SD of triplicate OD determinations are shown for each time point of blood sampling for two animals.



ScFv12			mAb12		
No.	Concentration [μM]	Mean diameter	Concentration [μM]	Mean diameter	No.
1	0.04	0mm	0.02	0mm	8
2	0.07	0mm	0.04	0mm	9
3	0.14	0mm	0.07	0mm	10
4	0.29	0mm	0.14	0mm	11
5	0.57	0mm	0.29	2mm	12
6	1.14	0mm	0.57	2.5mm	13
7	2.29	2mm	1.14	3mm	14

FIGURE 13. Effects of ScFv12 compared with mAb12 on basophils and mast cells are shown. *A*, Basophils were incubated with increasing concentrations (nM) of mAb12 (*upper panel*) and ScFv12 (*lower panel*, black bars) and with anti-human IgE mAb E-124.2.8 (Dε2) and buffer alone (Co) (*x*-axes). One set of cells that had been incubated with ScFv12 was super cross-linked with monoclonal anti-E tag Ab (*lower panel*, gray bars). Activation of basophils was measured by detection of the activation marker CD203c by flow cytometry. The degree of stimulation is expressed as stimulation index (*y*-axes). *B*, Human basophils were incubated with increasing concentrations of anti-IgE (Dε2), mAb12, or ScFv12. The percentage of histamine release is shown on the *y*-axis; for the *x*-axis, concentrations of Dε2 and mAb12 (0.001–1 nM) and of ScFv12 (0.002–2 nM) are shown. *C*, A nonallergic individual was skin prick tested on the forearm with 20-μl aliquots containing equimolar concentrations of IgE-binding domains of ScFv12 and mAb12, respectively, and, for control purposes, with histamine and PBS. The test solutions comprised purified ScFv12, diluted in PBS to concentrations of 0.04, 0.07, 0.14, 0.29, 0.57, 1.14, and 2.29 μM (1–7), and mAb12 at concentrations of 0.02, 0.04, 0.07, 0.14, 0.29, 0.57, and 1.14 μM (8–14). The mean diameters of the wheals are displayed in the table.

following an incubation period, with ScFv12 and IgG anti-E tag. The sequential addition and incubation scheme allowed α-chain binding before the excess of ScFv12 molecules would have had a chance to saturate both reactive sites on IgE Fc. Examples of the resultant complexes are shown in Fig. 7*B* along with interpretive diagrams.

ScFv12 targets IgE-bearing and -secreting cells

In flow cytometry studies, basophils could be strongly and specifically labeled using ScFv12 (Fig. 8). PBMCs were prepared from whole blood of a nonatopic individual, incubated with ScFv12 and a FITC-labeled detection Ab, giving rise to a distinct and intense

signal of a cell population that could be identified as basophils by gating with the basophil markers CD123 and CD203c (Fig. 8*A*). An identical result was obtained with another anti-IgE Ab that reacts with basophil-bound IgE but does not inhibit IgE binding to FcεRI (Fig. 8*B*). The specificity of the association of ScFv12 with this particular population was assured by backgating to the whole PBMC preparation displayed in the forward scatter and side scatter plots (data not shown).

Likewise, DCs that had been isolated from specimens of the oral mucosa could be specifically targeted with ScFv12 (Fig. 9*A*). Additionally, it was demonstrated that preincubation of IgE with ScFv12 blocked binding of IgE to FcεRI on the surface of DCs (Fig. 9*B*).

To determine whether ScFv12 also targets CD23-bound IgE, EBV-infected human B cells were loaded with IgE from an allergic individual (35) and incubated with ScFv12. ScFv12 stained IgE bound to the low-affinity receptor (Fig. 10*A*) similarly as did an anti-IgE Ab with known specificity to CD23-bound IgE (Fig. 10*A*, anti-IgE #1). No binding was observed in the control experiments when ScFv12 was omitted. Unlike the interaction between IgE and FcεRI, association of IgE with CD23 could not be blocked by preincubation of IgE with ScFv12 (Fig. 10*B*).

Finally, we studied whether ScFv12 also labels IgE-secreting plasma cells using the IgE-producing human cell line U266. Despite the low expression of IgE on the surface of the plasma cell line, flow cytometric analysis showed that 3.29% of the gated cells were specifically stained (Fig. 11*A*). In the control experiments omitting ScFv12 (Fig. 11*B*), the anti-E tag Ab (Fig. 11*C*), or ScFv12 and anti-E tag Ab (Fig. 11*D*), no labeling of cells was detected.

In vivo half-life of ScFv12

To estimate the increase and the elimination of ScFv12 from blood after in vivo application, 40 μg of ScFv12 was administered i.p. into BALB/c mice ($n = 2$). Blood sampling was done 15 min, 45 min, and 1 h 15 min after injection and thereafter at hourly intervals.

Peak values of serum concentrations of ScFv12 were detected 45 min (Fig. 12, orange curve) or 75 min (Fig. 12, blue curve) after injection of ScFv12, respectively. In the declining phase of the curve, serum half-life was calculated to be 70 min.

ScFv12 lacks allergenic activity as demonstrated by in vitro basophil activation assays and in vivo skin test experiments

PBMCs obtained from a nonallergic individual were incubated with corresponding dilutions of mAb12 and ScFv12 containing equimolar, 10-fold increasing concentrations of IgE-binding paratopes. In an additional set of experiments, cross-linking of IgE/FcεRI complexes targeted by ScFv12 was induced by addition of monoclonal anti-E tag Ab. Activation of the basophils was determined by measuring up-regulation of CD203c (Fig. 13*A*). ScFv12 alone induced almost no activation of basophils (Fig. 13*A*, *lower panel*, black bars), whereas after addition of anti-E tag Ab, the cells that had been preincubated with ScFv12 showed activation (Fig. 13*A*, *lower panel*, gray bars). The experiments were repeated twice with cells from the same donor, yielding almost identical results (data not shown). Complete mAb12 induced activation of basophils similarly as mAb Dε2 (Fig. 13*A*, *upper panel*).

Results from the CD203c activation experiments were confirmed by histamine release experiments (Fig. 13*B*). ScFv12 did not induce any relevant histamine release in basophils, whereas monoclonal anti-IgE Dε2 and complete mAb12 induced dose-dependent histamine release (Fig. 13*B*).

The in vivo allergenic activity of ScFv12 was compared with that of mAb12 by skin prick testing (Fig. 13*C*). Again, dilutions of mAb12 and ScFv12 were prepared to contain the same molar concentrations of IgE-binding paratopes. ScFv12 induced a weak skin reaction (i.e., 2 mm wheal diameter) only at the highest concentration tested (2.29 μM, corresponding to 64 μg/ml), whereas mAb12 started to induce a comparable immediate type skin reaction at a concentration of 0.29 μM (i.e., 43.2 μg/ml). The diluent (PBS) did not induce any skin reaction; histamine (concentration of 1.7 mg/ml) elicited a wheal reaction of 6-mm mean diameter.

Discussion

In the present study we describe the generation and characterization of a recombinant ScFv that may be useful for the therapeutic targeting of IgE Abs, IgE-bearing and IgE-secreting cells in allergic patients. The recombinant ScFv12 was obtained from a monoclonal anti-human IgE Ab that exhibits high affinity and specificity to IgE, prevents IgE-binding to FcεRI (24, 42), and, unlike other anti-human IgE Abs such as the therapeutic anti-IgE Ab omalizumab (43), is able to recognize cell-bound IgE.

To obtain the authentic DNA sequences of V_H and V_L of the mAb and to avoid potential PCR errors, we constructed a cDNA library from the hybridoma cell line and isolated the V_H and V_L cDNAs by direct oligonucleotide screening. Interestingly, the V_H and V_L sequences show <1% somatic mutations when compared with the corresponding germline sequences considering that the mAb exhibits an extremely high affinity for IgE and was obtained by an active immunization protocol.

To eliminate the basophil/mast cell triggering activity of the mAb resulting from bivalent recognition and consequent cross-linking of receptor-bound IgE, V_H and V_L of mAb12 were expressed as monovalent ScFv in *E. coli*. The recombinant ScFv could be purified to homogeneity as a folded, 28-kDa protein. ScFv12 shows high specificity to human IgE and does not react with other Ab classes or subclasses. Accordingly, the ScFv could be used for the specific detection of allergen-specific IgE Abs in fluid phase and hence may be used for the in vitro diagnosis of allergy by tracing allergen-specific IgE in serum samples or other body fluids or tissue specimens. Through the use of SPR technology, we could show that the recombinant ScFv exhibits fast association and stable binding to isolated as well as to FcεRI-bound human IgE with an extremely high affinity that is in the range of the affinity of the complete Ab as well as of that between IgE and FcεRI. It could also be shown that preincubation of IgE with ScFv12 efficiently prevents the binding of IgE to the α-chain of FcεRI, indicating that the ScFv may be used for therapeutic purposes in vivo to prevent the binding of newly synthesized IgE to FcεRI-bearing cells. However, in contrast to the therapeutic anti-IgE Ab omalizumab, which is already in clinical use for the treatment of allergies, ScFv12 offers an additional therapeutic option. Unlike omalizumab, ScFv12 can also efficiently target IgE that is bound to FcεRI and to FcεRII (CD23) on the surface of different cell types, such as basophils, DCs, or B cells and to membrane-standing IgE on IgE-secreting plasma cells. This particular property could be studied at the molecular level by means of negative stain electron microscopy, a technology that allows the direct visualization of the interaction of single molecules. It could be demonstrated that ScFv12, similarly as mAb12 (24), binds on either side of the IgE molecule close to the Cε2–3 domains that are involved in the interaction between IgE and FcεRI. When binding to FcεRI, only one of the two Cε2 domains of IgE is engaged, leaving the second Cε2 domain accessible for ScFv12 binding. Therefore, ScFv12 reacts with receptor-bound IgE and blocks IgE binding to FcεRI after preincubation of IgE with ScFv12 before

exposure to FcεRI. We further succeeded in forming and visualizing trimolecular complexes consisting of the FcεRI α-chain, human IgE, and ScFv12. This observation demonstrates that only one molecule of ScFv12 can bind to IgE once IgE is bound to FcεRI. Until now, only the structures of IgE fragments bound to the high-affinity receptor have been resolved (11, 44). Attempts to co-crystallize a complete IgE Ab with the α-chain have thus far been unsuccessful. Therefore, these images provide for the first time a direct view of a complex consisting of the high-affinity receptor and a complete IgE Ab molecule.

The results obtained with the purified molecules could be confirmed in cell-based experiments. Human basophils and DCs that are bearing FcεRI-bound IgE on the surface could be strongly and specifically labeled with ScFv12. Likewise, using ScFv12 we successfully targeted B cells via CD23-bound IgE and even IgE-secreting plasma cells, which are considered the most important producers of IgE (reviewed in Ref. 45), despite only low-level expression of membrane-standing IgE.

A crucial prerequisite for a potential in vivo use of ScFv12 is that the ScFv does not cross-link effector cell-bound IgE Abs and does not induce degranulation of basophils or mast cells. In fact, the lack of basophil/mast cell triggering activity of ScFv12 could be demonstrated by in vitro and in vivo experiments. Dilutions of ScFv12 and complete bivalent mAb12 containing equimolar concentrations of IgE-binding paratopes were exposed to basophils, and cell activation was measured via the up-regulation of CD203c and the release of histamine. In both in vitro assays the strongly reduced allergenic activity of ScFv12, compared with mAb12, could be demonstrated. That ScFv12 had indeed reacted with cell-bound IgE could be shown by super cross-linking with an anti-E tag Ab (Fig. 13*A*, *lower panel*, gray bars). These results were confirmed in vivo by skin testing in a human volunteer.

The various experimental results suggest several applications of ScFv12. ScFv12 may be used for diagnostic purposes to detect allergen-specific and total IgE in serum, body fluids, or tissues. When prepared as an immunoadsorber, ScFv12 may be used in a therapeutic approach for the extracorporeal depletion of IgE and/or IgE-bearing cells from the circulation of allergic patients.

ScFv12 may also be used for injection into allergic patients similar to omalizumab to complex IgE Abs and to prevent them from binding to effector cells. Such an approach would not only remove circulating IgE but also reduce the expression of IgE receptors. Due to the fact that ScFv12 also reacts with IgE-bearing and IgE-secreting cells, it may be conjugated with toxins, radioactive isotopes, or apoptosis-inducing agents and then be used as a compound to target and eliminate IgE-bearing and IgE-secreting cells. In fact, ScFv molecules are widely used as specific components of immunotoxins, as they combine the high specificity and affinity of an Ab with low molecular mass and therefore exhibit better tissue penetration than native Abs (46–48). Consistent with previous data, we found that ScFv12 has a relatively short in vivo half-life. Furthermore, ScFv12 molecules do not activate the complement system or other immune cells. A chimeric protein consisting of the toxic component of *Pseudomonas* exotoxin and the receptor binding portion of IgE (Cε2 and Cε3) already has been studied in a murine system in vitro (49) and in vivo (50). The toxin was selectively delivered to cells bearing the high-affinity IgE receptor and induced apoptosis without triggering the release of histamine (51). ScFv12 could be used as an alternative targeting vehicle delivering *Pseudomonas* exotoxin to all cells bearing IgE on their surface, that is, not only to the effector cells of anaphylaxis but also to IgE-producing cells. In this context it may be worth considering the targeting of IgE memory cells, which have been demonstrated to reside in the nasal and respiratory mucosa and that seem to mediate the disease aggravating increases of allergen-specific serum IgE levels in allergen-exposed patients (21). The precise nature of IgE-secreting cells in allergic patients has not been definitely elucidated, but available data indicate that plasma cells could be the prominent IgE-secreting cells (45). As no human B cell line-secreting allergen-specific IgE has been available to us, we demonstrated targeting of IgE-secreting cells by ScFv12 using the IgE-secreting plasma cell line U266 (36). Since ScFv12 could even trace plasma cells containing few membrane-bound IgE Abs, we would expect that local application of ScFv12 might block the repeated boosting of IgE production originating from IgE memory cells. However, in the case of in vivo applications the development of an immune response to administered ScFv12 is to be taken into account. To avoid the induction of an anti-ScFv12 response, we consider only few local applications per patient with the goal of eliminating IgE-producing cells. As has been demonstrated in studies using ScFv-based immunotoxins (52, 53), ScFvs are poorly immunogenic molecules. In most cases of an antiimmunotoxin immune response, patients' Abs were directed against the toxic part of the immunotoxin, even if the mouse-derived ScFv part had not been humanized. Therefore, the concern that clinically relevant anti-ScFv Abs are induced should be mitigated. Furthermore, immunogenicity of ScFv12 could be further reduced by humanization (54) or modifications, for example, by addition of polyethylene glycol. It should also be considered that a clinically relevant production of anti-ScFv Abs can be easily and quickly monitored before a consecutive application of ScFv12 by skin prick testing of patients. The short half-life of ScFvs (Fig. 12 and Ref. 47) and the availability of drugs that antagonize the effects triggered by cross-linking of cell-bound ScFv12 by anti-ScFv Abs (i.e., antihistamines, corticosteroids, and other drugs) provide a possibility to control potential adverse effects.

In conclusion, we have developed a molecule that, due to its high affinity and specificity to free, receptor-bound, and the membrane-standing forms of IgE, may be used as a tool to elucidate the role of IgE in health and disease and to develop new therapeutic agents for the therapy of IgE-mediated diseases.

Disclosures

The authors have no financial conflicts of interest.

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