

Generation of Human Monoclonal Allergen-Specific IgE and IgG Antibodies from Synthetic Antibody Libraries

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Background: Allergen-specific IgE and IgG antibodies play pivotal roles in the induction and progression of allergic hypersensitivity reactions. Consequently, monoclonal human IgE and IgG4 antibodies with defined specificity for allergens should be useful in allergy research and diagnostic tests. We used combinatorial antibody libraries and subsequent recombinant production to make and assess IgE, IgG1, and IgG4 allergen-specific antibodies.

Methods: We used phage display to select a synthetic single-chain antibody fragment (scFv) library against 3 different allergens, from bee venom, bovine milk, and apple. The scFv obtained were converted into IgG1, IgG4, and IgE antibody formats and assessed for their biochemical properties by ELISA, immunoblotting, and fluorescence-activated cell sorting.

Results: Two different antibody formats for each IgG1, IgG4, and IgE antibody were produced in mammalian cells as disulfide-linked and glycosylated Ig, which were usable in allergen-specific ELISA assays and immunoblots. In addition, the recombinant IgE antibodies mediated the binding of allergens to HEK-293 cells transfected with the high-affinity IgE receptor, and this binding was blocked by corresponding IgG antibodies.

Conclusions: The use of synthetic libraries for the generation of allergen-specific recombinant IgE and IgG

antibodies should have broad applications in allergological research and diagnosis.

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It is well established that the development of several immune-mediated diseases is linked to circulating concentrations of IgE, the antibody class responsible for allergic hypersensitivity (1). IgE antibodies bound to their high affinity receptor (FcεRI)³ on mast cells and basophils mediate receptor cross-linking by allergens and trigger degranulation and release of proinflammatory mediators responsible for immediate-type hypersensitivity reactions. Long-term exposure to higher concentrations of allergens or therapeutic intervention by specific immunotherapy (SIT) results in a T-helper cell type-1 shift in the immune response, leading to an increase in production of allergen-specific IgG antibodies, particularly of the IgG4 subclass. These IgG antibodies are thought to exert their function by blocking the IgE allergen interaction (2–4), by recruitment of Fcγ receptors that inhibit FcεR mediated activation (5, 6), or by inhibition of IgE-facilitated allergen presentation to T cells (7).

The in vitro measurement of allergen-specific IgE and IgG in serum that are involved in induction and progression of disease has become a major diagnostic and prognostic goal to describe the immune status of an allergic patient and to assess the success of SIT. However, reliable determination of allergen-specific serum IgE or IgG4 antibodies is limited by the fact that human IgE antibody serum pools are not standardized and cannot be reproducibly prepared. The lack of availability of allergen-

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³ Nonstandard abbreviations: FcεRI, high-affinity receptor for IgE; SIT, specific immunotherapy; scFv, single chain fragment variable; FACS, fluorescence activated cell sorting; C_H, immunoglobulin heavy chain constant domain; MPBS, milk powder phosphate buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Ap, alkaline phosphatase; and FITC, fluoresceine isothiocyanate.

specific IgG4 antibody pools of patient sera further complicates standardized methods. Standardization will likely become more important with the use of recombinant allergens for advanced diagnostics and intervention.

Our aim was to establish the generation of reproducible allergen-specific antibodies for the most relevant isotypes for allergy diagnostics and research. During the last decade combinatorial technologies have enabled the selection of a variety of monoclonal single-chain antibody fragments (scFv) against all types of antigens and epitopes (8–10). Although the selection of antibodies is often performed by using libraries built from lymphoid sources such as peripheral blood, bone marrow, or tonsils (11–13), this approach is of limited use for identifying allergy-relevant antibodies because of the evanescent number of IgE-producing cells and the highly individual reactivities of donors. In contrast, specificities against an almost unlimited panel of target molecules can be provided by synthetic library formats (14, 15), rendering their use attractive for the generation of antibodies against broad panels of allergens. To allow for their applicability in common laboratory methods, library-derived antibody fragments must be fused to Fc regions to overcome the monovalent format and the lack of constant region (Fc) effector domains. Such constructs were employed for recombinant production of scFv-Fc-fusions in mammalian cells (16–18) in *Pichia pastoris* (19), and to produce complete antibodies (20, 21).

We generated fully human IgE, IgG4, and IgG1 antibodies with defined specificity for 3 different allergens. From a human synthetic library, we selected scFv against the allergens and established their expression as human IgE, IgG4, and IgG1 antibodies. Expression of homodimeric or heterotetrameric recombinant proteins was performed in HEK-293 cells, and the antibodies were analyzed for allergen binding and formation of the allergen/IgE/IgE receptor complex. To our knowledge this is the first example of such a panel of IgE and IgG4 allergen-specific antibodies.

Material and Methods

MATERIALS

Target proteins for selection were phospholipase A2 from *Apis mellifera* (honey bee) venom, (Api m1) (Latoxan), milk β -lactoglobulin (Bos d5; Sigma), and the recombinant allergen from apple (Mal d1; Biomay). The antigen for the murine scFv that was used in some constructs with human constant regions was hen egg lysozyme (Gal d4) (Sigma).

PANNING PROCEDURE

For generation of monoclonal antibody fragments we used the human synthetic antibody library Griffin-1 (10), which comprises scFv-formatted variable regions of human origin, providing a diversity of approximately 2×10^9 . The allergens were employed for selection according to established protocols (10). Briefly, immunotubes

coated with the particular allergen and blocked with 20 g/L milk powder phosphate buffered saline (MPBS; 50 mmol/L sodium phosphate, 100 mmol/L sodium chloride, 20 g/L milk powder, pH 7.4) were incubated with a total volume of 4 mL of 20 g/L MPBS containing 10^{12} to 10^{13} phages for 90 min under continuous rotation at room temperature. The immunotubes were then washed, 1 mL of 100 mmol/L triethylamine was added to elute the bound phages, and neutralization was performed by addition of 0.5 mL of 1 mol/L Tris-HCl, pH 7.4. After reinfection of *Escherichia coli* TG1 and overnight growth on agar plates, the phages were rescued by growth of scraped cells in liquid culture, infection with M13KO7 helper phage, and another period of overnight growth. Phages were then subjected to the next round of selection. Immunoreactivity of polyclonal or monoclonal phages was assessed in ELISA with anti-M13-horseradish peroxidase conjugate (Amersham Pharmacia Biotech) and 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) di-ammonium salt for detection.

AMPLIFICATION AND CLONING OF IMMUNOGLOBULIN CONSTANT REGIONS

The human immunoglobulin constant regions were synthesized from cDNA derived from human peripheral blood mononuclear cells employing standard reaction conditions.

The genes for human IgG1 and IgG4 heavy chain constant regions (*IGHG1* and *IGHG4*⁴) were amplified using PCR primers containing an *AscI* and a *KpnI* site ($\gamma 1$: GAT CGG TAC CGA TCG GCG CGC CCA AAT CTT GTG ACA AAA CT CAC, $\gamma 4$: GAT CGG CGC GCC TTC CAC CAA GGG CCC ATC CGT CTT CCC CCT) and a *SfiI* site ($\gamma 1$: GAT CGG CCC AGC CGG CCT CAT TTA CCC GGA GAC AGG GAG AGG CTC TTC, $\gamma 4$: GAT CGG CCC AGC CGG CCT CAT TTA CCC AGA GAC AGG GA), the $\gamma 1$ C_H2–3 and $\gamma 4$ C_H2–3 domains using primers containing an *AscI* and a *KpnI* site ($\gamma 1$: GAT CTC TAG ATC ATT TAC CCG GAG ACA GGG AGA GGC TCT TC, $\gamma 1$: GAT CGG CGC GCC CAG CAA CAC CAA GGT GGA CA) and a *XbaI* site ($\gamma 1$: GAT CGG CGC GCC AGC CTC CAC CAA GGG CCC AT, $\gamma 1$: GAT CTC TAG ATC ATT TAC CCA GAG ACA GGG A).

For amplification of the genes for the human IgE heavy chain constant regions (*IGHG*) we used primers containing an *AscI* site (GAT CGG CGC GCC CAT CCG TCT TCC CCT TGA), an *SfiI* site, a 4xhis-tag (GAT CGG CCC AGC CGG CCT CAT TTA CCG GGA TTT ACA GAC AC), and for the ϵ C_H2–4 domains we used primers containing an *AscI* site (GAT CGG CGC GCC CAC CGT

⁴ Human genes: *IGHG1* human IgG1 heavy chain constant region; *IGHG4* human IgG4 heavy chain constant region; and *IGHG*, human IgE heavy chain constant region; *FCER1A*, Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide; *FCER1G*, Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide; *MS4A2*, membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for beta polypeptide).

GAA GAT CTT AC), an *Xba*I site, and a 4xhis-tag (GAT CTC TAG ATC AAT GGT GGT GAT GTT TAC CGG GAT TTA CAG ACA CCG).

The signal sequence of a gene for rat κ light chain was synthesized by PCR primers containing a *Nhe*I site (GTA CGC TAG CAA GAT GGA ATC ACA GAC CCA GGT CCT CAT GTC CCT GCT GCT CTG GAT TTC) and a *Kpn*I site (CAT GTC CCT GCT GCT CTG GAT TTC TGG TAC CTG TGG GGT GAG TCC TTA CAA CGC GTG TAC).

After introduction of the leader sequence into the mammalian expression vector pcDNA3.1-zeo (Invitrogen Life Technologies), we inserted the individual Ig domains, the Fc domains, and the entire heavy chains γ 1, γ 4, and ϵ via the *Xba*I and the *Asc*I sites. Transfer of the particular scFv into the scFv-C_H2-3 or scFv-C_H2-4 format was performed by introduction by PCR of a *Bsi*WI site at the N-terminus and an *Asc*I site at the C-terminus. Subsequently, the DNA was ligated into the vectors containing the signal sequence and the particular constant regions.

For expression of the heterotetrameric IgG and IgE formats we used the mammalian expression vector pBudCE4.1 (Invitrogen Life Technologies). The human κ light chain constant domain (IGKC) was amplified using one PCR primer containing an *Xba*I site and another primer containing an *Sgf*I site (GAT CTC TAG ACT AAC ACT CTC CCC TGT TGA AGC and GAT CGC GAT CG C AC G AAC TGT GGC T GC ACC ATC T GT C). The 2 human signal sequences VH3-64 and V κ I were synthesized by PCR using primers with an *Not*I and an internal *Swa*I or an *Sal*I and an internal *Sbf*I site for insertion of the variable regions (AGA ATG CGG CCG CTA TGG AAT TGG GGC TGA GCT GGG TTT TCC TTG TTG C TAT ATT TAAA TGT GTC CAG TGT and GAT CGT CGA CAT GGA CAT GAG GGT CCC CGC TCA GCT CCT GGG GCT CCT GCT ACT CTG CCT GCA GGG TGC CAG ATG T). After assembly of the leader sequences and the constant regions, the variable regions were then introduced via the *Sgf*I and the *Sbf*I sites, or the *Asc*I and *Swa*I sites, respectively. Finally, the entire light chain sequence including the leader sequence was introduced via the *Xba*I and the *Sal*I sites and the entire heavy chain including the leader sequence via the *Not*I site and the *Sfi*I site into the expression vector pBudCE4.1.

AMPLIFICATION AND CLONING OF IGE HIGH-AFFINITY RECEPTOR SUBUNITS

Sequences coding for the subunits of the human IgE receptor (*FCER1A*, *MS4A2*, and *FCER1G*) were amplified from a cDNA library derived from human peripheral blood mononuclear cells. The α subunit was amplified with PCR primers containing an *Xba*I site (GAT CTC TAG AAT GGC TCC TGC CAT GGA ATC) and a *Bam*HI site (GAT CGG ATC CTC AGT TGT TTT TGG GGT TTG GC), the β subunit using primers containing a *Not*I site (GAT CGC GGC CGC TAT GGA CAC AGA AAG TAA TAG GAG) and an *Sfi*I site (GAT CGG CCC AGC CGG CCT

CAT AAA TCA ATG GGA GGA GAC ATT TC) and the γ subunit using primers containing an *Nhe*I site (GAT CGC TAG CAT GAT TCC AGC AGT GGT CTT G) and a *Bam*HI site (GAT CGG ATC CCT ACT GTG GTG GTT TCT CAT GC). Subsequently, the DNA was inserted into the expression vectors pcDNA3.1-neo or pBudCE4.1. The expression of the γ chain is a prerequisite for surface expression of the α subunit and results in formation of functional receptor complexes of the $\alpha\beta\gamma$ 2 type.

TRANSFECTION OF HEK-293 CELLS

HEK-293 cells (22) (ATCC number CRL-1573) were cultivated in DMEM supplemented with 100 mL/L fetal calf serum, 10 kIU/L penicillin, and 100 mg/L streptomycin. Tissue culture reagents were obtained from Invitrogen Life Technologies. HEK-293 cells were transfected with 2 μ g of the particular expression vector by use of polyethylene imine (Sigma). The secreted immunoglobulins were purified from the culture medium by affinity chromatography using protein A-agarose (Santa Cruz Biotechnologies.) or Ni-NTA-agarose (Qiagen) according to the manufacturers' recommendations.

ASSESSMENT OF IMMUNOREACTIVITY IN ELISA AND ALABLOT

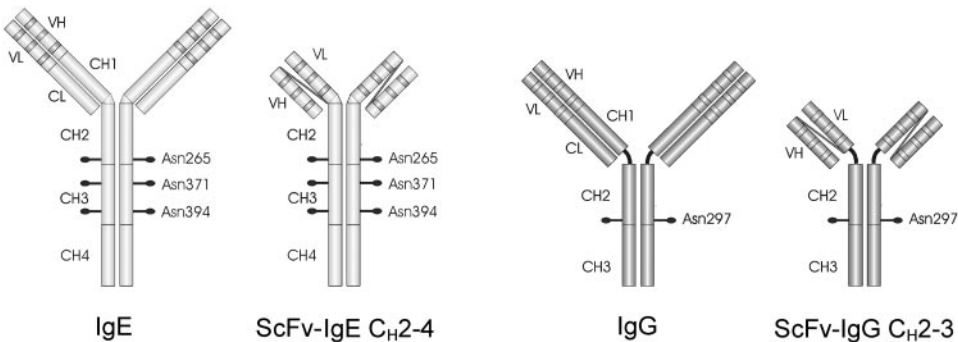
For assessment of immunoreactivity in ELISA the particular proteins (diluted with 20 g/L MPBS) were applied to microtiter plates coated with particular antigens at 4 °C overnight and blocked with 50 g/L MPBS at room temperature for 1 h. Thereafter, ELISA was performed according to established protocols. For immunoblot procedures, the particular recombinant antibodies were diluted using DMEM supplemented with 100 mL/L heat-inactivated fetal calf serum and applied to the corresponding Alablots (F1, hen egg white; F2, bovine milk white; I1, whole bee venom; and F49, apple extract; DPC-Biermann) with native allergens, separated by SDS-PAGE, and immobilized onto nitrocellulose strips in the form of extracts or purified allergens. Visualization of bound IgE was then performed with monoclonal antihuman IgE conjugated to alkaline phosphatase (AP) and nitrotriazolium blue chloride/5-bromo-4-chloro-3-indoyl phosphate according to recommendations of the manufacturer.

ASSESSMENT OF REACTIVITY WITH RECEPTORS

Interaction of the IgE antibodies with its receptor and with allergens was followed by flow cytometric analyses performed by use of a FACS Calibur and CellQuest software (both Becton Dickinson). Labeling of antibodies and allergens with fluorescein isothiocyanate (FITC) (Sigma) was performed according to standard protocols. Fc ϵ RI-expressing HEK-293 cells were resuspended in DMEM containing 100 mL/L fetal calf serum and seeded into 6-well microtiter plates at a density of 5×10^5 cells/well. After overnight growth, FITC-labeled antibody constructs in a volume of 400 μ L were added and cells were incubated for 120 min at 4 °C and then washed

Fig. 1. Schematic representation of the recombinant immunoglobulins.

Entire heterotetrameric IgE and IgG and homodimeric scFv-IgEC_H2-4 and scFv-IgG C_H2-3 molecules. The constant regions of the heavy and light chains and the variable regions are shaded grey, the linker in the scFv darker. Indicated are the different domains of the antibodies, the complementary determining regions (CDR), and the Fc glycosylation sites.



3 times with DPBS. Concentration of the antibody constructs was usually 2.5 nmol/L. Employing nonlabeled antibodies, cells were resuspended in 400 μ L DMEM containing 100 mL/L fetal calf serum and FITC-conjugated allergen. The resuspended cells were incubated for 120 min at 4 $^{\circ}$ C and then washed 3 times with DPBS. Controls consisted of cells resuspended in 500 μ L DPBS only.

OTHER METHODS

SDS-PAGE, immunoblotting, and ELISA as well as standard procedures in molecular biology were performed according to established protocols (23).

Results

SELECTION OF RECOMBINANT ANTIBODY FRAGMENTS

To generate monoclonal antibody fragments with specificity for different major allergens, we subjected the human synthetic antibody library Griffin-1 (10) to selection against the 3 purified allergens Api m1, Bos d5, and Mal d1 immobilized on polystyrene tubes. After iterative panning, significant enrichment in the 2nd and 3rd rounds of selection was assessed by polyclonal ELISA (data not shown). Subsequently, the individual phage clones exhibited signals 3–12 times background in the monoclonal ELISA. For each of the allergens individual reactive fragments were identified by sequence analysis (see Fig. 1 in the Data Supplement that accompanies the online version of this article at <http://www.clinchem.org/content/vol53/issue6>).

EXPRESSION OF IGE AND IGG ANTIBODIES

For expression of selected scFv as different Ig isotypes in mammalian cells, we constructed a set of modular cassettes containing leader sequences, constant Ig regions, and restriction sites for the incorporation of scFv or separate variable regions. The γ 1, γ 4, and ϵ heavy chain constant regions and a corresponding light chain constant region were amplified from a cDNA library derived from human peripheral blood mononuclear cells (data not shown). Fusion proteins were produced by use of modular cassettes containing signal sequence and restriction sites for cloning of variable and constant regions of both entire heterotetrameric antibodies as well as homodimeric scFv-C_H2-4 or scFv-C_H2-3 (Fig. 1). The entire panel of 6 expression formats was developed for the library-derived human antibody fragments Bos d5-1 and Api m1-9. Moreover, human scFv-based homodimeric formats were produced on the basis of antibody fragments selected against Api m1, Mal d1, and Bos d5 (Table 1). A murine scFv was employed for the primary establishment of the complete set of 6 different antibody types and formats against the allergen Gal d4 (one of each format for the human isotypes IgG1, IgG4, and IgE).

After purification from culture supernatants of transfected HEK-293 cells, SDS-PAGE demonstrated homogeneity of the antibody molecules, and immunoblotting verified the presence of the particular Ig chains (Fig. 2). Molecular masses were found to be 130 kDa and 150 kDa for the homodimeric scFv-based IgG- and IgE-analogous molecules, respectively, and 160 kDa and 190 kDa for the

Table 1. Antibody formats constructed and produced from antibody fragments.

Allergen	Antibody fragment	IgG1	IgG4	IgE	scFv-IgG1 C _H 2-3	scFv-IgG4 C _H 2-3	scFv-IgE C _H 2-4
Gal d4 (Lysozyme)	Gal d4	+	+	+	+	+	+
Bos d5 (β -Lactoglobulin)	Bos d5-1	+	+	+	+	+	+
	Bos d5-4				+	+	+
	Bos d5-6				+	+	+
Api m1 (Phospholipase A2)	Bos d5-10				+	+	+
	Api m1-2				+	+	+
	Api m1-9	+	+	+	+	+	+
Mal d1 (Apple)	Mal d1-2				+	+	+
	Mal d1-7				+	+	+

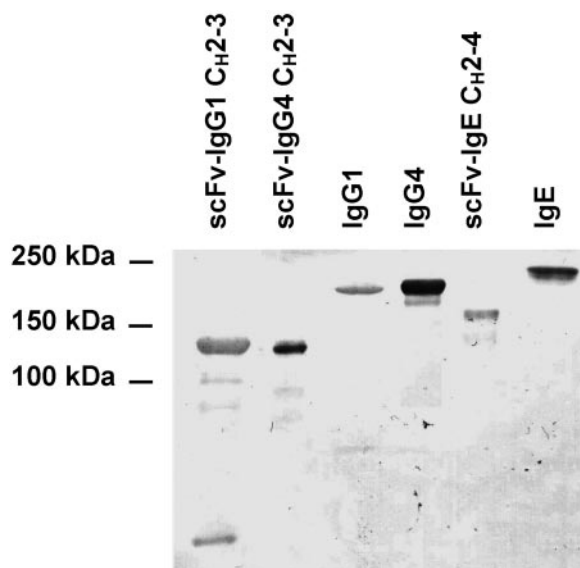


Fig. 2. Immunoblot analysis of recombinant proteins.

Purified scFv-based and entire antibody proteins were separated by non-reducing 7.5% SDS-PAGE and transferred to a PVDF-membrane and incubated with either antihuman IgG-AP conjugate or antihuman IgE-AP conjugate (diluted 1:10,000 or 1:1,000). Bound antibodies were visualized using NBT/BCIP as substrate.

heterotetrameric IgG and IgE, respectively. The expected molecular masses in SDS-PAGE suggest that the majority of antibody secreted from the HEK-293 cells is properly folded and glycosylated, in particular the extensively glycosylated IgE.

ASSESSMENT OF IMMUNOREACTIVITY

Immunoreactivity of the resulting proteins was assessed by several approaches. All recombinant proteins detected their particular target allergen by ELISA (Fig. 3A). Moreover, specific immunoreactivity of the recombinant scFv-IgE antibodies was demonstrated by detection of Api m1, Bos d5, and Gal d4 with commercial AlaBLOT immunoblot strips that contain a plethora of extracted antigens (Fig. 3B) routinely employed in allergy diagnostics. The lack of reactivity by antibody Mal d1-7 in the AlaBLOT

likely reflects recognition of a conformational epitope lost during immunoblotting. Most of these antibodies also bound to the appropriate allergen extracts immobilized to paper discs (data not shown).

Recombinant antibodies were added to serum from nonallergic individuals to produce human serum with defined reactivity. Dilutions of these supplemented sera in ELISA mimicked signals from individual sera from allergic individuals (Fig. 4A). The immunoreactivity of the scFv-based antibodies added to nonallergic serum remained stable for at least 1 week at room temperature (Fig. 4B).

ACTIVITY OF THE IGE ANTIBODIES IN CELLULAR ASSAY SYSTEMS

After stable transfection of HEK-293 cells with the α , β , and γ subunits of the human Fc ϵ RI, we identified transfectants expressing the functional receptor by use of FACS using an α chain-specific antibody (Fig. 5A). Functionality of the receptor and binding of the recombinant antibodies were demonstrated by use of FITC-conjugated scFv-based IgE (data not shown). Specific binding of allergens via receptor-bound recombinant IgE could be shown by use of nonlabeled recombinant IgE antibodies and FITC-labeled Gal d4 (Fig. 5B) or FITC-labeled Api m1 (Fig. 5C). To mimic the decrease of allergen binding to receptor-bound IgE in the presence of blocking allergen-specific IgG4 antibodies, the allergen was applied in the presence of IgE and IgG4 antibodies; subsequent FACS analyses confirmed the inhibition of allergen binding to receptor-bound IgE (Fig. 5B, D-F).

Discussion

In general, the presence of IgE vs IgG1 and IgG4 antibodies in allergy reflects the T-helper cell type 1/2 type immune milieu. Although the role of allergen-specific IgG responses after allergen exposure (24, 25) has remained controversial (26, 27), the production of high-affinity IgG antibodies during SIT is of particular interest because

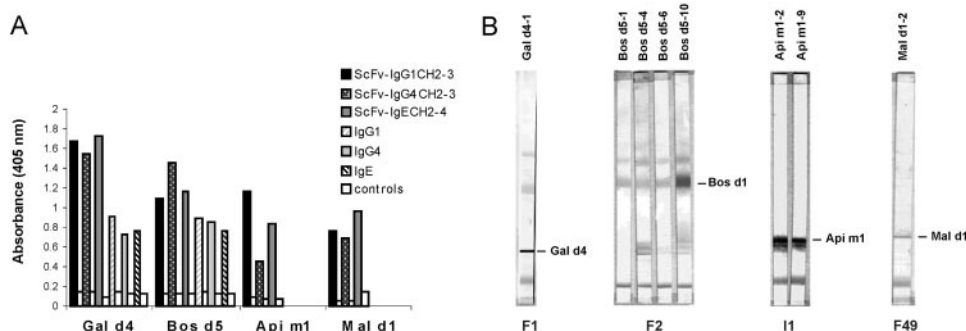


Fig. 3. Immunoreactivity of the recombinant antibodies in diagnostic approaches.

(A), immunoreactivity of representative different recombinant antibody constructs produced on the basis of different scFv was assessed by ELISA. The entire set of antibodies was established for the Gal d4- and a Bos d5-specific scFv, while only scFv-based constructs were assessed for scFv specific for Api m1 and Mal d1. Controls for each construct were performed by omission of antigen. Bound constructs were visualized using monoclonal anti-human IgG1, IgG4, and IgE AP conjugates. (B), recombinant scFv-based IgE antibodies were employed in immunoblot-based allergen detection by applying antibodies to the particular AlaBLOTs according to the manufacturers recommendations. The indicated AlaBLOT names contain protein extracts as described in methods.

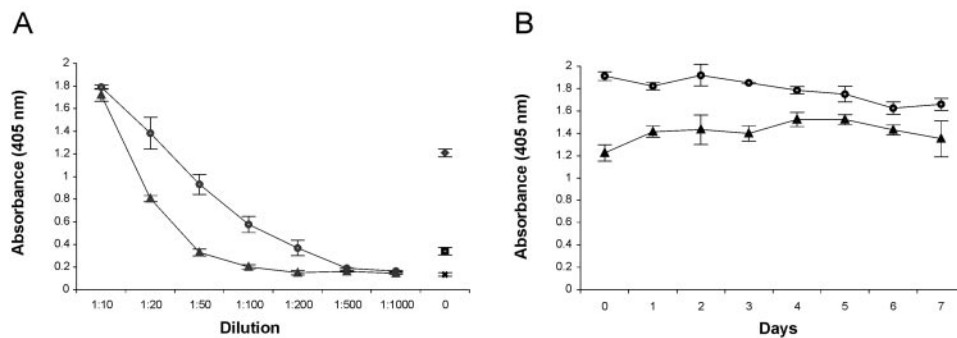


Fig. 4. Immunoreactivity of human serum supplemented with recombinant antibodies.

(A), reactivities of recombinant scFv-based IgE antibodies (Bos d5-1, triangles, and Bos d5-4, circles) against the allergen were assessed by ELISA. Cellular supernatants were used to supplement serum of a non-allergic individual (diluted 1:10 in PBS). Serum of two allergic individuals (square and diamond) and the non-allergic individual (cross) without supplementing antibodies are shown as reference. Bound IgE constructs were visualized using monoclonal antihuman IgE AP conjugate. Data represent the mean (SD) absorbance of triplicate measurements. (B), long-term stability of scFv-based IgE antibodies Bos d5-1 (triangles) and Bos d5-4 (circles) in serum diluted as above was assessed by ELISA. Bound constructs were visualized as above. Data represent the mean (SD) absorbance of triplicate measurements.

such antibodies are thought to interfere with the IgE allergen interaction (2, 3) and could act as blocking antibodies (4). Their activity may rely either on molecular excess or on affinity maturation during vaccination (28). The ratio of serum concentrations of allergen-specific IgE and IgG4 antibodies is thought to reflect the immune status of an allergic patient and the success of SIT.

The unavailability of monoclonal IgG and IgE antibodies has thus far prohibited detailed analyses of their characteristics in pathophysiology as well as their molecular interplay. Approaches to generating human allergen-specific IgE-secreting hybridomas from immunized donors have not been successful (29). Furthermore the production of allergen-specific antibodies by conventional hybridoma technology is hampered by the low immunogenic potential of allergens, and the resulting murine antibodies are neither compatible with established human

specific assay formats nor allow differentiation of different human isotypes.

Recently, the establishment of combinatorial approaches and the relatively easy generation of avian antibody phage libraries have enabled the production of allergen-specific chicken monoclonal scFv; however, this approach is also limited by time-consuming immunization procedures (30). Chimerization of animal-derived antibodies by addition of desired human constant regions can open alternatives but is very time-consuming and is not specific for allergenic epitopes. Nonetheless, the re-cloning of murine hybridomas to chimeric IgE has been reported (31). Indeed, we used a murine antibody fragment with specificity for Gal d4 to express recombinant human Fc antibodies. This approach supports the idea that available allergen-specific hybridomas may also provide suitable binding moieties for recombinant IgE and

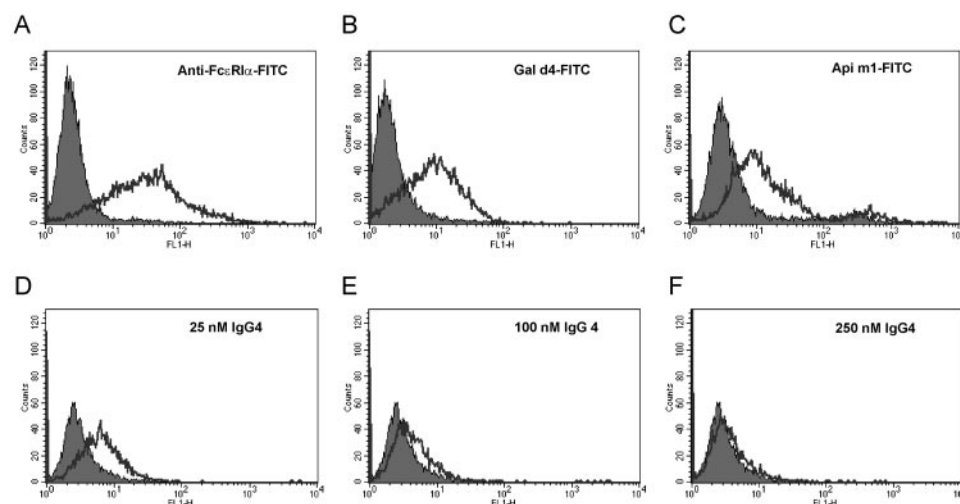


Fig. 5. Interaction of recombinant IgE with the high affinity IgE receptor.

After stable transfection of HEK-293 cells expression of all 3 subunits of the IgE receptor was assessed by FACS using an anti-FcεRIα specific monoclonal antibody together with anti-mouse IgG-FITC conjugate (A), interaction of recombinant IgE with the receptor was shown by using FITC-labeled Gal d4 (B) and FITC-labeled Api m1 (C) in combination with scFv-based IgE constructs. Competitive blocking of this interaction is shown in a dilution series of recombinant anti-Gal d4 IgG4 (B: 0 nmol/L; D: 25 nmol/L; E: 100 nmol/L; F: 250 nmol/L).

IgG antibody formats. The selection of IgE-derived antibody fragments from immune repertoires of human donors (32, 33) is labor-intensive because of the evanescent number of IgE-producing cells, the highly individual reactivities of donors and the limited number of resulting clones makes this method unsuitable for minor allergens.

In contrast, as demonstrated in this study, synthetic libraries of human origin can quickly provide reactive antibody fragments against broad panels of available allergens by employing a high-diversity library of human scFv-formatted variable regions. The epitopes recognized by such antibody fragments may differ from the epitopes of allergens recognized by natural IgE antibodies. However, biochemical and structural characteristics may render particular epitopes favorable for both natural IgE antibodies and scFv obtained by selection processes. A possible example is the cross-reaction of the major allergen from the apple fruit Mal d1 with natural IgE specific for Bet v 1 and other homologs, which causes birch pollen related food allergies. For the recombinant antibody Mal d1-2 in our study, however, assessment using AlaBLOT revealed no evidence for such cross-reactivity with Bet v1. Supporting the potential of the phage-display technology, our selection process yielded a panel of reactive human antibody fragments for several common allergens. In honey bee venom, Api m1 represents the dominating major antigen in immunity as well as in allergy (34). Bos d5 (β -lactoglobulin) is a principle allergen in cow's milk. Compared to the conversion to entire heterotetrameric Igs (20, 21), a more convenient approach relies on the expression of homodimeric scFv-C_H2-3 IgG fusions (16, 19) that are easy to generate and purify and possess full binding and effector functions (19). For analogous IgE and IgG4 Fc fusion proteins of scFv, expression has not previously been produced.

Our transfection of HEK-293 cells yielded a set of scFv-based as well as entire heterotetrameric antibodies that are allergen-specific IgE, IgG1, and IgG4 antibodies. The immunoreactivity in direct ELISA of these recombinant IgE and IgG4 antibodies when added to nonallergic human serum and their reactivity in commercial immunoblots demonstrates their potential use in different types of immunological and allergy diagnostic methods.

Our analyses of IgE binding to the recombinant high-affinity receptor Fc ϵ RI also demonstrate the compatibility of the recombinant proteins with cellular assay systems. Moreover, allergen binding to the Fc ϵ RI could be blocked by IgG antibodies of the same allergen specificity. Because the recombinant IgE and IgG4 antibodies exhibit identical affinities due to their identical scFv moieties, the set of antibodies may mimic the circulating antibodies after affinity maturation of IgG antibodies that may occur after SIT.

Controversy exists regarding the affinities of allergen-specific IgE and IgG antibodies and the physiological relevance of their affinities (35). The overall affinity of allergen-specific polyclonal IgG is thought to be signifi-

cantly lower than that of allergen-specific polyclonal IgE (28). In contrast, antibody fragments isolated from immune libraries of allergic donors demonstrated comparably high affinities (36), and analyses of IgG antibodies during SIT revealed no increase in IgG affinity (37). These inconsistent findings may be partially explained by circulating IgE and IgG antibodies affinities that are highly variable without being discernible in immunoassays. The recombinant antibodies most likely exhibit high affinities attributable to the nature of the selection process and therefore correspond to the natural antibodies.

In summary, we used both combinatorial selection of synthetic antibody libraries and antibody technology to generate different formats of monoclonal allergen-specific IgE, IgG1, and IgG4 antibodies. The use of such reliable and precise reagents may help to establish novel reagents to aid in the standardization of allergen-specific diagnostic immunoassays and to minimize interassay variance. In addition, such defined antibodies are attractive tools for basic and applied research to evaluate the complex molecular interplay of allergens, different allergen-specific antibodies, and the different types of Fc ϵ and Fc γ receptors to better understand modulation of the allergic reaction. Finally, such approaches may have therapeutic applications including recruitment of negatively regulating receptors (38) as well as passive or adjuvant immunotherapy (39).

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Supplemental Data Figure 1

V_H	1	10	20	30	40	50	60	70	80	90	100	110
Bos d5-1	: EVQLVESGGGLVQPFGSLRLSCAASGFTFSSYAMS... <u>WVRQAPGKGLEWVSAISGS</u> ...GGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARKKF.....GHWGQGT LVTV											
Bos d5-4	: EVQLVESGGGLVQPFGSLRLSCAASGFTFSSYAMS...WVRQAPGKGLEWVSAISGS...GGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARLRSRQRTS...NFWGQGT LVTV											
Bos d5-6	: EVQLVEPGGGVVQPGRSLRLSCAASGFTFSNYGMH...WVRQAPGKGLEWVAISYD...GSNKYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARGWHL.....RVWGQGT LVTV											
Bos d5-10	: EVQLVESGGGLVQPFGSLRLSCAASGFTFSSYAMS...WVRQAPGKGLEWVSAISGS...GGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARYYSKATANRWSWGQGT LVTV											
Api m1-2	: QVQLGQSGAEVKKPGATVKISKVSGYTFDDYYMH...WVQQAPGKGLEWMLVDP...EDGETIYAEKFQGRVTITADTSTDTAYMELSSLRSED TAVYYCARRLAAK....TNWGQGT LVTV											
Api m1-9	: QVQLGQSGAEVKKPGATVKISKVSGYTFDDYYMH...WVQQAPGKGLEWMLVDP...EDGETIYAEKFQGRVTITADTSTDTAYMELSSLRSED TAVYYCARKFYGT....TDWGQGT LVTV											
Mal d1-2	: EVQLVESGGVVQPFGSLRLSCAASGFTFSNAWMS...WVRQAPGKGLEWVGRIKSKTDGGTTDYAAPVKGRTISRDDSKNTLYLQMNSLTKTD TAVYYCARTGK.....QPWGQGT LVTV											
Mal d1-7	: QVQLVESGGGLVQPFGSLRLSCAASGFTFSSYAMS...WVRQAPGKGLEWVSAISG...SGGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVYYCARQYRPLHS...SNWGQGT LVTV											
				CDRH1			CDRH2				CDRH3	
				31-35B			50-65				95-102	
linker	SSGGGGSGGGSGGSA											
V_L	1	10	20	30	40	50	60	70	80	90	100	
Bos d5-1	: LSSELTQDPFAVSVALGQTVRITCQGD SLR...YYASWYQKPGQAPVLVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYCNSRDSSG.....TVFGGGTKLTVLG											
Bos d5-4	: DIQMTQSPSSLSASVGD RVTITCRASQ SIS...SYLNWYQKPGKAPKLLIYAASLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQOSYST.....PTFGGQTKVEIKR											
Bos d5-6	: LQSVLTQPPSASGTPGQRVTISCSGSSSNIGS...NTVNWYQQLPGTAPKLLIYSNDQRPSGVDPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSL.....VVFSGGTKLTVLG											
Bos d5-10	: LQSVLTQPPSASGTPGQRVTISCSGSSSNIGS...NTVNWYQQLPGTAPKLLIYSNNQRPSGVDPDRFSGSKSGTSASLAISGLQSEDEADYYCAAWDDSL.....PVFGGGTKLTVLG											
Api m1-2	: LQSALTQPASVSGSPGQSITISCTGTSSDV...GGYNYVSWYQQHPGKAPKLMIEVSNRPSGVSNRPSGSKSGNTASLTISGLQAEDEADYYCSSYSSS.....TVFGGGTKLTVLG											
Api m1-9	: LQSVLTQPPSASGTPGQRVTISCSGSSSN...IGSNTVNWYQQLPGTAPKLLIYSNNQRPSGVDPDRFSGSKSGTSASLAISGLRSEDEADYYCAAWDDSLA.....LVFGGGTKLTVLG											
Mal d1-2	: LSSELIQDPFAVSVALGQTVRITCQGD SLR...SYIASWYQKPGQAPXXVIYGKNNRPSGIPDRFSGSSSGNTASLTITGAQAEDEADYYCNSRDSS.....GTVFGGGTKLTVLG											
Mal d1-7	: LQSVLTQPPSSGTPGQRVTISCSGSSSN...IGSNVYVWYQQLPGTAPKLLIYRNNQRPSGVDPDRFSGSKSGTSASMAISGLRSEDEADYYCAAWDNSLNR.....MVFGGGTKVTVLG											
				CDRL1			CDRL2				CDRL3	
				24-34			50-56				89-97	

Supplemental Data Figure 1 Legend: Sequence analysis of isolated allergen-specific scFv

Numbering and determination of the CDR (underscored) of the variable regions of heavy (V_H) and light chains (V_L) were performed according to Kabat (40).