

Isolation of high-affinity human IgE and IgG antibodies recognising Bet v 1 and *Humicola lanuginosa* lipase from combinatorial phage libraries

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Abstract

Allergen-specific Fab fragments isolated from combinatorial IgE and IgG libraries are useful tools for studying allergen–antibody interactions. To characterise the interaction between different allergens and antibodies we have created recombinant human phage antibody libraries in the Fab format. Human IgE and IgG libraries have been created from patients allergic to birch pollen or lipase. These libraries have been used to select binders recognising the major birch pollen allergen Bet v 1 and *Humicola lanuginosa* lipase. A panel of allergen-specific IgE and IgG antibodies were identified; these were further characterised by allergen binding studies using Biacore and competition studies using human sera and antibodies purified from human sera. Affinities in the nM range were recorded and a competition with human sera for allergen binding was observed.

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1. Introduction

IgE is the least abundant class of immunoglobulins in human serum (10–400 ng/ml), making it difficult to characterise allergen–antibody binding. Display of antibodies on filamentous bacteriophage followed by selection provides a powerful tool for this characterisation. The phage display technology offers the ability for generation of large surface displayed antibody repertoires (Marks et al., 1991; Hoogenboom and Winter, 1992; Griffiths, 1993; Vaughan et al., 1996) while proper selection procedure permits the isolation of individual antibodies. Steinberger et al. (1996) have described the successful cloning of Phl p 5-specific human IgE Fab fragments using the combinatorial library approach and mouse IgE Fab phage libraries have been created from mice immunised with Tricosanthin (Li and Yeh, 1997). Allergen-specific IgG Fab fragments from a large

human IgG phage repertoire have also been identified (de Lalla et al., 1996) and their affinities to a rye-grass pollen allergen, Lol p 2, have been determined.

The only curative approach for the IgE dependent allergy is allergen-specific immunotherapy (SIT) which was first introduced in 1911 (Noon et al., 1911). The effects of SIT are well characterised in terms of reduced symptoms and medication scores, but the underlying biomolecular processes are less well understood. SIT seems to have little effect on the amount of allergen-specific IgE in serum. Instead an increased production of allergen-specific IgG, especially a rise in allergen-blocking IgG of the IgG4 class is observed (Cooke et al., 1935; Loveless, 1940; Reid et al., 1986; Wetterwald et al., 1985; Svenson et al., 2003). These data support the notion that a therapy of allergy might be obtained using therapeutic antibodies blocking the allergen–IgE interaction. These antibodies can be either of the IgG subclass or IgE Fab fragments, which do not bind to the FcεRI receptor. Thereby the allergic symptoms may be prevented, and the risk of anaphylaxis related to allergen-specific immunotherapy could be reduced or eliminated.

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In the present study we have constructed human IgE and IgG combinatorial libraries in the Fab format based on RNA obtained from blood lymphocytes of a non-allergic person and from patients allergic to birch pollen or *Humicola lanuginosa* lipase. These libraries have been used to select binders recognising the major birch pollen allergen Bet v 1 and *H. lanuginosa* lipase. The selected IgE and IgG antibodies were further characterised in relation to allergen binding using surface plasmon resonance (Biacore) and in competition studies using human sera and antibodies purified from human sera. Affinities in the nM range were recorded and a competition with human sera and purified serum antibodies for allergen binding was observed suggesting that the selected antibodies recognise the same epitope pattern as human sera.

2. Materials and methods

2.1. Characterisation of birch pollen and lipase allergic patients

For the construction of human IgE and IgG combinatorial libraries, peripheral blood mononuclear cells were obtained from a non-allergic donor (donor A), a patient who had developed occupational allergy to *H. lanuginosa* lipase (donor B), and 2 different patients allergic to birch pollen (donor C and D). After diagnosis donor B was moved to a lipase-free area, and he was free of symptoms at the time of blood collection. He reported himself to be free of allergy to birch pollen. The blood samples from donor C and D were obtained during the birch pollen season 2002. Donor C had received immunotherapy for 2.5 years (Bodtger et al., 2002). The data of the patients are shown in Table 1. The study was approved by the local ethics committee of Copenhagen, Denmark, and written informed consent was obtained from all patients prior to admission to the study.

2.2. Bacterial strains and cloning

Electrocompetent amber codon suppressing *E. coli* strain TG1 (Stratagene, *supE thi-1 D(lac-proAB) D(mcrB-hsdSM)5*

Table 1
Characteristics of the donors selected for this study

Patient	Donor A	Donor B	Donor C	Donor D
Gender	Female	Male	Female	Male
Age	26	35	34	31
Sensitised to	None	<i>H. lanuginosa</i> lipase	Birch	Birch
Allergy duration (years)	0	2	16	9
SPT diameter (mm)	–	n.d.	6.0	7.5
Specific IgE (kU/l)	–	15	44.1	6.37
Immunotherapy	–	No	Yes	No
Duration of IT	–	–	2.5	–

n.d.: not determined.

(*rK–mK–*) [*F' traD36 proAB lacIqZDM15*]) containing *F'* episome for single-strand rescue was used for transformations, preparations of phagemids and phage display library preparation. The transformations of phagemid DNA were performed as described in Sambrook et al. (1989).

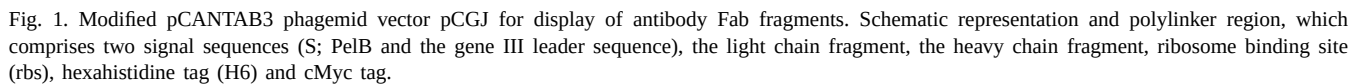
2.3. pCGJ phagemid vector construction

A new phagemid vector pCGJ (Fig. 1) for creation of the human Fab libraries was designed and constructed by modifying the pCANTAB3 (Amersham Biosciences, UK) phagemid vector. pCANTAB3 was digested with the restriction endonucleases *Apa*LI and *Not*I (New England Biolabs, MA) and gel-purified. Simultaneously, the polylinker region of pCGJ was established by annealing the eight oligonucleotides (DNA Technology, Denmark) described in Table 2, by mixing them in equal amounts and heating them to 95°C followed by slowly cooling to 4°C. Ligation of the insert containing the polylinker region into the gel-purified pCANTAB3 vector fragment was performed by overnight incubation at 16°C (vector:insert ratio 1:4) in the presence of T4 DNA ligase (New England Biolabs, MA). The ligation mixture was desalted and used for electroporation of *E. coli* strain TG1 to produce the pCGJ phagemid.

The pCGJ vector allows stepwise cloning of antibody fragments in the Fab format. The heavy chain is linked to the phage coat protein gIIIp of the filamentous phage fd and carries a hexahistidine tag and a c-Myc tag for detection and purification. Two ochre stop codons (UAA) are situated immediately upstream of the *Apa*LI restriction endonuclease site, resulting in expression of the light chain as a separate fragment, secreted into the periplasm, where it can pair with the heavy chain. Two different signal sequences (gIIIp and pelB) were chosen to avoid recombination which has previously been observed (Courtney et al., 1995).

2.4. Isolation and stimulation of human B-cells

For each donor, human peripheral blood mononuclear cells (PBMCs) were isolated from 20 ml blood using Lymphoprep (Invitrogen, CA). B-cells were further isolated from the PBMCs using magnetic anti-human-CD19 MACS microbeads (Miltenyi Biotec, UK) and MACS High Gradient Magnetic LS Separation Columns (Miltenyi Biotec, UK). The cells were resuspended in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal calf serum (FCS), 0.5% BSA, 2 mM L-glutamine, 0.2 mg/ml gentamicin, 1x ITS Liquid Media Supplement, 1 µg/ml oleic acid, 1 µg/ml linoleic acid, 1 µg/ml palmitic acid (Sigma–Aldrich, MO), and 2×10^5 cells/well were cultured in 24 well plates (Nunc, Denmark). B-cells were stimulated with 25 ng/ml human IL-4 (Sigma–Aldrich, MO) and 1 µg/ml anti-human-CD40 (Beckman Coulter, CA) to achieve a class switch from IgM to IgE.



The switch from IgM to IgE after stimulation with IL-4 and anti-CD40 was determined at the cellular level by flow cytometry. Following stimulation with human IL-4 and anti-human-CD40 for 6 days, human PBMCs were labelled with different monoclonal antibodies (mAb) recognising specific cellular markers. Briefly, PBMCs (1.25×10^5 cells per 100 μ l) were incubated in PBS supplemented with 2% FCS and 0.1% sodium azide for 30 min at 4°C, in the presence of fluorescein isothiocyanate (FITC)-conjugated anti-CD19 mAb (BD Biosciences, CA), phycoerythrin (PE)-conjugated anti-CD3 mAb (BD Biosciences, CA), PE-conjugated anti-CD23 mAb (BD Biosciences, CA), or biotin-conjugated anti-human-IgE mAb (PharMingen, CA). After washing in PBS supplemented with 2% FCS and 0.1% sodium azide (5 min, $300 \times g$, 4°C) the cells labelled with biotin-anti-human-IgE were incubated with PE-conjugated

2.6. Reverse transcription

Total RNA from the harvested cells was purified using Trizol (Invitrogen, CA) following the instructions of the manufacturer. Reverse transcription was performed on 5 µg total RNA. Following heat-denaturation for 10 min at 65 °C in the presence of 1.25 µg of a dN6 oligo (DNA Technology, Denmark), a poly-T oligo (DNA Technology, Denmark), or a human IgE specific oligo ([HuCH-IgE FOR], [Table 3](#))

Cassette 1 sense	5' <u>TGCACAGGTCCAAGCGGCCGCTCGACCTAATAAGG</u> 3'
Cassette 1 antisense	5' <u>CGCGCCTTATTAGGTCGACGCGGCCGCTTGACCTG</u> 3'
Cassette 2 sense	5' <u>CGCGCCAATTCTATTTC</u> AAGGAGACAGTCATAATGAAATACCTATTGCCTACGGCAGCCGCTGGATTGTT-ATTACTCGCGGCCAGC3'
Cassette 2 antisense	5' <u>GGGCGCGAGTAATAACA</u> ATCCAGCGGCTGCCGTAGGCAATAGGTATTTCAATTATGACTGTCTCCTTGAAAT-AGAATTGG3'
Cassette 3 sense	5' <u>CGGCCATGGCCAGGTGCAGCCTCGAGGAGAGCGGCCA</u> 3'
Cassette 3 antisense	5' <u>CCGGTGCGCGCTCTCCTCGAGGCTGCACCTGGCCATGGCCGGCT</u> 3'
Cassette 4 sense	5' <u>CCGGTGTCTATCATCATCATCATC</u> ACGGGGCCGAGAAACAAAACTCATCTCAGAAGAGGATCTGAATGG3'
Cassette 4 antisense	5' <u>GGCCCCATT</u> CAGATCCTCTTCTGAGATGAGTTTTTGTCTGCGGCCCGTGATGATGATGATGATGAC <u>A</u> 3'

Partial restriction sites are underlined.

Table 3
Oligonucleotides used for human library construction

HuVH-back	
SfiI	
HuVH1A/1D/7A-BACK	5'-CGAATAGGCCAGCCGGCC CAG RTB CAG CTG GTG CAR TCT GG-3'
HuVH1B/1C-BACK	5'-CGAATAGGCCAGCCGGCC SAG GTC CAG CTK GTR CAG TCT GG-3'
HuVH2A/2B-BACK	5'-CGAATAGGCCAGCCGGCC CAG RTC ACC TTG ARG GAG TCT GG-3'
HuVH3A/3B-BACK	5'-CGAATAGGCCAGCCGGCC SAR GTG CAG CTG GTG GAG TCT GG-3'
HuVH3C-BACK	5'-CGAATAGGCCAGCCGGCC GAG GTG CAG CTG TTG GAG WCY GG-3'
HuVH4A-BACK	5'-CGAATAGGCCAGCCGGCC CAG STG CAG CTG CAG GAG TCS GG-3'
HuVH4B-BACK	5'-CGAATAGGCCAGCCGGCC CAG GTG CAG CTA CAG CAG TGG GG-3'
HuVH5A-BACK	5'-CGAATAGGCCAGCCGGCC GAR GTG CAG CTG GTG CAG TCT GG-3'
HuVH6A-BACK	5'-CGAATAGGCCAGCCGGCC CAG GTA CAG CTG CAG CAG TCA GG-3'
HuCH-for	
SgrAI	
HuCH-IgE FOR	5'-TGGCTCACC ^u GGTG GCT GAA GGT TTT GTT GTC GAC CCA GTC-3'
HuCH-IgG1-4 FOR	5'-TGGCTCACC ^u GGTG GTC CAC CTT GGT GTT GCT GGG CTT-3'
HuVK-back	
ApaLI	
HuVK1A/1B/1C/4A-BACK	5'-CTCCTGTGCACTT RMC ATC CRG WTG ACC CAG TCT CC-3'
HuVK1D-BACK	5'-CTCCTGTGCACTT GTC ATC TGG ATG ACC CAG TCT CC-3'
HuVK2A/2B/6B-BACK	5'-CTCCTGTGCACTT GAT RIT GTG ATG ACH CAG TCT CC-3'
HuVK3A/3B/3C-BACK	5'-CTCCTGTGCACTT GAA ATW GTR WTG ACR CAG TCT CC-3'
HuVK5A-BACK	5'-CTCCTGTGCACTT GAA ACG ACA CTC ACG CAG TCT CC-3'
HuVK6A-BACK	5'-CTCCTGTGCACTT GAA ATT GTG CTG ACT CAG TCT CC-3'
HuCK-for	
Ascl	
HuCK-FOR	5'-TCTGGCGCGCCTTATTA ACA CTC TCC CCT GTT GAA GCT CTT TG-3'
HuVL-back	
ApaLI	
HuVL1A-BACK	5'-CTCCTGTGCACTT CAG TCT GTG CTG ACT CAG CCA CC-3'
HuVL1B/1C-BACK	5'-CTCCTGTGCACTT CAG TCT GTS BTG ACG CAG CCG CC-3'
HuVL2-BACK	5'-CTCCTGTGCACTT CAR TCT GCC CTG ACT CAG CCT-3'
HuVL3A/3B-BACK	5'-CTCCTGTGCACTT TCC TAT GWG CTG ACW CAG CCA CC-3'
HuVL3C-BACK	5'-CTCCTGTGCACTT TCT TCT GAG CTG ACT CAG GAC CC-3'
HuVL3D-BACK	5'-CTCCTGTGCACTT TCC TAT GAG CTG ATG CAG CCA CC-3'
HuVL4-BACK	5'-CTCCTGTGCACTT CAC GTT ATA CTG ACT CAA CCG CC-3'
HuVL5-BACK	5'-CTCCTGTGCACTT CAG GCT GTG CTG ACT CAG CCG TC-3'
HuVL6-BACK	5'-CTCCTGTGCACTT AAT TTT ATG CTG ACT CAG CCC CA-3'
HuVL7/8-BACK	5'-CTCCTGTGCACTT CAG RCT GTG GTG ACY CAG GAG CC-3'
HuVL9-BACK	5'-CTCCTGTGCACTT CWG CCT GTG CTG ACT CAG CCM CC-3'
HuCL-for	
Ascl	
HuCL-FOR	5'-TCTGGCGCGCCTTATTA AGA GCA TTC TGC AGG GGC CAC TG-3'
HuVH-back 5'UTR	
HuVH1-BACK, 5'UTR	5'-CTC CTG TGT CCT CTC CAC-3'
HuVH2-BACK, 5'UTR	5'-GCT GAC CAT CCC TTC ATG-3'
HuVH3-BACK, 5'UTR	5'-DAA RCA GTG GAT WTG TGT GGC-3'
HuVH4-BACK, 5'UTR	5'-GGC AGC TCC CAG ATG TGA G-3'
HuVH5-BACK, 5'UTR	5'-CTC CCC CAC AGG AGT CTG-3'
HuVH6-BACK, 5'UTR	5'-GCA GCA TTC ACA GAC TGA GG-3'
HuVH7-BACK, 5'UTR	5'-CTT CAT GCA CTC CCA TCT CC-3'

Restriction sites are indicated by underlining. Some positions are degenerated according to the following: R = (A or G), Y = (C or T), M = (A or C), K = (T or G), S = (C or G), W = (A or T).

(TAG Copenhagen, Denmark). Buffer, dithiothreitol (DTT) (Invitrogen, CA), 250 μ M dNTP (Invitrogen, CA), 20 units of RNasin (Promega, Germany) and 25 units of Superscript-II reverse transcriptase (Invitrogen, CA) were added, and incubation in a total volume of 50 μ l was performed for 2 h at 42 °C. The enzymatic reaction was stopped by incubation at 70 °C for 15 min.

2.7. Amplification of cDNA by a semi-nested PCR

After heat-inactivation the cDNA was used for PCR amplification with sets of primers designed to cover human IgG and IgE V_LC_L and V_HC_H regions (Table 3). The primers used for amplification of heavy and light chains, respectively, were taken from the literature

(Steinberger et al., 1996) and V-BASE (<http://www.mrc-cpe.cam.ac.uk/vbase-ok.php>), or were designed based upon sequences in the International Immunogenetics Database (<http://imgt.cines.fr:8104>), or the GenBank sequence database (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>). Occasionally, primers were degenerated in a few positions in order to get all possible combinations. All primers were from TAG Copenhagen, Denmark.

All PCR reactions were performed in a volume of 25 μ l using Ready-To-GoTM PCR beads (Amersham Biosciences, UK), 10 pmol of each primer and 1 μ l cDNA. For amplification of the human IgG and IgE heavy chain a semi-nested PCR was performed using primers in the 5'UTR and a primer covering IgE or a primer covering all four IgG isotypes. First seven separate IgG or IgE V_HC_H amplifications (95 °C for 1 min, 30 cycles (95 °C for 20 s, 65 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min) were performed using 1 μ l cDNA, the [HuVH-back 5'UTR] primers plus [HuCH-IgE FOR] or [HuCH-IgG1-4 FOR] to cover all V_H families. From this reaction 2 μ l was used as template in a second PCR (95 °C for 1 min, 3 cycles (95 °C for 20 s, 58 °C for 30 s, 72 °C for 1 min), 27 cycles (95 °C for 20 s, 65 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min) using [HuVH-back] and again [HuCH-IgE FOR] or [HuCH-IgG1-4 FOR] primers.

Light chains were amplified in six separate κ and 11 separate λ V_LC_L amplifications (95 °C for 1 min, 35 cycles (95 °C for 20 s, 58 °C for 30 s, 72 °C for 1 min), 72 °C for 5 min) to cover all V_L families. All different light chain and heavy chain PCR products were purified using GFXTM PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK).

2.8. Library construction

The construction of the Fab libraries was sequential. First, purified light chain PCR-fragments were mixed in equal amounts and digested with *Apa*LI and *Asc*I (New England Biolabs, MA). The digested fragments of the correct size (~650 bp) were gel-purified using GFXTM PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK) and cloned into the phagemid vector pCGJ (Fig. 1) also digested with *Apa*LI and *Asc*I. Plasmid DNA, containing light chain fragments, was purified from 50 ml overnight culture of transformed TG1 cells using Qiagen Plasmid Midi Kit (Qiagen, Norway). This light chain repertoire was digested with *Sfi*I and *Sgr*AI (New England Biolabs, MA) and gel-purified.

Subsequently, the different Fab libraries were obtained by cloning the heavy chain PCR-fragments of correct size (~670 bp), which were mixed in equal amounts and digested with *Sfi*I and *Sgr*AI, into the light chain plasmid repertoire.

2.9. Production of phage libraries

An overnight culture of TG1 cells containing Fab antibody library was diluted 1:100 in 50 ml 2 $\times$ TY containing

1% glucose and 100 μ g/ml ampicillin, and was incubated at 37 °C to OD₆₀₀ = 0.5. To 10 ml of this culture, a 20:1 molar excess of KM13 helper phage (Kristensen and Winter, 1998) was added. Following incubation for 30 min at 37 °C, cells were sedimented by centrifugation at 2500 $\times g$ for 10 min. The pellet was resuspended in 100 ml 2 $\times$ TY containing 100 μ g/ml ampicillin and 25 μ g/ml kanamycin and incubated overnight at 30 °C. Subsequently the cells were sedimented by centrifugation at 2500 $\times g$ for 10 min. The supernatant was mixed with 1/5 volume of 20% polyethylenglycol (PEG) 6000 containing 2.5 M NaCl and incubated for 1 h at 4 °C to pellet phage particles. Following centrifugation at 10,500 $\times g$ for 30 min, the phage pellet was resuspended in 15 ml H₂O. PEG precipitation was repeated and pellet was finally resuspended in 1.5 ml H₂O and sterilised by filtration over a 0.45 μ m filter.

2.10. Determination of display levels by titration

Display levels were determined as the relative amount of transducing units (TU) observed after versus before trypsin treatment. Phages were trypsinised for 10 min at room temperature in 50 mM Tris-HCl pH 7.4, 1 mM CaCl₂ supplemented with trypsin (Sigma-Aldrich, MO) to a final concentration of 1 mg/ml. Subsequently a log phase TG1 culture (OD₆₀₀ = 0.5) was infected with phages and TUs determined on 1 $\times$ TY agar plates supplemented with 100 μ g/ml ampicillin and 1% glucose.

2.11. Selection of monoclonal phage antibodies

M-280 Tosylactivated Dynabeads (Dyna, Norway) were coated with *H. lanuginosa* lipase (Novozymes, Denmark) or recombinant Bet v 1 (Biomay, Austria) according to the manufacturer's recommendations. The coated beads were washed three times in 10 ml PBS containing 0.5% (v/v) Tween20 and blocked with 2% (w/v) skimmed milk powder in PBS for 2 h at room temperature. The library was added to 100 μ l (2 $\times$ 10⁸) beads in PBS with 0.5% (w/v) skimmed milk powder and incubated for 2 h at 37 °C with slow tilt rotation. Subsequently the beads were washed twice for 10 min and three times shortly with 10 ml PBS containing 0.5% (v/v) Tween20, followed by washing twice for 10 min and three times shortly with 10 ml PBS. The sedimented beads were resuspended in 500 μ l of a 1 mg/ml trypsin solution in PBS, and bound phages were eluted by incubation for 10 min at room temperature with rotation. The eluted phages were added to 1 ml TG1 (OD₆₀₀ = 0.5) and incubated for 30 min at 37 °C without shaking. Plated on 1 $\times$ TY agar plates supplemented with 100 μ g/ml ampicillin and 1% glucose. The selections using the IgG-A and IgG-B libraries were propagated for a second round of selection by rescue with the helper phage KM13 as described above.

2.12. Production of monoclonal phages

Individual TG1 colonies were inoculated into 96 well plates (Nunc, Denmark) containing 100 μ l/well 2 $\times$ TY with 100 μ g/ml ampicillin and 1% glucose. The cultures were grown overnight at 37 °C while shaking (250 rpm). An inoculum of 2 μ l was transferred from each well to a second 96 well plate containing 200 μ l/well 2 $\times$ TY with 100 μ g/ml ampicillin and 1% glucose. Following incubation at 37 °C for 2 h while shaking (250 rpm), 25 μ l 2 $\times$ TY containing 100 μ g/ml ampicillin, 25 μ g/ml kanamycin and 10⁹ helper phages were added to each well and the plates were incubated at 37 °C for 1 h. The plates were centrifuged for 10 min at 1800 $\times g$ and the pellets were resuspended in 200 μ l 2 $\times$ TY containing 100 μ g/ml ampicillin and 25 μ g/ml kanamycin. These cultures were incubated overnight at 30 °C while shaking. Cells were sedimented by centrifugation at 1800 $\times g$ for 10 min, and the phages in the supernatant were then used for direct phage ELISA.

2.13. Monoclonal phage ELISA

H. lanuginosa lipase or recombinant Bet v 1 were coated overnight at 4 °C on an ELISA maxisorp plate (Nunc, Denmark) at 1 μ g/ml in PBS (100 μ l/well) followed by blocking with 300 μ l 2% (w/v) skim milk in PBS for 2 h at room temperature. 50 μ l monoclonal phages and 100 μ l PBS with 0.5% (w/v) skim milk were added per well, and the plate was incubated 2 h at RT. After three washes with PBS with 0.1% (v/v) Tween20, bound phages were detected with HRP-conjugated mouse anti-M13 (Amersham Biosciences, UK). Staining was performed using 100 μ l 3,3',5,5'-tetramethylbenzidine (TMB+) substrate (Kem-En-Tec, Denmark) per well. The colour reaction was stopped with 100 μ l 2 M H₂SO₄ and the optical density at 450 nm was measured.

The clones which were positive in the monoclonal phage ELISA and did not react with skimmed milk were tested again but with known amount of phages based on TUs.

Competitive ELISA was performed by adding an amount of monoclonal phages, previously determined in direct phage ELISA to give an OD value =1 at 450 nm, together with serum or purified IgG or IgE in dilution series, followed by incubation for 2 h at room temperature. Washing and staining was performed as described above. Polyclonal sera from rabbits immunised with lipase or subtilisin were used as controls.

2.14. Screening and sequencing of clones

Individual clones from the different libraries before and after selections were sequenced by amplifying the inserted fragments in a colony PCR using the primers M13-back (5'-GAA TTT TCT GTA TGA GG-3') and

M13-rev (5'-GAA ACA GCT ATG ACC ATG-3') (TAG Copenhagen, Denmark). The gene segments were identified using the International Immunogenetics Database (<http://imgt.cines.fr:8104>), the GenBank sequence database (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) and V-BASE (<http://www.mrc-cpe.cam.ac.uk/DNAPLOT.php>).

2.15. Surface plasmon resonance (Biacore)

Recombinant Bet v 1 and *H. lanuginosa* lipase was immobilised in 10 mM sodiumacetate, pH 4.0 at a concentration of 15 μ g/ml on the CM5 biosensor chip using the *N*-hydroxy-succinimide (NHS)/*N*-ethyl-*N'*-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) kit (Biacore, Sweden) yielding a surface of approximately 1000 RU. Phages were diluted two-fold in HBS-EP running buffer (10 mM HEPES, 3.4 mM EDTA, 150 mM NaCl, 0.05% (v/v) surfactant P20, pH 7.4) and binding characteristics were analysed at a flow rate of 4 μ l/min.

The kinetics of binding was analysed using the BIAevaluation software.

2.16. Purification of IgE and IgG from serum

Human IgE and IgG was purified by affinity chromatography from sera obtained from the patients donating the B-cells for library production. Five mg of mouse anti-human IgE (ATCC clone HB121) was coupled to 1.5 g sepharose 4B (Amersham Biosciences, UK). NaCl was added to 10 ml serum resulting in a final concentration of 0.5 M. The sample was applied to the column, followed by washing with 100 mM phosphate buffer, pH 8.0 with 0.5 M NaCl. Bound human IgE was eluted with 0.1 M citric acid. Fractions containing IgE were pooled, buffer was changed to 100 mM phosphate, pH 8.0, and the antibodies were concentrated to ~2 ml using Ultracel Amicon YM30 Ultrafiltration Discs (Millipore, MA).

The effluent from the anti-IgE column was loaded on a protein A column (Amersham Biosciences, UK) to purify total IgG. The protein A column was washed with 100 mM phosphate buffer, pH 8.0 with 0.5 M NaCl. Bound human IgG was eluted with 0.1 M citric acid. Fractions containing IgG were pooled, buffer was changed to 100 mM phosphate, pH 8.0, and the antibodies were concentrated to ~25 ml as described for IgE.

3. Results

3.1. Determination of the fraction of B-cells contributing to IgE production

The transcription level of human IgE in B lymphocytes is very low and therefore it has proven necessary to stimulate the B lymphocytes using IL-4 and anti-CD40 to promote

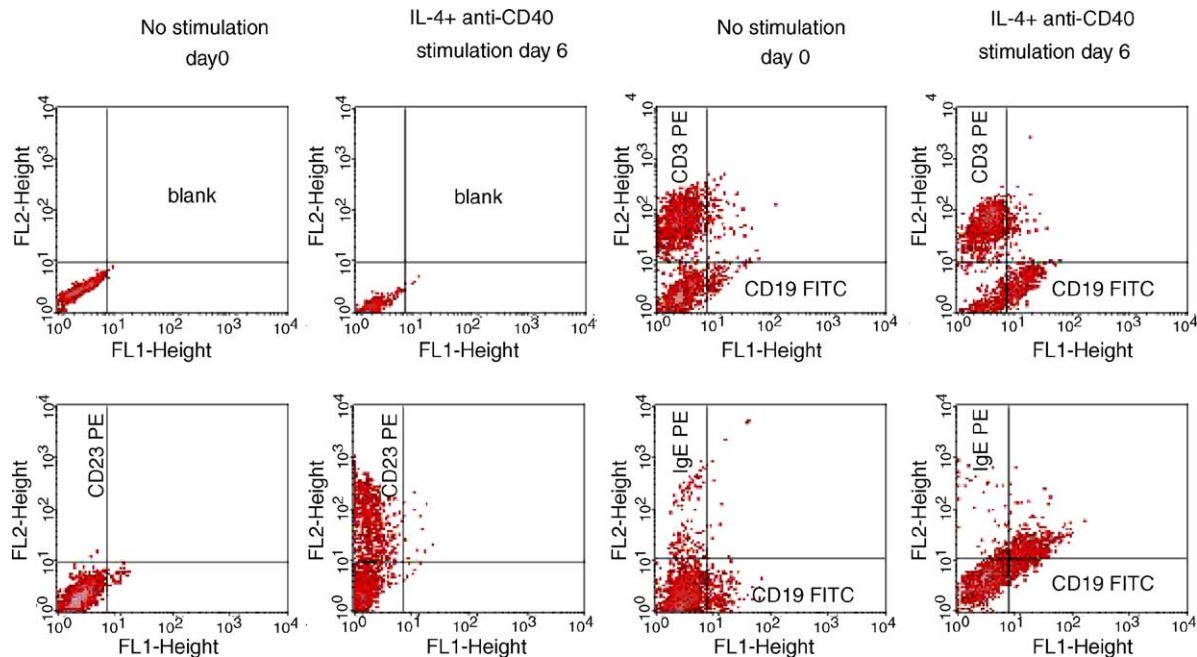


Fig. 2. Effect of IL-4 + anti-CD40 induction of human PBMCs. Isolated PBMCs cultured with IL-4 and anti-CD40 for 6 days were stained with anti-CD3 PE, anti-CD19 FITC, anti-CD23 PE, or anti-human-IgE PE and analysed by flow cytometry.

the class switch to IgE to ensure an adequate mRNA level allowing library construction.

Isolated human PBMCs stimulated with human IL-4 and anti-CD40 were analysed with flow cytometry (Fig. 2). After stimulation for 6 days the number of B-cells (CD19+) had increased from 4% to 22% whereas the T-cell (CD3+) population had increased from 43% to 50%. The low affinity IgE receptor, FcεRII (CD23), a B-cell marker involved in the regulation of human IgE synthesis (Bonnefoy et al., 1995) was shown to be induced after stimulation of B-cells with IL-4 and anti-CD40 (Defrance et al., 1987; Saeland et al., 1993). The same was observed in this experiment with an increase from 0.06% to 36% of the PBMCs expressing CD23, and it was also seen that the amount of IgE producing B-cells (CD19+/IgE+) had increased from 0.4% to 23%.

3.2. Library construction

The Fab libraries were constructed in two steps as described in Section 2. All combinations of light chain primers amplified variable and constant domains. In contrast not all combinations of heavy chain primers were able to amplify IgE and IgG variable and constant domains. Nevertheless, though PCR products were not obtained from all V_H families, the resulting PCR products were pooled and cloned into the pCGJ phagemid vector.

For the IgG-B library it was mainly the [HuCH4A-BACK] and [HuCH4B-BACK] primers (Table 3) that gave rise to

amplification, whereas for the other libraries more primer combinations were able to amplify the heavy chain genes. After insertion of the light chain fragments, 100% of the clones in the IgG-A and IgG-B library contained an insert (showed by colony-PCR and sequencing), whereas for the IgE-C, IgG-C, and IgG-D light chain libraries only about 85% of the clones contained a light chain insert, which also lead to a lower percentage of clones in the Fab libraries containing two inserts. An overview of the different libraries is given in Table 4.

3.3. Selection of phage-displayed Fab antibodies against lipase and Bet v 1

H. lanuginosa lipase was chosen for selection with the human libraries from a non-allergic individual (IgG-A) and a patient allergic to lipase (IgG-B), whereas the major allergen of *Betula verrucosa*, Bet v 1, was used for selections with all the human libraries.

Two rounds of selections were performed with the first two libraries whereas only one round of selection was performed with the three human libraries from patients allergic to birch pollen as these libraries were smaller than the others, and already after one selection round the amount of selected phages was low (Table 5). No positive antibodies were isolated from the library from the non-allergic person (IgG-A) but clones with specificity for either lipase or Bet v 1 were isolated from all other libraries (Table 5). The selected clones were analysed in direct phage ELISA. Only positive clones were further analysed.

Table 4
Size and composition of the human phage Fab antibody libraries

Library	Description	Library	Insert (%)	Size (TU)	Display level (%)
IgG-A	Human IgG1-4 Fab library made from a non-allergic donor	V _L C _L	100	$\sim 1 \times 10^6$	0.5
		Fab	92	$\sim 1 \times 10^6$	
IgG-B	Human IgG1-4 Fab library made from a donor allergic to <i>H. lanuginosa</i> lipase	V _L C _L	100	$\sim 2 \times 10^5$	5
		Fab	85	$\sim 1 \times 10^6$	
IgE-C	Human IgE Fab library made from a donor allergic to birch pollen	V _L C _L	86	n.d.	0.2
		Fab	n.d.	$\sim 1 \times 10^3$	
IgG-C	Human IgG1-4 Fab library made from a donor allergic to birch pollen	V _L C _L	86	n.d.	0.1
		Fab	63	$\sim 4 \times 10^4$	
IgG-D	Human IgG1-4 Fab library made from a donor allergic to birch pollen	V _L C _L	83	n.d.	0.3
		Fab	50	$\sim 1 \times 10^4$	

n.d.: not determined.

3.4. Antibody sequences

The DNA encoding the selected antibodies was sequenced and the amino acid sequences were deduced (Table 6). The four human IgG antibodies recognising lipase (IgG-B-1G1, -1G2, -1H10, and -1H11) used light chain fragments from two different V κ families and from one V λ family. The heavy chains were almost identical except for a few bases. The CDR3 regions of the heavy chains contained two amber stop codons but as the phages were produced in the amber codon suppressing *E. coli* strain TG1, the stop codons were read through and glutamine was inserted.

The three human IgE antibodies recognising Bet v 1 (IgE-C-H1, -H2 and -H7) had different light chains but IgE-C-H1 and -H7 had identical heavy chains except for the CDR3 region. The six human IgG antibodies recognising Bet v 1 (IgG-C-1A2, -1A12, -1D12, -2D1, IgG-D-1D2 and -1G12) used light chain fragments from two different V κ families and from one V λ family. The three light chains from the V κ 3 family were very homologous and the same was the case for the three heavy chains from the IgG-C library belonging to the V_H4 family and the two heavy chains from the IgG-D library, respectively.

The CDR3 regions of all the selected antibodies were rich in aromatic residues.

3.5. Affinity measurements

The affinity to Bet v 1 and lipase were determined for antibodies displayed on phages (Table 7) by surface plasmon resonance.

The four lipase-specific IgG Fab clones had binding affinities (K_a) to lipase at $\sim 10^9 \text{ M}^{-1}$ with the IgG-B-1G2 clone having the highest affinity and IgG-B-1G1 the lowest. Compared to a polyclonal rabbit-anti-lipase antibody the affinities of the selected clones were 20–100-fold higher.

The binding affinities to Bet v 1 of the three IgE clones from the IgE-C library were about 10^9 to 10^{10} M^{-1} with the IgE-C-H1 clone having the highest Bet v 1 affinity. The six clones from the two IgG libraries, IgG-C and IgG-D, had affinities to Bet v 1 at 10^8 to 10^9 M^{-1} .

3.6. Competitive ELISA

The selected antibodies were tested in competitive ELISA. The binding between antibody-displaying phages and their antigens was tested in the presence of different

Table 5
Outcome of selections performed using the different libraries

Library		Selection round	Input titer	Output titer	Positive clones	Name of clones
IgG-A	Lipase	1	4×10^{10}	2350	0/96	–
IgG-A	Lipase	2	1.5×10^8	52	0/52	–
IgG-A	Bet v 1	1	4×10^{10}	1800	n.d.	–
IgG-A	Bet v1	2	5×10^7	44	0/44	–
IgG-B	Lipase	1	2×10^{10}	1250	n.d.	–
IgG-B	Lipase	2	4.4×10^8	24	4/24	IgG-B-1G1, IgG-B-1G2, IgG-B-1H10, IgG-B-1H11
IgG-B	Bet v 1	1	2×10^{10}	850	n.d.	–
IgG-B	Bet v 1	2	7.5×10^5	38	0/38	–
IgE-C	Bet v 1	1	1.5×10^8	8	3/8	IgE-C-H1, IgE-C-H2, IgE-C-H7
IgG-C	Bet v 1	1	3.8×10^{10}	152	$\geq 4/152$	IgG-C-1A2, IgG-C-1A12, IgG-C-1D12, IgG-C-2D1
IgG-D	Bet v 1	1	3×10^{10}	198	$\geq 4/198$	IgG-D-1D2, IgG-D-1G12

n.d.: not determined.

Table 6
Deduced antibody sequence of the CDR3 regions of the isolated antibodies

Clone	Specificity	Ab isotype	VH family	VH segment	CDR3	VL family	VL segment	CDR3
IgG-B-1G1	Lipase	IgG2	VH4	VH4-34	CGLGTH Q DQEAQDPKNLEPAD Q SHYCGLDV	Vκ1	L8	QQTYDFLAYT
IgG-B-1G2	Lipase	IgG2	VH4	VH4-34	CGLGTH Q DQEAQDPKNLEPAD Q SHYCGLDV	Vκ1	O2	QQSYITPWT
IgG-B-1H10	Lipase	IgG2	VH4	VH4-34	CGLGTH Q DQEAQDPKNLEPAD Q SHYCGLDV	Vκ3	A27	QQYAAPPRT
IgG-B-1H11	Lipase	IgG2	VH4	VH4-34	CGLGTH Q DQEAQDPKNLEPAD Q SHYCGLDV	Vλ2	V1-2	SSYAADKNV
IgE-C-H1	Bet v 1	IgE	VH7	VH7-81	RVPSRPYDYIWGSYPDL	Vκ1	O12	QQSYSTPRT
IgE-C-H2	Bet v 1	IgE	VH1	VH1-18	RSPVVVPAADAFDI	Vλ1	1c	AAWDDSLSGRVV
IgE-C-H7	Bet v 1	IgE	VH7	VH7-81	SQGEAFDI	Vκ3	L6	QQRSNWPLT
IgG-C-1A2	Bet v 1	IgG2/3	VH4	VH4-34	GIGRFLEWSYPQPRYYHYGMDV	Vκ3	L6	QQRSNWFLT
IgG-C-1A12	Bet v 1	IgG3	VH4	VH4-34	GRPGANSYYYYGMDV	Vκ3	L6	QQRSNWPLT
IgG-C-1D12	Bet v 1	IgG1/2	VH6	VH6-1	RGWQLDY	Vκ3	L6	QQRSNWFLT
IgG-C-2D1	Bet v 1	IgG1	VH4	VH4-34	GIGRFLEWSYPQPRYYHYGMDV	Vκ1	L8	QQLSNYPWT
IgG-D-1D2	Bet v 1	IgG1	VH4	VH4-39	DRGGIALGTLDI	Vλ2	2a2	SSYTTSSTRV
IgG-D-1G12	Bet v 1	IgG1	VH4	VH4-39	DRGGIALGTLDI	Vκ1	O2	QQSYSTPRS

Stop codons read as Q are indicated in bold.

concentrations of human sera from persons allergic to Bet v 1 or lipase, or in the presence of human total IgE or total IgG purified from sera. As additional controls rabbit-anti-lipase and rabbit-anti-subtilisin were used.

The binding to lipase of the antibodies selected from the human IgG library, IgG-B, was reduced to 40% of the original binding for all four clones when purified IgG was added. The concentration of the purified IgG necessary for seeing an inhibition was below 10 ng/ml. Human serum from the individual the library was created from, and also sera from lipase-immunised rabbits inhibited the binding of the phages, though a higher immunoglobulin concentration was required than for the purified IgG. One exception was clone, IgG-B-1H10, which required a very low concentration of rabbit Ig in serum to see an inhibition (<1 ng/ml). The binding of all four phage antibody clones was also diminished

by addition of serum from a patient allergic to birch pollen (donor C) though it was not supposed to contain antibodies recognising lipase. However, this non-specific inhibition was always observed when using high concentrations of human or rabbit serum.

Fig. 3 shows that the binding to Bet v 1 of the three antibodies selected from the human IgE library, IgE-C, was decreased with increasing concentrations of competing antibodies. Especially for the IgE-C-H1 clone, where the binding was reduced to at least 20% when purified IgE or serum from a person allergic to birch pollen was added and to 60% when purified IgG was added. The binding of IgE-C-H2 was only reduced to 75% when purified IgE or IgG were added but the IgE/IgG concentration needed to see this binding reduction was below 1 pg/ml whereas a high concentration of antibodies in human serum was necessary. The binding

Table 7
Surface plasmon resonance

Clone	k_{on} ($M^{-1} s^{-1}$)	k_{off} (s^{-1})	K_a (M^{-1})
Lipase			
IgG-B-1G1	$(2.50 \pm 1.65) \times 10^6$	$(1.63 \pm 0.81) \times 10^{-3}$	$(1.47 \pm 0.28) \times 10^9$
IgG-B-1G2	$(3.56 \pm 1.42) \times 10^7$	$(4.18 \pm 0.35) \times 10^{-3}$	$(8.40 \pm 2.71) \times 10^9$
IgG-B-1H10	$(7.98 \pm 7.52) \times 10^6$	$(2.57 \pm 2.63) \times 10^{-3}$	$(3.39 \pm 0.55) \times 10^9$
IgG-B-1H11	$(9.08 \pm 1.87) \times 10^5$	$(2.31 \pm 0.22) \times 10^{-3}$	$(4.00 \pm 1.19) \times 10^9$
Rabbit-anti-lipase	2.27×10^3	3.03×10^{-5}	7.49×10^7
WT phage	0	0	–
Bet v 1			
IgE-C-H1	$(5.77 \pm 0.93) \times 10^6$	$(6.00 \pm 0.81) \times 10^{-4}$	$(9.80 \pm 2.86) \times 10^9$
IgE-C-H2	$(6.07 \pm 2.00) \times 10^5$	$(7.78 \pm 2.81) \times 10^{-4}$	$(8.84 \pm 5.77) \times 10^8$
IgE-C-H7	$(3.03 \pm 2.31) \times 10^6$	$(1.03 \pm 0.08) \times 10^{-3}$	$(2.86 \pm 2.00) \times 10^9$
IgG-C-1A2	$(2.47 \pm 1.66) \times 10^6$	$(3.10 \pm 0.30) \times 10^{-3}$	$(8.25 \pm 6.15) \times 10^8$
IgG-C-1A12	$(1.34 \pm 0.28) \times 10^6$	$(5.07 \pm 3.08) \times 10^{-4}$	$(3.45 \pm 2.66) \times 10^9$
IgG-C-1D12	$(1.81 \pm 0.68) \times 10^6$	$(3.97 \pm 1.39) \times 10^{-3}$	$(5.16 \pm 3.43) \times 10^8$
IgG-C-2D1	$(6.66 \pm 0.34) \times 10^5$	$(5.33 \pm 1.65) \times 10^{-3}$	$(1.30 \pm 0.34) \times 10^8$
IgG-D-1D2	$(3.08 \pm 3.79) \times 10^6$	$(5.45 \pm 4.31) \times 10^{-4}$	$(4.24 \pm 3.60) \times 10^9$
IgG-D-1G12	$(2.35 \pm 1.54) \times 10^6$	$(4.90 \pm 4.79) \times 10^{-4}$	$(6.25 \pm 2.96) \times 10^9$
WT phage	0	0	–

Binding affinity K_a (M^{-1}) = k_{on}/k_{off} to lipase and Bet v 1 for the Fab antibodies displayed on phages. All affinities are an average of at least two experiments.

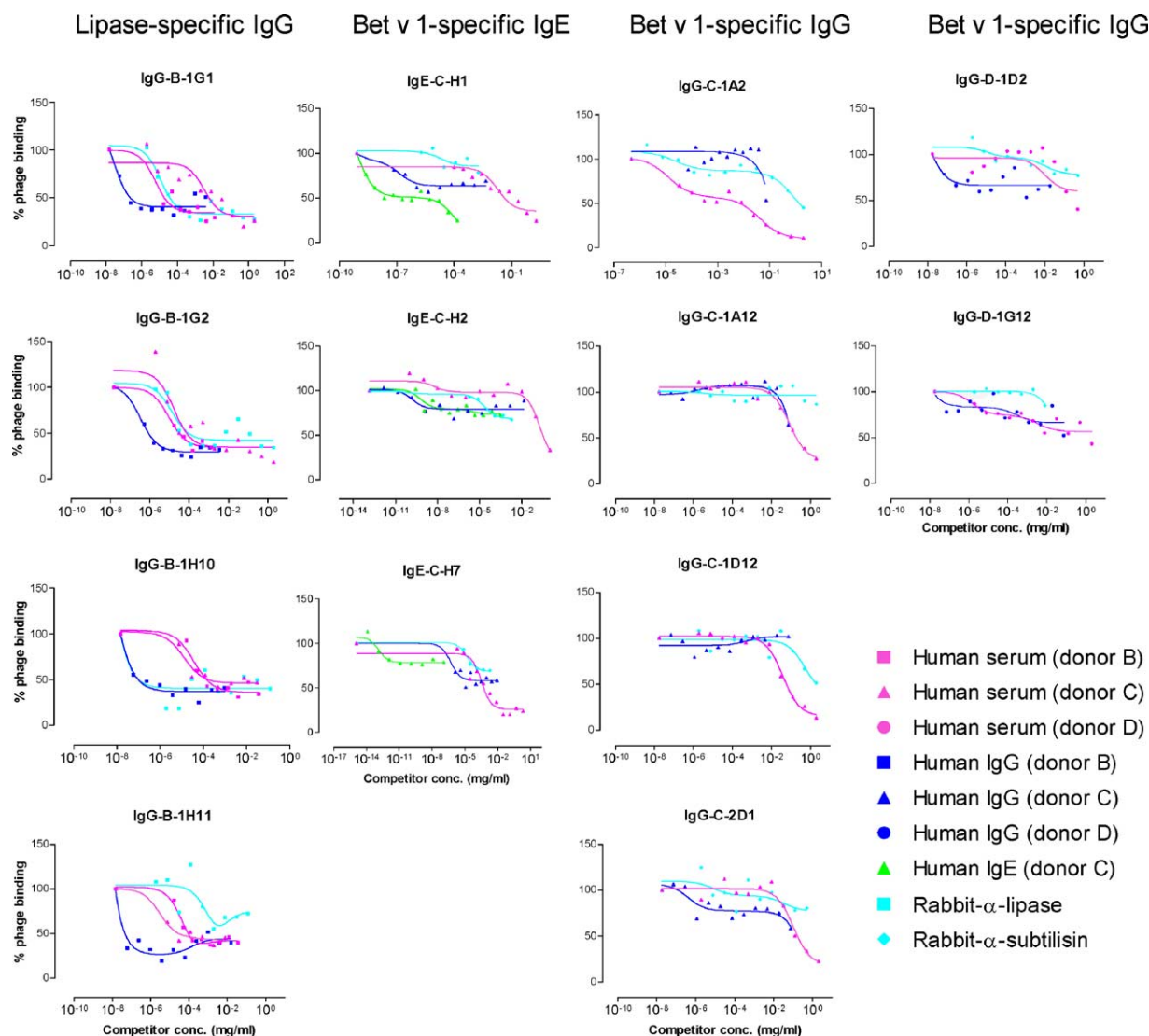


Fig. 3. Competition ELISA with IgG and IgE phage antibodies recognising lipase and Bet v 1. The same amount of phages was added to each well coated with allergen in competition with varying concentrations of competitor (serum or purified human IgG/IgE). The percent of phages binding to the allergen is plotted against the competitor concentration. No competitor is 100%.

to Bet v 1 of the IgE-C-H7 clone was also inhibited with the addition of extra antibodies. When using IgE the binding was reduced to 75% of the original binding whereas the binding was reduced to 55% when using IgG. Serum from a rabbit immunised with subtilisin also gave a small decrease in the phage binding for all three clones at relatively high Ig concentrations compared to the purified antibodies.

The binding to Bet v 1 of the six antibodies selected from the two human IgG libraries, IgG-C and IgG-D, was also reduced with increasing serum Ig concentrations (Fig. 3). IgG-C-1A12, -1D12, -2D1, and IgG-D-1D2 required a high serum Ig concentration (10 μ g/ml), whereas the binding of IgG-C-1A2 and IgG-D-1G12 was reduced at a much lower serum Ig concentration ($\sim$ 1 ng/ml) though the binding was almost totally inhibited for all clones. IgG-C-1A2, -1A12, and -1D12 needed a relatively high IgG concentration

before binding was reduced compared to the IgE clones from the IgE-C library, whereas the competition curves of IgG-C-2D1, IgG-D-1D2, and -1G12 more resembled the curves of the clones from the IgE library though the binding was not inhibited more than $\sim$ 50%.

Though IgG-C-1A2 and IgG-C-1D12 showed both sequence and affinity similarity, they had different inhibition curves.

Again a binding reduction with non-specific anti-sera was observed at high competitor concentration.

The titer of the Bet v 1-specific IgG in serum from donor C having received immunotherapy was $\sim$ 500 times higher than in the serum from donor D (data not shown). However, the affinity of the total IgG from donor C used for binding-competition of the IgG-C clones was $\sim 5 \times 10^6$ M $^{-1}$, whereas the affinity of the IgG from donor D

used for binding-competition of the IgG-D clones was $\sim 5 \times 10^7 \text{ M}^{-1}$, which might explain the lower concentration of donor D-IgG needed for inhibiting the binding of the IgG-D clones.

4. Discussion

One of the most important and promising applications of phage display has been the capability to generate antibodies in vitro. In contrast to time-consuming and expensive immunisation of animals and subsequent immortalisation of B-lymphocytes expressing the sought monoclonal antibody, a panel of phage-generated monoclonal antibodies can ideally be obtained in a few weeks at reduced costs.

Five different human IgE and IgG phage display libraries from the in vivo rearranged V-gene repertoire of human allergic and non-allergic donors have been constructed. The display levels fall within the “normal” range which is between 1:9 and 1:1000 for polypeptide-pIII fusions depending on the type of phagemid, growth conditions, the nature of the polypeptide fused to pIII, and proteolytic cleavage of antibody-pIII fusions (Azzazy and Highsmith, 2002).

Using the trypsin sensitive helper phage KM13 (Kristensen and Winter, 1998) in phage antibody selections it was possible to isolate a panel of antibodies from the different libraries applying only one or two rounds of selection. The selected antibodies were characterised by affinity measurements and competitive ELISA using different sera and purified antibodies.

The library from a person allergic to lipase (IgG-B) mainly contained heavy chains from the V_H4 family and was of isotype IgG2, which of course also resulted in selecting antibodies of this type. The other libraries contained heavy chains from more families. However, the clones selected from the two IgG libraries all derived from the V_H4 family, whereas the IgE clones belonged to the V_H1 and V_H7 families. This may support different V_H usage in IgG and IgE antibodies from the same patient. It has previously been observed that antibodies of some atopic individuals exhibit a broad usage of V_H -gene families (Eibenstein et al., 2000; Tilgner et al., 1997), whereas for others the V_H usage is biased towards a few families (Demaison et al., 1995; Snow et al., 1995; Edwards et al., 2002). The V_H usage in donor B was probably biased against V_H4 , as the amplification of other V_H families was tried several times and under different conditions without success.

The three libraries produced from two different persons allergic to birch pollen were very small, which were probably due to low transformation efficiencies. Nevertheless there was some diversity in the libraries, and it was still possible to select different high affinity Bet v 1-binding antibodies. The affinities of the lipase-specific clones, which were selected from a larger library, are slightly higher than the Bet v 1 specific clones, but as it is two different allergens and

different libraries it might be difficult to draw any conclusions on that.

In the two studies performed with human IgE phage libraries (Edwards et al., 2002; Steinberger et al., 1996), the affinities of the selected Fab antibodies remain to be determined, but the affinities of the IgE antibodies that was selected in this study are in the same range as the selected IgG antibodies, and they are also in agreement with what has previously been observed (Sheets et al., 1998; Vaughan et al., 1996).

If the selections using the library from a non-allergic donor (IgG-A) had been repeated under less stringent conditions, it might have resulted in antibodies of lower affinity. The reason for the high-affinity antibodies in the libraries from allergic donors is probably somatic hypermutation after allergen exposure.

The CDR3 regions of all the selected IgE and IgG Fab antibodies are rich in aromatic residues. Consistent with this, other studies have shown, that CDR3 regions that were rich in aromatic amino acids were found to sustain the allergen cross-reactivity of specific IgE (Droupadi et al., 1994), and previous results have also shown a high percentage of aromatic residues in allergen-specific antibodies (IgE and IgG) (Steinberger et al., 1996; Li et al., 1997). By molecular modelling they were predicted to interact with the antigen (Li et al., 1997).

With further developments the constructed libraries might be used for allergenicity testing of both new and existing proteins. Bet v 1 is known to be very allergenic. More than 95% of the patients suffering from IgE-antibody-mediated hypersensitivity to birch pollen produce IgE directed against the major birch pollen allergen, Bet v 1, and about 60% of these react exclusively with this allergen (Jarolim et al., 1989; Niederberger et al., 1998). On the contrary *H. lanuginosa* lipase is known to be less allergenic; at least when administered alone (Sarlo et al., 1997) possibly explaining why no positive binders were isolated from the human library created from a non-allergic person but only from the library created from a person known to be allergic to lipase. However, if all the libraries had been larger, it might have been possible to analyse less allergenic proteins for their binding to antibodies in the libraries.

If more antibodies had been selected, mixtures of the allergen-specific recombinant monoclonal Fabs blocking the allergen-IgE interaction might have been considered as potential therapeutic tools in allergic patients as they recognise the same epitope pattern as human polyclonal serum with a high affinity. The binding of the three IgE clones to Bet v 1 is inhibited by purified human polyclonal IgE and IgG, suggesting that IgE epitopes overlapped by IgG epitopes are recognised. Especially the IgE-C-H1 clone, whose binding is diminished to less than 20% in competition with polyclonal IgE seems to recognise an important IgE epitope on Bet v 1. It has been shown that IgE and IgG epitopes on allergens can be overlapping and share sites (Zhang et al., 1992) or totally distinct (Kobayashi et al., 1996). This might explain

why the binding of some of the IgE-displaying phages are inhibited solely by either IgE or IgG, whereas the binding of others are inhibited by both IgE and IgG. If the genes encoding the different antibodies were derived from B-cells, which had undergone class switching, this shift may have been from IgG, giving rise to IgE antibodies recognising IgG epitopes.

The lipase and Bet v 1 specific IgG Fab clones look as if they recognise IgG epitopes as their binding is inhibited by purified human polyclonal IgG. Since the clones have similar K_a values, but between the clones none of the competition graphs are alike, they probably recognise different IgG epitopes on lipase and Bet v 1.

In conclusion five different human IgE and IgG phage libraries were created and a panel of high-affinity allergen-specific Fabs were isolated from these libraries. The binding between the allergen and the isolated antibodies was further characterised by competitive ELISA.

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