A Large Non-immunized Human Fab Fragment Phage Library That Permits Rapid Isolation and Kinetic Analysis of High Affinity Antibodies*

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We report the design, construction, and use of the first very large non-immunized phage antibody library in Fab format, which allows the rapid isolation and affinity analysis of antigen-specific human antibody fragments. Individually cloned heavy and light chain variable region libraries were combined in an efficient two-step cloning procedure, permitting the cloning of a total of $3.7 \times 10^{10}$ independent Fab clones. The performance of the library was determined by the successful selection of on average 14 different Fabs against 6 antigens tested. These include tetanus toxoid, the hapten phenyl-azochrome, the breast cancer-associated MUC1 antigen, and three highly related glycoprotein hormones: human chorionic gonadotropin, human luteinizing hormone, and human follicle-stimulating hormone. In the latter category, a panel of either homone-specific or cross-reactive antibodies were identified. The design of the library permits the monitoring of selections with polyclonal phage preparations and to carry out large scale screening of antibody off-rates with unpurified Fab fragments on BIAcore. Antibodies with off-rates in the order of $10^{-2}$ to $10^{-4}$ s$^{-1}$ and affinities up to $2.7 \times 10^{	ext{i}}$ nM were recovered. The kinetics of these phage antibodies are of the same order of magnitude as antibodies associated with a secondary immune response. This new phage antibody library is set to become a valuable source of antibodies to many different targets, and to play a vital role in target discovery and validation in the area of functional genomics.

Display on filamentous phage in combination with selection forms a powerful tool for the identification of peptide- or protein-based drugs (1, 2). Of these, antibodies are especially of interest, due to their capacity to recognize a variety of targets with high specificity and affinity. In particular, the use of partially or completely human antibodies, which elicit no or minimal immune response when administered to patients, is yielding an increasing list of FDA-approved protein-based drugs (3). Phage display technology enables the generation of large repertoires of human antibodies (4–7), while the biopanning procedure permits the selection of individual antibodies with a desired specificity.

Key to the success of the technology were two critical observations: (i) the expression of functional antibody fragments by secretion into the periplasm of Escherichia coli (8, 9), and (ii) the rapid access to variable region gene pools by the polymerase chain reaction (10–12). For the construction of antibody libraries, V-genes are amplified from B cell cDNA and heavy and light chain genes are randomly combined and cloned to encode a combinatorial library of single-chain Fv (scFv)1 or Fab antibody fragments (4, 13–15). The natural primary (unscreened) antibody repertoire within B cells contains a large array of antibodies that recognize a variety of antigens; this array can be cloned as a "naive" repertoire of rearranged genes, by harvesting the V-genes from the IgM mRNA of B cells of unimmunized human donors, isolated from peripheral blood lymphocytes (4), from bone marrow or tonsils (7), or from similar animal sources (16). This procedure provides access to antibodies that have not yet encountered antigen, although the frequency of those genuine "germline" antibodies will depend heavily on the source of B cells (17). A single naive library, if sufficiently large and diverse, can indeed be used to generate antibodies to a large panel of antigens, including self, non-immunogenic and relatively toxic antigens (4, 6). In a different approach, antibodies may be built artificially, by in vitro assembly of V-gene segments and D(J) segments, yielding "synthetic" antibodies (5). A major drawback of these procedures is that from the initial naive and synthetic libraries, only moderate affinity antibodies were isolated (4, 18). Over the last few years, more efficient techniques have been developed to build larger libraries of antibody fragments, using sophisticated in vivo recombination methods (6) or brute force cloning procedures (7, 19). Such large libraries have yielded a greater number of human antibodies per antigen tested, with on average much higher affinity (up to subnanomolar). However, technical restrictions on the size of libraries that may be obtained or handled in selection, the loss of library diversity upon library amplification, and the relatively long downstream analysis path of the selected antibodies, i.e. large scale affinity analysis, have limited the spread of these libraries as generic tools in antibody generation.

We describe here the generation of a very large antibody library based on the display of Fab fragments on phage. The choice for the Fab format was based on the notion that the monomeric appearance of the Fab should permit the rapid screening of large numbers of clones on kinetics of binding

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1 The abbreviations used are: scFv, single-chain Fv fragment; PCR, polymerase chain reaction; PBL, peripheral blood lymphocyte; BSA, bovine serum albumin; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; RU, resonance units; hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; hFSH, human follicle-stimulating hormone; CTP, carboxyl-terminal peptide.
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subsequently, buffer and dithiothreitol were added according to the

DNA was sheared to completion by passing through a narrow syringe

min at 65 °C in the presence of 20

of spleen RNA was used as template. RNA was heat-denatured for 5

Polytron homogenizer in 20 ml of 8 M guanidinium thiocyanate, 0.1 M

were isolated from 2 liters of blood on a Ficoll-Paque gradient. For

also carried out using

against the highly homologous glycohormones, demonstrating

was analyzed by the selection with an extended panel of anti-

37 billion different Fab clones. The performance of the library

multimerization of scFv on phage with respect to the

selection of fragments with very low affinities has indeed been

observed previously (6). Therefore, compared with scFv libraries,

selection, with Fab phage may be more governed by avidity

rather than avidity, even when performing selections by

panning on immobilized antigen (24) or with soluble multiva-

itly rather than avidity, was checked by analysis of 4 μl of the "unamplified" PCR mixture on agarose gel.

Construction of the Primary and Secondary Repertoires—For the

construction of the primary heavy chain and the two primary light

chain repertoires, the PCR products, appended with restriction sites,

were gel-purified prior to digestion and the different VH, Vk, and Va families combined into three groups. The VeCx and ViCa fragments were digested with ApoLi and Ascl, and cloned into the phagemid vector pCES1 (Fig. 1). The VH fragments, 1.5 μg in total, were digested with SfI and BstEII and ligated in a 100–200-μl reaction mixture with

9 units of T4-DNA ligase at room temperature to 4 μg, gel-purified vector pUC19-CES1 (similar to vector pCES1, but with the PII gene deleted). The desalted ligation mixture for light or heavy chain pools was used for electroporation of the E. coli strain TG1, to create the one-chain libraries.

The Fab library was obtained by cloning of VH fragments, digested from plasmid DNA prepared from the heavy chain repertoires, into the plasmid collection containing the light chain repertoires. Plasmid DNA, isolated from at least 3 × 10^9 bacteria of the VH library, was digested with SfI and BstEII for cloning in the vector that already contained a light internal bacteriophage phage helper. To retain clones with approximate equal abundance in the library, the cloning of VHChi in the light chain repertoire containing vector was also carried out using SfI and NorI cloning sites, to create a less restriction-biased Va library.

The Rescue of phagemid particles with helper phage M13-K07 was performed according to (4) on a 10-liter scale, using representative numbers of bacteria from the library for inoculation, to ensure the presence of at least 10 bacteria from each clone in the start inoculum. For selections, 10^10 colony-forming units were used with antigens immobilized in immunotubes (Maxisorp tubes, Nunc) (4) or with soluble biotinylated antigens (28). The amount of the immobilized antigens tetanus toxoid and the hapten phenyl-oxazolone (conjugated to BSA in a ratio of 17 to 1) was reduced 10-fold during subsequent selection rounds, starting at 100 μg/ml at round 1. Capture with biotinylated antigen in solution was used for a 100-mer peptide encoding five copies of the tandem repeat of MUC1 (29), or with human chorionic gonadotropin (hCG), human luteinizing hormone (hLH), human follicle-stimulating hormone (hFSH) and its chimeric derivative (hFSH-CTP), containing the carboxyl-terminal peptide from the hCG β-subunit fused to the β-subunit of hFSH. Antigens were biotinylated at a ratio of 10–20 molecules of NHS-Biotin (Pierce) per molecule of antigen according to the supplier's recommendations. Unless stated otherwise, the antigens were used for selection at concentrations of 100, 30, and 10 nm during rounds 1, 2, and 3 respectively. For hFSH-CTP, 50, 15, and 10 nm was used, respectively; for MUC1 peptide, 500, 100,
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**TABLE I**

| Oligonucleotide primers used for construction of the library |
|-------------------------------------------------------------|
| **H** back                                               |
| HuVH1B/7A-BACK                                            |
| HuVH1C-BACK                                              |
| HuVH2B-BACK                                              |
| HuVH3B-BACK                                              |
| HuVH3C-BACK                                              |
| HuVH4B-BACK                                              |
| HuVH4C-BACK                                              |
| HuVH5B-BACK                                              |
| HuVH6A-BACK                                              |

| **V**<sub>k</sub> back                                     |
| HuVx1B-BACK                                              |
| HuVx2-BACK                                               |
| HuVx3B-BACK                                              |
| HuVx4B-BACK                                              |
| HuVx5-BACK                                               |
| HuVx6-BACK                                               |

| **V**<sub>a</sub> back                                     |
| HuVa1A-BACK                                              |
| HuVa1B-BACK                                              |
| HuVa1C-BACK                                              |
| HuVa2-BACK                                               |
| HuVa3A-BACK                                              |
| HuVa3B-BACK                                              |
| HuVa4-BACK                                               |
| HuVa5-BACK                                               |
| HuVa6-BACK                                               |
| HuVa7/8-BACK                                             |
| HuVa9-BACK                                               |

A Primary amplifications

| IgM heavy chain constant region                          |
|---------------------------------------------------------|
| HuIgMFOR                                                |
| k light chain constant region                           |
| HuCxkFOR                                                |
| l light chain constant region                            |
| HuCa2-FOR                                               |
| HuCa7-FOR                                               |

20, and 5 nM was used.

**Screening and Sequencing of Clones—Soluble Fab was produced from individual clones as described before (4).** Culture supernatants were tested in ELISA with directly coated antigen or indirectly captured biotinylated antigen via immobilized biotinylated BSA-streptavidin. Tetanus toxoid and phOx-BSA were coated at 10 μg/ml in 0.1 M NaHCO₃, pH 9.6, for 16 h at 4 °C. For coating of hCG and hFSH-CTP, a concentration of 4 μg/ml in 50 mM NaHCO₃, pH 9.6, was used. For capture of biotinylated antigens, biotinylated BSA was coated at 2 μg/ml in PBS during 1 h at 37 °C. After 3 washes with PBS, 0.1% (v/v) Tween 20, plates were incubated during 1 h with streptavidin (10 μg/ml in PBS/0.5% gelatin) (30). Following washing as above, biotinylated antigen was added for an overnight incubation at 4 °C at a concentration of 0.5 μg/ml for MUC-1 peptide, 3 μg/ml for hLH, and 0.6 μg/ml for hFSH (binding to hCG was tested with directly coated antigen). The plates were blocked during 30 min at room temperature with 2% (w/v) semi-skim milk powder (Marvel) in PBS. The culture supernatant was diluted 1- to 5-fold in 2% (w/v) Marvel/PBS and incubated 2 h. Bound Fab was detected with anti-myc antibody 9E10 (5 μg/ml) recognizing the myc-peptide tag at the carboxyl terminus of the heavy Fd chain, and rabbit anti-mouse-HRP conjugate (Dako) (4). Following the last incubation, staining was performed with tetramethylbenzidine and H₂O₂ as substrate and stopped by adding 0.5 volume of 2 M H₂SO₄; the optical density was measured at 450 nm. Clones giving a positive signal in ELISA (over 2 times the background), were analyzed by BstNI fingerprinting of the PCR products obtained by amplification with the oligonucleotide primers M13-reverse and geneIII-forward (4).

Large scale induction of soluble Fab fragments from individual clones was performed on a 50-ml scale in 2× TY containing 100 μg/ml ampicillin and 2% glucose. After growth at 37 °C to an OD₆₀₀ of 0.9, the cells were pelleted (10 min at 2500 × g) and resuspended in 2× TY with ampicillin and 1 mM isopropyl-1-thio-β-D-galactopyranoside. Bacteria were harvested after 3.5 h of growth at 30 °C by centrifugation (as before); periplasmic fractions were prepared by resuspending the cell pellet in 1 ml of ice-cold PBS. After 2–16 h of rotating head-over-head at 4 °C, the spheroplasts were removed by two centrifugation steps; after spinning during 10 min at 3,400 × g, the supernatant was clarified by an additional centrifugation step during 10 min at 13,000 × g in an Eppendorf centrifuge. The periplasmic fraction obtained was directly used for determination of fine specificities by surface plasmon resonance or for Western blot studies (described below).

For screening, plasmid DNA was prepared from 50-ml cultures grown at 30 °C in LB-medium, containing 100 μg/ml ampicillin and 2% glucose, using the Qiagen Midi-kit (Qiagen). Sequencing was performed with the thermocycling kit (Amersham Pharmacia Biotech) with CyS-labeled primers CH1FOR (5'-GTC TCT GAT CAG GCA GCC GCG GCG-3') and M13REV (5'-CAG GAA GAC ACA GCT ATG AC-3'); samples were run on the ALF-Express (Amersham Pharmacia Biotech). V-gene sequences were aligned to V-BASE or the Sanger Center.

**Determination of Fine Specificities of the Anti-hormone Fabs by Western Blot and Surface Plasmon Resonance—An hCG preparation purified from urine and immuno-affinity-purified recombinant hLH, hFSH, and hFSH-CTP produced in CHO cells (30, 31) were used for Western blot studies as described elsewhere (32). Between 0.5 and 1 μg of each hormone was loaded per lane; proteins were diluted in non-reducing sample buffer and boiled during 5 min or directly applied on gel without heat treatment; proteins were transferred to blotting membrane by electrotransfer. Blots were subsequently incubated for 16 h at room temperature with a 10-fold diluted periplasmic fraction in PBS, 4% Marvel. Bound Fab was detected with anti-myc antibody 9E10 (5 μg/ml) and 4,000-fold diluted anti-mouse alkaline phosphatase-conjugate (Promega), using the substrates 5-bromo-1-chloro-3-indolyl phosphate and nitro blue tetrazolium (Roche Molecular Biochemicals) for visualization.

The specificity of the Fab was further characterized by surface plasmon resonance (BLAcore 2000, Biacore). Recombinant hLH, hFSH, and the urinary hCG were immobilized on the flow cells of a CM chip using the NHS/EDC kit (Biacore AB, Uppsala, Sweden), yielding a surface of 1806 RU for hLH, 1529 RU for hFSH, and 1375 RU for hCG.

2 V-BASE is available via the World Wide Web (Medical Research Council Center for Protein Engineering, 1997; http://www.mrc-cpe.cam.ac.uk/imt-doc/pblic/public/INTRO.html); Sanger Center is also available via the World Wide Web (Sanger Center Germline Query, 1997; http://www.sanger.ac.uk/Data Search/gq-search.html).
Periplasmic fractions were diluted 3-fold in Hepes-buffered saline (10 mM Hepes, 3.4 mM EDTA, 150 mM NaCl, 0.05% (v/v) surfactant P20, pH 7.4) and analyzed using a flow rate of 10 μl/min.

**Purification of Soluble Fab Fragments**—Fabs were obtained by re-folding of the total bacterial proteins from a 50-ml culture (33). Briefly, the pelleted cells from a 50-ml induced bacterial culture were resuspended in 8 ml of 8 M urea (in PBS). After sonication, the mixture was rotated head over head for 30 min and insoluble material was removed by centrifugation for 30 min at 13,000 g. The supernatant was dialyzed against PBS with four buffer changes. Insoluble proteins were removed by centrifugation and the flow-through fraction, obtained by rotation head over head for 30 min and insoluble material was removed by centrifugation and the flow-through fraction, obtained by filtration through a 0.2-μm membrane, was immediately loaded on an hCG column (bed volume 0.3 ml). The column material was prepared by coupling 8.4 mg of protein to 1 g of Tresyl-Sepharose according to the
supplier's instructions (Pierce). The column (1 ml column material) was washed with 10 volumes of 100 mM Tris, 500 mM NaCl, pH 7.5; then subsequently with 10 volumes of 100 mM Tris, 500 mM NaCl, pH 9.5; and 2 volumes of 0.9% NaCl. Bound Fab was eluted in a batchwise fashion with 2 volumes of 0.1 M triethylenamine. After a 10-min incubation, the effluent was collected and immediately neutralized with 0.5 volume of 1 M Tris, pH 7.5. The Fab fraction was diaлизed against PBS using a Microcon 30 spin dialysis filter (Amicon). Finally, a gel filtration analysis was carried out on a Superdex 75HR column (Amersham Pharmacia Biotech). The yield was determined by measuring the optical density at 280 nm (using a molar extinction coefficient of 13 for Fabs).

**Determination of On- and Off-rate Using Surface Plasmon Resonance with Crude Fab Preparations**—The kinetics of binding were analyzed by surface plasmon resonance on three different hCG surfaces (303, 615, and 767 RU immobilized, with 4955 RU of BSA on a separate flow cell as a negative control). Obviously, ranking of the off-rates of the individual clones needs to be done by analysis with the BIAlevaluation software. Fab present in crude periplasmic extracts was quantified on a Microcon 30 spin dialysis filter (Amicon). Finally, a gel filtration analysis was carried out on a Superdex 75HR column (Amersham Pharmacia Biotech). The yield was determined by measuring the optical density at 280 nm (using a molar extinction coefficient of 13 for Fabs).

**RESULTS**

**Design of the Non-immunized Phage Antibody Library**—We considered a number of variables to address in the construction of a novel, very large phage antibody library: (i) the primer design was optimized for amplification of variable gene pools to maintain maximum diversity; (ii) a highly efficient two-step cloning method was developed to obtain a very large non-immunized library; (iii) an antibody format and compatible cloning vector were chosen, which should permit the rapid downstream analysis of selected clones.

In order to achieve access to as many different human heavy and light chain V-region gene segments as possible, a new set of oligonucleotide primers was developed (Table I), the design of which was based on the most recent sequence information provided by the V-base (see “Materials and Methods”). The primers should allow efficient amplification of all commonly used V-gene segments. Further, to obtain large sized libraries (over 1010 in diversity), we used a two-step cloning procedure; heavy and light chain variable genes were first separately cloned as digested PCR products, and then combined by restriction fragment cloning to form a large library of Fab fragments. This cloning procedure should be a more efficient route for library construction than the relatively inefficient direct cloning of as many different human heavy and light chain variable genes were first separately cloned as digested PCR products, and then combined by restriction fragment cloning to form a large library of Fab fragments. In this vector system, the variable heavy chain region genes are cloned as VH-gene fragments; the vector supplies all Fabs with a human gamma-1 CH1 gene. The Fd fragment is fused to two tags for purification and detection (a histidine tail for immobilized metal affinity chromatography and a c-myc-derived tag), followed by an amber stop codon and the minor coat protein III of filamentous phage fd. The antibody light chain is cloned as full VLCL fragment, for directed secretion and assembly with the VHCH1 on the phage particle.

**Library Construction**—The Fab library was constructed in two steps. In the first step, variable region gene pools were amplified from approximately 4 × 108 B cells from the PBLS of four healthy donors, and, as a source of possibly more heavily mutated IgM antibodies, from a segment of a (tumor-free) spleen removed from a patient with gastric carcinoma, containing approximately 1.5 × 108 B cells (40). Only IgM-derived VH segments were amplified by using an amplification with an oligonucleotide primer located in the first constant domain of this isotype. These products were cloned into phagemid vector pCES1 for VL, and in pUC119-ES1 for VH (cloning was more efficiently in the smaller sized vector, in which gene III was deleted). The PBL- and spleen-derived VH, Vx, and Va libraries were cloned separately to maintain diversity, to yield one-chain libraries in size typical for libraries made by cloning of PCR fragments (4): 1.75 × 108 individual clones for the heavy chain, 9.4 × 107 individual clones for Vx, and 5.2 × 107 individual clones for Va. An overview is given in Table II. In the second step, the heavy chain fragments were digested from plasmid DNA isolated from the primary VH repertoire, and cloned into the vector containing the light chain repertoire (again separately for PBL- and spleen-derived repertoires; Table II). The libraries were combined using this efficient cloning procedure, to create a non-immunized Fab repertoire with 3.7 × 1010 individual clones (4.3 × 1010 recombinant clones, 86% of which have a full-length Fab insert), with 70% of clones harboring a light chain, 30% a λ chain. All of 20 clones with full-length Fab insert
tested scored positive in dot-blot analysis with the 9E10 antibody indicating an expression level of soluble Fab of at least 0.2 mg/liter.

Quality Control of the Library by Selection with a Panel of Antigens—We evaluated the library by selection with different antigens, the screening data of which are summarized in Table III. First, the results from three model antigens, the protein tetanus toxoid, the hapten 2-phenyloxazol-5-one (phOx) (41), and the peptide MUC1, are discussed. Three rounds of biopanning on tetanus toxoid yielded a diverse set of ELISA-positive Fabs; in a series of 47 tetanus toxoid binding Fabs, at least 21 were different with regard to BstNI fingerprint. Similarly, an extensive panel of phOx-specific Fabs was retrieved after three rounds of panning; at least 24 different clones were identified in a series of 50 ELISA-positive clones. Solution capture with biotinylated MUC1 peptide resulted in the selection of 14 different antibody fragments out of 37 ELISA-positive clones selected after 3 rounds (Table III).

Rapid Dissociation Rate Determination—With such large panels of antibodies isolated, it is crucial to have methods available to readily determine the kinetic parameters of each individual antibody-antigen interaction. Such an assay should be robust and ideally employ non-purified antibody fragments. We tested whether it would be feasible to use periplasmic fractions prepared from small scale cultures for a rapid and accurate determination of the off-rate of the antibodies using surface plasmon resonance. An example of an overlay plot with the sensorgrams from a series of tetanus toxoid-specific Fabs is shown in Fig. 2. The plot of ln(R0/R) versus time (Fig. 2, lower graphs) reveals a linear relation with slope koff (off-rate), thereby confirming a monophasic dissociation, which can be expected for a truly monomeric Fab fragment binding to a low density antigen surface. At the beginning of the dissociation phase, the relation is not linear due to a difference in composition of the BIACore running buffer (Hepes-buffered saline) and the buffer solution of the Fab samples (phosphate-buffered saline); this may be avoided by pre-dialysis. Using this off-rate screening assay, we determined the off-rates for the best tetanus toxoid- and MUC1-specific Fabs to be in the order of 10^-2 to 10^-4 s^-1 (Fig. 2).

Selection of Fab Antibodies against Related Glycoprotein Hormones—As a more stringent test panel of antigens to assay the performance of the library, we chose to derive antibodies to three structurally related glycoproteins: hCG, hLH, and hFSH (reviewed in Ref. 42). These hormones are heterodimers sharing an identical α-chain with 92 amino acid residues, but have β-subunits of different composition and length. The β-chain of hCG contains 145 amino acid residues, and the one from hLH only 121 residues, the latter showing 85% homology to β-hCG. The β-chain of hFSH is only 111 amino acids and shares 36% of the residues with hCG. Antibodies that specifically detect hCG have been used extensively in pregnancy tests (42) and for cancer diagnosis (43, 44). A large set of antibodies to these targets would extend the limited number of hormone-specific antibodies (especially against hLH), obtained using the hybridoma technology (42). The human origin of the antibodies might be beneficial when using these for imaging or therapy of testicular and bladder cancer (43, 44).

Selections were thus performed on biotinylated urinary hCG, recombinant hLH, hFSH, and hFSH-CTP (the latter is a chimeric molecule containing the carboxyl-terminal peptide of β-hCG fused to the β-chain of FSH; Ref. 45). The highest degree of enrichment with respect to the increase in the number of eluted phage particles in round 3 versus round 1 was found for hCG (10,000-fold), followed by hFSH-CTP (1,000-fold), hFSH (300-fold), and hLH (150-fold). Polyclonal phage of selected
populations were tested for binding using sensor chips containing immobilized hormones (46). Polyclonal phage populations from rounds 1, 2, and 3 (R1, R2, and R3, respectively) of the selection with hCG, were analyzed on flow cells with hCG, hLH, and hFSH at $t = 70$ s, $t = 400$ s, and $t = 690$ s, phage was injected, ending at $t = 120$ s, $t = 450$ s, and $t = 740$ s, respectively (A). Analysis of phage from round 3 selected with the antigens hCG, hFSH-CTP, hFSH, and hLH (the latter was selected with 100 nM hormone (coded LH(1)) or 10 nM (coded LH(2)) at round 1), using flow cells with hCG and streptavidin (upper sensorgram) or with hFSH-CTP, and hCG (lower sensorgram); injection was started at $t = 60$ s, $t = 270$ s, $t = 500$ s, $t = 720$ s, and $t = 950$ s, and terminated at $t = 120$ s, $t = 330$ s, $t = 560$ s, $t = 780$ s, and $t = 1010$ s, respectively (B).

**Fig. 3.** Monitoring of selections with polyclonal phage using surface plasmon resonance. Polyclonal phage populations from rounds 1, 2, and 3 (R1, R2, and R3, respectively) of the selection with hCG, were analyzed on flow cells with hCG, hLH, and hFSH, at $t = 70$ s, $t = 400$ s, and $t = 690$ s, phage was injected, ending at $t = 120$ s, $t = 450$ s, and $t = 740$ s, respectively (A). Analysis of phage from round 3 selected with the antigens hCG, hFSH-CTP, hFSH, and hLH (the latter was selected with 100 nM hormone (coded LH(1)) or 10 nM (coded LH(2)) at round 1), using flow cells with hCG and streptavidin (upper sensorgram) or with hFSH-CTP, and hCG (lower sensorgram); injection was started at $t = 60$ s, $t = 270$ s, $t = 500$ s, $t = 720$ s, and $t = 950$ s, and terminated at $t = 120$ s, $t = 330$ s, $t = 560$ s, $t = 780$ s, and $t = 1010$ s, respectively (B).
hFSH and hCG (250 RU, Fig. 3B). In this case, the control surface was coated with streptavidin; no specific signals were obtained (<50 RU between the signal before and after injection of the phage preparation). Selections on hLH yielded antibodies reactive with hFSH and hCG (thus most likely anti-a-chain antibodies; marked LH (1) in Fig. 3B). When hLH was used at lower concentrations (at 10 nM in round 1 and 3 nM during the subsequent selection rounds), a signal was seen with streptavidin only (marked LH(2) in Fig. 3B), due to the selection of streptavidin-specific antibodies. Thus, this polyclonal phage screening provides a rapid test to check the overall quality of the clones in the selected repertoire, and may also be used to guide the choice of the conditions for the next selection round (46).

Specificity Analysis of the Selected Monoclonal Fabs—ELISA

ELISA of monoclonal phage antibodies revealed that three rounds of selection with hCG indeed resulted in the isolation of a high percentage (74%) of clones positive for the gonadotropin. 27% of these clones were hLH-cross-reactive; none were reactive against streptavidin. BstNI fingerprint analysis of the ELISA-positive clones revealed a high degree of diversity (8 different patterns). From a representative hCG-specific (coded CG#4F) and hLH-cross-reactive (CG#5C) clone, the specificity was tested in BLAcore using unpurified soluble Fab fragments (Fig. 4). Clone CG#4F gave a high response on hCG, with no visible binding to either hLH or hFSH-CTP. In contrast, clone CG#5C bound to hCG and hLH, but not to hFSH-CTP. Western blots, with the different hormones in non-reduced form, showed the specific recognition of the β-subunit of hCG by clone CG#4F, while the cross-reactive clone CG#5C reacted with the β-subunit of both hCG and hLH (Fig. 5).

Selection with the hormone hLH resulted in the isolation of hLH-specific and hCG-cross-reactive clones. Examination of individual clones from selection round three in ELISA revealed a large fraction of hLH-specific clones (69%), and a minor group of cross-reactive clones (16%); no streptavidin-reactive clones were selected. Within the group of specific clones, a large array of different species (>21) could be discriminated by fingerprint analysis; however, all cross-reactive species had a single pattern. The unique hLH specificity was confirmed for representative clones LH#2H and LH#3G, shown in surface plasmon resonance (shown for clone LH#3G in Fig. 4); and on Western blot (illustrated for clone LH#3G in Fig. 5). This Fab only recognizes the intact α/β-heterodimer of hLH. Two representative clones of a pan-reactive antibody in ELISA, coded LH#1C and LH#3F, reacted in BLAcore with hFSH-CTP, hCG, and hLH (shown for clone LH#3F in Fig. 4), and in Western blot analysis with the α-chains from all three hormones (data not shown).

When hFSH was used as antigen during selection, six different antibodies were isolated from the library, with one type, represented by clone FS#8B, dominating the selected population. This Fab only recognized hFSH in BLAcore (Fig. 4), and, as Western blot analysis demonstrated, in particular its β-unit (Fig. 5). Further, the specificity of an α-chain binding clone, SC#2B, was confirmed in BLAcore (Fig. 4) and Western blot (Fig. 5).

Upon selection with FSH-CTP, seven different α-chain-specific Fabs were identified by fingerprint analysis, from which the clones coded SC#2B, SC#2F, SC#2G, and SC#4G were examined in more detail. Immunoblot analysis with the recombinant Fab as detecting antibody confirmed the α-chain specificity (blot incubated with clone SC#2B is shown in Fig. 5).

Phage-selected Antigen-specific Clones Are Intact Fab Fragments—There have been some reports on the isolation from scFv or Fab libraries of antigen-specific single-domain or other artificial antibody fragments (47, 48). Therefore, we tested the integrity of the selected Fabs. First, the nature of the Fab...
fragments in periplasmic fractions was determined in Western blot. When incubated in non-reducing sample buffer, two products were detected with the 9E10 antibody, which recognizes the myc-tag at the end of the CH1 domain (Fig. 6A); the major product is the intact Fab molecule, in which an intermolecular disulfide bridge covalently links heavy and light chain fragments; the low molecular product is most likely derived from non disulfide bridge linked heavy chains. Analysis with anti-light chain sera reveals a similar pattern and shows that the clones use a nearly equal percentage of \( k \) and \( l \) chains (found in 6 and 7 clones, respectively, of a total of 13 tested) (Fig. 6, B and C). From the densities of the blots, it would be unlikely that all of the light chain would be complexed as Fab. Instead, it appears that in many cases more light than heavy chain fragment is produced, which may be expected from the design of the bicistronic expression cassette. Upon purification of functional antigen-binding fragments using denaturation and refolding, followed by affinity chromatography, this excess of light chain disappers, as expected (shown with a Coomassie blue-stained protein gel, for five clones in Fig. 7). Upon reduction, equal amounts of heavy and light chain are seen, while under non-reducing conditions (shown for one clone only), the main product is represented by the disulfide linked Fab-molecule, with an equal amount of the (most likely) non-covalently linked VHCH1 and VLCL products visible. Production yields of selected hormone-specific Fabs varied between 160 mg and 1.43 mg of Fab/liter of culture, which was in the same range as was found for the unselected Fabs (data not shown).

Use of Diverse Germline Sequences—A panel of 14 antigen-specific Fabs was fully sequenced (Table IV; 3 anti-MUC1 antibodies positive in BIAcore on 100-mer peptide, and 11 anti-gonadotropin antibodies). The heavy chain genes are derived from the four largest VH families (VH1, VH3, VH4, and VH6); the VL genes belong to one of four \( V_k \) families or one of three \( V_l \) families. Chain promiscuity is seen for the \( a \)-chain-specific clone SC#4G, the \( a/\beta \)-LH-specific clones LH#2H and LH#3G, and \( \beta \)-FSH-specific clone FS#8B, which all used a highly homologous \( V_2 \) light chain gene segment (A19, previously coded DPK15) combined with different heavy chain fragments. This promiscuity for A19 was previously found in antibodies derived from a synthetic Fab repertoire (35). The three anti-MUC1 antibodies use heavy and light chain genes derived from two different VH and \( V_k \) families; clone MUC#9 uses a VH with a cross-over of two segments. It is remarkable that both MUC#4 and MUC#9 VH genes use the same reading frame of the same D-segment (D6–13; with a stretch of 13 or 16 bp from this segment, respectively; Ref. 49), encoding an alanine-glycine stretch (AAAG; Table IV). This may reflect a similar mode of binding to MUC1, despite the use of a different light chain by these two clones.

Measurement of Affinities with Purified Anti-hCG Fabs—The affinities and off-rates of affinity-purified hCG-reactive Fabs LH#1C, SC#2B, LH#3F, and CG#5C were determined. The off-rates for most Fabs were in the order of \( 10^{-2} \) and \( 10^{-3} \) s\(^{-1}\) (Table V). The off-rate values obtained using crude periplasmic fractions were in good agreement with the values found for the purified Fabs, validating the utility of the off-rate screen with unpurified Fab fragments. The affinities, 23 and 38 nm for the \( a \)-subunit-specific antibody LH#1C and the \( \beta \)-subunit hCG/hLH-cross-reactive antibody CG#5C, respectively, are comparable to the affinity of antibodies selected from a murine immune phage antibody library\(^3\); the top affinity, 2.7 nm for the \( a \)-chain-specific Fab SC#2B (Table IV), approaches the values

\(^3\) H. J. de Haard and B. Kazemier, unpublished results.
donors and its evaluation by selection with a panel of hapten and protein antigens. The source of antibody producing B cells was twofold: peripheral blood lymphocytes, which are mainly IgM-positive cells, and B cells from a human spleen. The theoretical diversity of a combinatorial antibody library made from the PBLs of one donor is much larger than what can be practically made or accessed (10^14 combinations with 10^7 individual B cells). However, there may be a strong bias in the diversity introduced because of the donor’s recent immune history and major differences in mRNA contents and clonal outgrowth. Therefore, in previously constructed very large non-immunized libraries, B cells from many different donors were used (7). Most probably the repertoire will be limited in diversity by using random priming because plasma cells (mostly of the IgG type) will produce 10,000-fold more mRNA compared with non-activated B cells; a better source for non-immunized libraries are IgM primed V-genes (4). We have successfully used B cells from only a few donors (four for the PBLs and one spleen), but have aimed to access a more diverse pool (spleen and PBL-IgM in this library, versus tonsil and PBL random primed V-genes). In the PBL pool of adults, over 60% of the B cells are unmutated IgM+IgD+ naive B cells, while the remaining cells are memory cells that have acquired somatic mutations (50). Since the latter class contains more mRNA (17), most of the VH genes derived from cloning of this pool are expected to carry mutations. Similarly the IgM pool in spleen B cells will contain antibodies with mainly mildly mutated germline genes. Indeed, many of the selected antibodies carry a low level of mutations in the heavy chain genes. Some antibodies (i.e. clone LH#3G) are nevertheless completely germline encoded and yet of high affinity and specificity (similarly to what has been described for some B cell-derived antibodies (51)). There may be sources of B cells that will yield a truly naïve V-gene repertoire (possibly, bone marrow-derived and/or IgM+IgD+CD27– B cells), but it remains to be seen if these V-gene sources will also yield better libraries. Indeed, when comparing synthetic antibody libraries (35), which incorporate germline encoded V-gene segments with non-immunized human V-gene libraries such as the one presented here, it is difficult to pinpoint any performance differences with regard to affinity and specificity of selected antibodies.

We employed an efficient two-step cloning procedure with DNA fragments digested from plasmid DNA instead of PCR fragments, to obtain the largest non-immunized human Fab repertoire reported to date, with a theoretical diversity of 37 billion different clones.

The choice of the Fab format was based on the possibility to develop rapid affinity/kinetic screens. Most large libraries made to date use the single-chain format for display on phage (7, 19). One report described the use of a human non-immunized Fab library on phage (not permitting immediate screening of selected soluble Fab fragments) (35). scFv fragments have the tendency to form dimers and higher order multimers in a clone-dependent and relatively unpredictable way (20–22). As a consequence, the affinity assay used (such as BIAcore analysis) often necessitates purification of the selected antibody fragments. For example, ranking for off-rates using BIAcore is not easily possible with unpurified scFv fragments; the monomeric fraction of selected scFv clones first needs to be purified by affinity chromatography and gel filtration (19, 24). Our data suggest that the off-rate screening of individual Fab clones using non-purified bacterial preparations yield data similar to the off-rates determined with the purified Fab fragments. Therefore, provided sufficient Fab fragment is produced, the true monomeric appearance of Fabs allows a rapid initial screen for off-rate. In combination with a concentration of the best anti-hCG monoclonal antibodies.³

**DISCUSSION**

This report describes the construction of a phage display library from the in vivo rearranged V-gene repertoire of human

FIG. 6. Examination of the integrity of produced Fab fragments on Western blot. The periplasmic fractions from the indicated glycoprotein-specific Fab clones were boiled in non-reducing sample buffer and loaded on 12.5% SDS-PAGE. The blots were incubated with the anti-myc mAb 9E10 (A), anti-human α polyclonal antibodies (upper panel of B), or with anti-human kappa polyclonal antibodies (C). Affinity-purified Fab was used as a control on the blot incubated with anti-myc mAb (indicated with pur Fab).

FIG. 7. SDS-PAGE of affinity-purified Fabs. hCG binding Fabs produced by clones LH#1C, LH#3F, CG#5C, SC#2B, and SC#2F were purified by affinity chromatography on hCG-Sepharose, and analyzed on a Coomassie-stained 12.5% SDS-PAGE gel under reducing conditions, and for Fab SC#2B also under non-reducing conditions. As positive control, a Fab fragment made by proteolytic digestion of a human monoclonal antibody was included (marked with +). B is a 4-fold dilution of A of the same sample.
2.9

parison of the first non-immunized scFv repertoire containing the selection of high affinity antibodies to the antigen. Com-

(35), the size of the antibody library dictates the probability of chain after the recombination event suggests instability of the low percentage (28%) of clones having both a heavy and a light system does not allow a rapid screening procedure, while the screening of individual clones had to be performed after reclon-

measurements without further purification by gel filtration, (with few randomized CDR3 residues) (35). Although the au-

pletely synthetic CDR3 sequences) and light chain repertoires bination system to combine separately cloned heavy (with com-

the off-rates from $10^{10}$ s$^{-1}$ to $10^{-4}$ s$^{-1}$ for fragments selected from the smaller sized library to $10^{-3}$ to $10^{-4}$ s$^{-1}$ for those from the larger library. This is in the same order of magnitude as we observe for the off-rates of our selected antibody fragments. SinceFab fragments lack the tendency to dimerize, Fab libraries could possibly display a lower fraction of avid phage than equivalent scFv libraries library. This does not appear to have reduced the number or average affinity of selected antibodies. An indication that antibodies from this library behave similarly or better with regards to affinity comes from a comparison of selections of two different libraries on the same two antigens under identical conditions. Antibodies to MUC1 selected from a large non-immunized scFv library (29) have faster off-rates than the best scFv (7).5

Most large libraries made to date use the single-chain format for display on phage (7, 19), which does not easily allow the rapid screening of large numbers of clones on kinetics of bind-

ing (off-rate) with crude protein fractions. One report described a very large human synthetic library with Fab fragments dis-

played on phage, which was constructed with an in vivo recombina-

tion system to combine separately cloned heavy (with com-

pletely synthetic CDR3 sequences) and light chain repertoires (with few randomized CDR3 residues) (35). Although the au-

lhors also used affinity-purified Fab fragments for affinity measurements without further purification by gel filtration, screening of individual clones had to be performed after recloning of the selected Fabs for soluble expression. Clearly, this system does not allow a rapid screening procedure, while the low percentage (28%) of clones having both a heavy and a light chain after the recombination event suggests instability of the library.

As was postulated and observed by Griffiths and colleagues (35), the size of the antibody library dictates the probability of the selection of high affinity antibodies to the antigen. Comparison of the first non-immunized scFv repertoire containing $2.9 \times 10^7$ clones (4), with recently constructed scFv repertoires of approximately $10^{10}$ clones (7, 19), confirms this postulation; increasing the library size 500-fold resulted in approximately 100-fold higher affinities. This increase is caused by lowering the off-rates from $10^{-1}$- $10^{-2}$ s$^{-1}$ for fragments selected from the smaller sized library to $10^{-3}$ to $10^{-4}$ s$^{-1}$ for those from the larger library. This is in the same order of magnitude as we observe for the off-rates of our selected antibody fragments. Since Fab fragments lack the tendency to dimerize, Fab libraries could possibly display a lower fraction of avid phage than equivalent scFv libraries library. This does not appear to have reduced the number or average affinity of selected antibodies. An indication that antibodies from this library behave similarly or better with regards to affinity comes from a comparison of selections of two different libraries on the same two antigens under identical conditions. Antibodies to MUC1 selected from a large non-immunized scFv library (29) have faster off-rates than the equivalent Fabs isolated from the library described in this study. Further, they show a very distinct V-gene usage and have a different fine specificity.4 Similarly, when comparing the off-rates of phage antibodies against the pancarcinoma marker epithelial glycoprotein-2, one of the Fabs selected from the present library appears to have a 10-fold slower off-rate than the best scFv (7).5

The affinities of the selected antibody fragments are, however, very dependent on the antigen used for selection. Sheets and colleagues reported an affinity varying between 26 and 71 nM for the selected scFv fragments specific for the anti-Clostridium botulinum neurotoxin type A fragments, whereas for antibodies to the extracellular domain of human ErbB-2, $K_d$ values between 0.22 and 4.03 nM were found (19). The affinities of the gonadotropin-specific Fabs selected from our library varied between 2.7 and 38 nM, which is comparable to the protein binding scFv fragments from the non-immunized library made
determination assay (which could also be carried out on BIA-
core; Ref. 34), this should allow the rapid affinity determination of large series of antigen-specific Fabs. The Fab format is therefore more amenable then scFv to high throughput affinity screening, and should be the preferred format when rapid affinity measurement is crucial (e.g. during affinity maturation studies).

Table IV

| Clone | Specification | VH family | VH segment | CDR3 | Amino acid changes from germline* | VL family | VL segment | CDR3 | Amino acid changes from germline*
|-------|---------------|-----------|------------|------|----------------------------------|-----------|------------|------|----------------------------------|
| MUC#4 | MUC1          | VH4       | 4–39       | AAAGMVD | 7     | Ve2                               | A17       | MQATHWPIT  | 1    |
| MUC#9 | MUC1          | VH4       | 4-34–39    | FSIAAAQQSY | 5   | Vx3                               | L6        | QQYWSIWL      | 10   |
| MUC#32 | MUC1          | VH3       | 3–30       | VGSGGMLYFD | 2   | Vx3                               | L6        | QQSHPWPIT  | 2    |
| CG#4F | β CG          | VH3       | 3–30.3     | EGTATPGEYD | 2   | Vx1                               | L5        | QGSYEPST    | 7    |
| CG#5C | β CG/LH       | VH4       | 4–04       | GAAASYFPDY | 0   | Vx6                               | 6a        | QSSHTAVV | 13   |
| LH#1C | a             | VH1       | 1–08       | GERSNFDY | 8   | Vx3                               | L6        | QHRRT    | 8    |
| LH#2H | α β-LH        | VH3       | 3–15       | DFGTIITYYGMV | 0   | Vx2                               | A19       | MQLAEPLT  | 4    |
| LH#3F | a             | VH3       | 3–30.5     | LLYGYVSAFDI | 2   | Vx7                               | 7b        | LLVYGGGAV  | 6    |
| LH#3G | αβ-LH         | VH3       | 3–15       | RIAAAAAAYGMDV | 0   | Vx2                               | A19       | MQLAQTPRT | 0    |
| FS#8B | β FSH         | VH6       | 6–01       | GEHYGTSS | 8   | Vx2                               | A19       | MQLQITPPT | 1    |
| SC#2B | a             | VH1       | 1–02       | GKVGAFFDY | 3   | Vx2                               | 2a        | SSTSNSGLV | 9    |
| SC#2F | a             | VH1       | 1–02       | ASYFPNDADFI | 6   | Vx1                               | 1c        | ESLDSSDGLVV | 20   |
| SC#2G | a             | VH1       | 1–e        | GEASYGVNWFDI | 5   | Vx2                               | 2e        | CSSYNGSTTV | 9    |
| SC#4G | a             | VH1       | 1–24       | GGGYGLA | 2   | Vx2                               | A19       | MQLQIPNW    | 2    |

* Amino acid differences in V-gene segment, excluding the FR-1 region encoded by the primers used for cloning, and CDR3.

Table V

| Clone | Preparation | $k_{\text{in}}$ | $k_{\text{off}}$ | $K_d$
|-------|-------------|-----------------|-----------------|-------|
| LH#1C | Purified    | $(1.04 \pm 0.04) \times 10^{-2}$ | $(4.50 \pm 0.37) \times 10^3$ | $(2.30 \pm 0.23) \times 10^{-8}$ |
| SC#2B | Periplasmic fraction | $(1.12 \pm 0.08) \times 10^{-8}$ | ND* | ND |
| CG#5C | Purified    | $(2.89 \pm 0.41) \times 10^{-3}$ | $(1.06 \pm 0.08) \times 10^6$ | $(2.71 \pm 0.25) \times 10^{-9}$ |
| LH#3F | Periplasmic fraction | $(2.90 \pm 0.57) \times 10^{-3}$ | ND | ND |
| CG#4F | Periplasmic fraction | $(1.46 \pm 0.27) \times 10^{-2}$ | $(3.76 \pm 0.71) \times 10^2$ | $(3.88 \pm 0.03) \times 10^{-8}$ |
| SC#2F | Periplasmic fraction | $(1.21 \pm 0.16) \times 10^{-2}$ | ND | ND |

* ND, not determined.
by Vaughan et al. (7) and Sheets et al. (19). It also approaches the values of the best antibodies in their kind.6

The size of the library is not only important for affinity, it also determines the success rate of selection of antibodies against a large set of different antigens. In this respect the library performs very well; over 24 antibodies to the hapten phOx, and on average 13 antibodies against the other antigens were selected. Furthermore, the specificities of the antibodies obtained by selections on the gonadotropins are unique; due to the high degree of homology between hLH and hCG, it has been very difficult to isolate hCG-specific monoclonal antibodies with the hybridoma technology, whereas there are very few hLH-specific antibodies (32, 42). Using a straightforward selection procedure, taking no precaution to avoid the selection of cross-reactive Fabs, we have readily isolated fragments with all possible specificities: Fabs specific for any of the three hormones hCG, hLH, and hFSH, and cross-reactive Fabs recognizing the common a-chain or epitopes on the b-chain shared by hCG and hLH. These selections demonstrated that antibodies directed against different epitopes within single antigen molecules can be retrieved from the library.

In the limited set of 14 clones that were sequenced, we identified antibodies with variable region genes from all large V-gene families, including VH1/3/4, V\(\text{l}\)2/7, and V\(\text{l}\)7 were also selected. Most likely, the use of an extended set of variable region gene primers, designed on the most recent sequence information of the germline V-regions, and/or the separate PCRs, combined with partially separate cloning, ensured access to a highly diverse sample of the human V-gene repertoire. The average amino acid mutation frequency of the selected human V-gene segments was calculated to be 4.0% for the VH segments and 7.3% for the VL (92 out of 1267) and 7.53 (mean 7.53 (50 amino acid mutations in 1267) and 7.3% for the VL (92 out of 1260). This mutation frequency is the reverse of that reported for IgG of 1260). This mutation frequency is the reverse of that re-

References—We thank colleagues at the Department of Pathology, in particular Dr. R. Hoet and C. Petracia for discussions, and collaborators (Dr. E. Krambovitis) for materials.

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6 H. J. de Haard and B. Kazemier, unpublished results.

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High Affinity Fabs from Non-immunized Phage Antibody Library

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