A model phage display subtraction method with potential for analysis of differential gene expression

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Received 31 May 1996

Abstract In order to establish a subtractive procedure that makes it possible to enrich selectively phage displayed antibodies directed against proteins constituting a difference between two populations of cells, a competitive selection strategy utilising two solid phases was developed and tested. Antibodies recognising a defined difference between two otherwise identical protein mixtures were isolated and their specificity confirmed. To test further the efficacy of selection inhibition during the competitive selections, selections towards a total cell extract were performed with and without competition from the same extract. An analysis of the resulting phage antibodies confirmed the subtractive nature of the system described.

Key words: Phage display; Phage display subtraction; Competitive selection; Phage antibody library; Antibody engineering; Single-chain Fv fragment

1. Introduction

Since 1985 when George Smith introduced the idea of displaying peptides in fusion with the minor coat protein, pIII, of filamentous phage [1], phage display technology has found many applications in the study of molecular recognition [2]. In recent years, libraries of antibody fragments have been cloned for display on the surface of filamentous phage, and phage antibodies have been selected against a variety of antigens, thus providing an important alternative to the hybridoma technology [3–5]. Phage display antibody libraries are constructed from V-gene repertoires, which can be obtained from either immunised [6,7] or non-immunised (naive) [8,9] sources. The advantage of large naive libraries over immunised is that phage antibodies against virtually any antigen can be identified from one single library, by using selection and amplification procedures to mimic the immune system [10–14].

Because antibodies bind specifically to their target molecules, they have wide applications as molecular probes for identifying a particular antigen in cells, tissues or biological fluids. Much effort has been put into identifying antibodies against specific biomarkers, since such reagents can be useful in studying the biological processes that define a given cell, or in targeting diagnostic and therapeutic agents [15–17]. Phage display libraries are likely to prove useful tools, as they offer the potential of selecting against many different ligands at the same time, thereby obtaining a complementary image of the molecules expressed. Accordingly, antibodies and peptides that are cell-selective have been isolated by panning phage display libraries against intact cells [18,19].

In the search for biomarkers, however, it is essential to use a subtractive strategy to provide information about differences, i.e. cell-specific markers, rather than similarities. Thus, by combining flow cytometry with selection from a naive phage display antibody library, it has been possible to separate sub-populations of cells, and to isolate sub-type-specific phage antibodies [20]. Other approaches, to enrich selectively for binders to new epitopes, have taken advantage of competitive elution with a known monoclonal antibody [21] or masking of an epitope with a known monoclonal antibody [22]. These techniques, however, rely on pre-existing monoclonal antibodies that bind specifically to the target molecules or target cells of interest, so a more general approach would be desirable. Hence, using a library constructed from peripheral blood lymphocytes of auto-immunised melanoma patients, phage antibodies that react with melanoma cells but not with melanocytes were isolated by adsorbing the panned phage against normal melanocytes, and cloning the unadsorbed phage [23]. Other investigators have used pre-adsorption of the naive library before panning to obtain a similar effect [18]. Finally, a very simple approach towards selection inhibition has been to perform selections in the presence of soluble competitor [13,24].

The specific aim of this study was to develop a simple procedure, by which phage antibodies can be raised against cytosolic cell population-specific proteins. A competitive biopanning procedure was developed and tested on two model systems, using a naive phagemid library of single-chain Fv antibody fragments (scFv) expressed on phage [11]. The results confirm that preferential selection can be obtained from naive phage display libraries, and suggest that the subtractive strategy presented is valuable in attempts to identify antibodies against known or unknown antigens in a given population of cells.

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Abbreviations: A_{490nm}, UV absorption at 490 nm; cfu, colony-forming units; EDTA, ethylenedinitrilotetraacetic acid; ELISA, enzyme-linked immunosorbent assay; IPTG, isopropyl-1-thio-β-D-galactopyranoside; LDH, lactate dehydrogenase; MES, 2-[*N*-morpholino]ethanesulphonic acid; MOPS, 3-[*N*-morpholino]propanesulphonic acid; MPBS, skimmed milk powder in PBS; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonyl fluoride; scFv, single-chain Fv fragment

PII S0014-5793(96)00703-X

2. Materials and methods

2.1. Library and bacteria

A naive phagemid library of approximately 10^8 clones of scFv expressed on phage [11] was kindly provided by Dr Greg Winter, MRC, Cambridge, UK. TG-1 (K12, Δ(*lac-pro*), *supE*, *thi*, *hsdD5*/F′*traD36*, *pro*A$^+$B$^+$, *lac*I^q, *lac*ZΔM15) and HB2151 (K12, *ara*, (*lac-pro*), *thi*/F′*pro*A$^+$B$^+$, *lac*^{q}ZΔM15) were used to produce phage displayed scFv and soluble scFv, respectively.

2.2. Proteins

α-Lactalbumin from bovine milk (L5385), β-lactoglobulin from bovine milk (L2506), soybean trypsin inhibitor (T9003), bovine catalase (C100), and bovine serum albumin (A4503) were all obtained from Sigma Chemical Co. Lactate dehydrogenase from rabbit muscle (127230) and creatine kinase from rabbit muscle (126969) were from Boehringer Mannheim GmbH.

2.3. Cell culture and cytosolic cell extracts

The melanoma cell line (FM55p) was a kind gift from Dr Jesper Zeuthen, Danish Cancer Society, Copenhagen, Denmark [25]. The cells were grown in RPMI 1640 medium (Dulbecco, Life Technologies) supplemented with 10% fetal calf serum. In order to prepare a cytosolic cell extract, the cells were harvested, pelleted, and resuspended on ice in 1 ml MES buffer (17 mM MES, pH 7.4, 2.5 mM EDTA, 250 mM sucrose, and 1 mM PMSF) per 1×10^7 cells. The cells were lysed on ice in a glass homogeniser with 60 strokes, and after centrifugation at $150\,000 \times g$ for 1 h at 4°C, the amount of protein was determined with bovine serum albumin as standard [26]. The cytosolic extract was stored at −80°C.

2.4. Competitive two solid phase biopanning-protein mixture

A protein mixture (MIX) was made, containing equal amounts (w/v) of the following proteins: α-lactalbumin, β-lactoglobulin, soybean trypsin inhibitor, bovine catalase, bovine serum albumin, and creatine kinase. This mixture was split into two aliquots and lactate dehydrogenase (LDH) was added to one of these (MIX+LDH). All coatings were with 5 μg/ml of each protein in 50 mM $NaHCO_3$, pH 9.6 overnight at 4°C. Blocking was with 4% skimmed milk powder in PBS (4% MPBS) at 30°C for 1 h.

A schematic presentation of the panning system is shown in Fig. 1. An aliquot of 10^{12} colony-forming units (cfu) from the naive library, and competitive soluble MIX proteins (5 μg/ml of each protein) in 4 ml 2% MPBS, were added to an immunotube (~1885 mm^2) (Nunc, Maxisorp) coated with competitive MIX proteins. After pre-incubation for 20 min at room temperature, an immunobead (~28 mm^2) (custom made by Nunc, Maxisorp) coated with the target MIX+LDH proteins was added and the incubation continued for 2 h at room temperature. The immunobead was washed 20 times in PBS with 0.2% Tween-20, and 20 times in PBS. The bound phage were eluted with 1 ml 100 μM triethylamine for 10 min at room temperature and neutralised with 0.5 ml 1 M Tris, pH 7.4. Exponentially growing TG-1 bacteria were infected with 1 ml of the eluate for 30 min at 37°C, and phage were produced by super-infection with the helper phage VCS-M13 (Stratagene) and growing the bacteria with shaking overnight at 30°C.

2.5. Competitive two solid phase biopanning-cytosolic cell extract from a melanoma cell line

In all the experiments the same preparation of cytosolic proteins from FM55p was used. All the coatings were with 25 μg/ml of FM55p proteins in 50 mM $NaHCO_3$, pH 9.6 overnight at 4°C. Blocking was with 4% MPBS at room temperature for 2 h. The naive library (10^{12} cfu) and competitive soluble FM55p proteins (25 μg/ml) were added in 4 ml 2% MPBS to an immunotube coated with competitive FM55p proteins. After preincubation for 20 min at room temperature, five immunobeads coated with FM55p proteins were added (see Fig. 1) and the incubation continued for 2 h at room temperature. The immunobeads were then washed by soaking 5 times for 2 min in PBS with 0.1% Tween-20, once for 10 min in PBS with 0.1% Tween-20, and 3 times for 2 min in PBS, and the bound phage were eluted and propagated as described above. A selection, without competition, was performed simultaneously against FM55p proteins coated on five immunobeads using the same washing conditions.

2.6. Soluble expression of scFv

Monoclonal scFv fragments were obtained from the periplasmatic fractions from 20 ml cultures of infected *E. coli* nonsuppressor strain HB2151 induced with 1 mM IPTG, and grown for 30 h at room temperature with shaking. Pellets of the bacterial cultures were resuspended in 300 μl MOPS buffer (20 mM MOPS pH 7.5, 0.5 mM EDTA, 20% sucrose) and left on ice for 15 min. Subsequently, 1.6 ml water was added to disrupt the outer bacterial membrane. The suspensions were centrifuged at $3000 \times g$ for 20 min at 4°C, and the supernatants were used as reagents in immunoblotting.

2.7. 9E10 anti-c-*myc* antibody

The 9E10 anti-c-*myc* tag mouse monoclonal antibody was obtained from a hybridoma cell line provided by the European Collection of Animal Cell Cultures (ECACC). The cells were grown in RPMI 1640 medium (Dulbecco, Life Technologies) supplemented with 5% Myoclone (Life Technologies). The antibody was purified on a protein G-column, and stored in small aliquots (1 mg/ml) at −80°C until used.

2.8. Test-panning assay

The binding of the isolated polyclonal phage pools was determined by the number of cfu rescued after panning against proteins (25 μg/ml) coated overnight on immunotubes at 4°C in 50 mM $NaHCO_3$, pH 9.6. Bacterial culture supernatants containing equal amounts of phage were added in 4 ml 2% MPBS and binding was allowed for 1 h at room temperature. The immunotubes were washed 10 times in PBS with 0.2% Tween-20, soaked 3 times for 5 min in PBS with 0.4% Tween-20, and were finally washed 10 times in PBS. The bound phage were eluted as described and an aliquot was used to infect TG-1 bacteria. The number of cfu were determined by plating a serial dilution of the infected bacteria onto agar plates containing ampicillin (100 μg/ml) and glucose (1% w/v), and incubating the plates overnight at 30°C.

2.9. Phage ELISA

Proteins were coated on microtitre plates (Greiner GmbH, Microlon) overnight at 4°C in 100 μl 50 mM $NaHCO_3$, pH 9.6 per well. The plates were blocked with 270 μl 4% MPBS per well at 30°C for 1 h. First, 50 μl of bacterial culture supernatants, containing either polyclonal phage or monoclonal phage produced from single ampicillin-resistant clones of infected TG-1 bacteria [8], were added together with 50 μl 4% MPBS. After 1 h incubation at room temperature

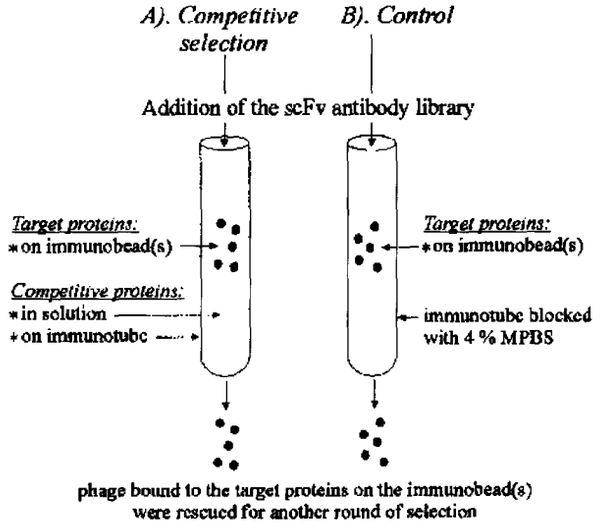

Fig. 1. Schematic presentation of the selection strategy. (A) In the competitive two solid phase system, the target proteins, MIX+LDH proteins or FM55p proteins, were coated on immunobead(s). The second solid phase support was an immunotube coated with competitive MIX proteins or competitive FM55p proteins, respectively. Moreover, the competitive proteins were added in solution. (B) In the control system, selections were performed on immunobeads in an immunotube previously blocked overnight in 4% MPBS.

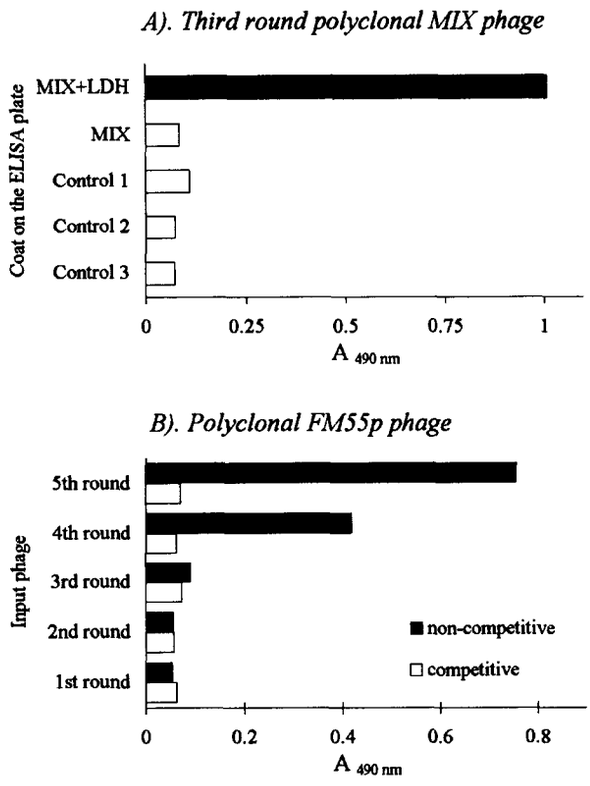

Fig. 2. Phage (10^{10} cfu) in 100 μl 2% MPBS were added to wells in microtitre plates coated with antigen. (A) The reactivity of polyclonal phage from the third round was determined from wells coated with either MIX+LDH (2 μg/well) or MIX (2 μg/well). Negative controls were performed by addition of phage to wells coated with 4% MPBS (control 1), or by addition of phage from the naive unselected library to wells coated with either MIX (control 2) or MIX+LDH (control 3). (B) The reactivities of polyclonal phage from selections with or without competition were compared for reactivity against FM55p proteins (2 μg/well).

the plates were washed 3 times in PBS with 0.05% Tween and 3 times in PBS, and horseradish peroxidase-conjugated anti-M13 antibody (Pharmacia Biotech) was added in a dilution of 1:1500 in 100 μl 2% MPBS for 1 h. After a final wash, the plates were developed with *ortho*-phenylenediamine (OPD) tablets (Kem-En-Tec), according to the manufacturer's instructions, and the A_{490nm} read. The specificity of the binding was assessed by ELISA assays using wells coated with the appropriate protein in dilution, and wells coated with irrelevant proteins.

2.10. Immunoblotting

The proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters (Amersham, Hybond-C Super) by semidry electroblotting. The blots were blocked in 4% MPBS overnight at 4°C.

In phage-immunoblotting, bacterial culture supernatants containing phage were added in 2% MPBS and binding was allowed for 40 min at room temperature. The blots were washed 5 times for 5 min in PBS containing 0.4% Tween-20. Horseradish peroxidase-conjugated anti-M13 antibody (Pharmacia Biotech) was added in a dilution of 1:1500 in 2% MPBS. After 1 h incubation the blots were washed as described, and developed using a chemiluminescent detection system (Pierce, SuperSignal), according to the instructions of the manufacturer.

In scFv immunoblotting, soluble scFv from the periplasm of infected HB2151 bacteria were added in 2% MPBS and binding was allowed for 1 h at room temperature. The blots were washed 5 times for 3 min in PBS containing 0.1% Tween-20, and 1:1000 of the anti-c-*myc* antibody (9E10) was added in 2% MPBS. After 1 h incubation, the blots were washed again, and 1:1500 horseradish peroxidase-conjugated anti-mouse antibody (DAKO) was added for 1 h. Following a final wash, the blots were developed by chemiluminescent detection as in phage-immunoblotting.

Table 1
The number of colony forming units by test panning

Input phage	MIX coat	MIX+LDH coat
10^{11} MIX phage[a]	8×10^5 cfu	2×10^8 cfu
10^{11} unselected phage	3×10^5 cfu	2×10^5 cfu

[a]Polyclonal phage from the third round. See Section 2 for details.

3. Results and discussion

We used a naive library of approximately 10^8 scFv antibody fragments displayed on phage to investigate the possibility of selecting preferentially against a particular protein differentially occurring in two pools of proteins. The goal is to establish a competitive panning procedure, which can be used to obtain phage antibodies against antigens expressed differentially in different cell populations. As this requires selection against various antigens simultaneously, we chose to work with a naive library, which is known to be robust and easy to use, and has been used to select antibodies towards several antigens [11]. The affinity of antibodies identified from this library is normally in the micromolar range, but can be improved by affinity maturation [27–31].

The binding of proteins to plastic surfaces is known to alter the structural conformation of some proteins, thereby changing the epitopes that are expressed [32]. In order to take this into account, a two solid phase system was developed, in which the competitive antigens were coated in the same manner as the protein of interest. Competitive proteins were, however, also added in solution as described by others [13,24]. We chose to conduct competition along with the selections, rather than adsorbing against the competing extract after selections [23], as it was found hard to eliminate binders once enrichment to high numbers had occurred (unpublished observations).

By selecting against a protein mixture containing six proteins plus lactate dehydrogenase (MIX+LDH), in the presence of the same six proteins (MIX) in competition, we were able to enrich preferentially for phage that were reactive against LDH. After three rounds of selection, the binding of the isolated polyclonal phage pools was tested by performing test-panning assays against MIX proteins and MIX+LDH proteins, respectively. The number of cfu calculated from the test panning against MIX proteins was comparable with that from the naive unselected library, suggesting non-significant binding against MIX proteins. In contrast, the test pan-

Table 2
Reactivity of monoclonal phage clones

Clone obtained from	Positives/total
MIX phage, 1st	0/96
MIX phage, 2nd	81/96 (84%)
MIX phage, 3rd	95/96 (99%)
FM55p phage, 5th, competitive	0/88
FM55p phage, 5th, non-competitive	59/88 (67%)

Individual clones were picked from colonies of infected TG-1 bacteria and 50 μl of bacterial supernatants was tested in ELISA coated with LDH (1 μg/well) for MIX phage and FM55p proteins (2 μg/well) for FM55p phage.

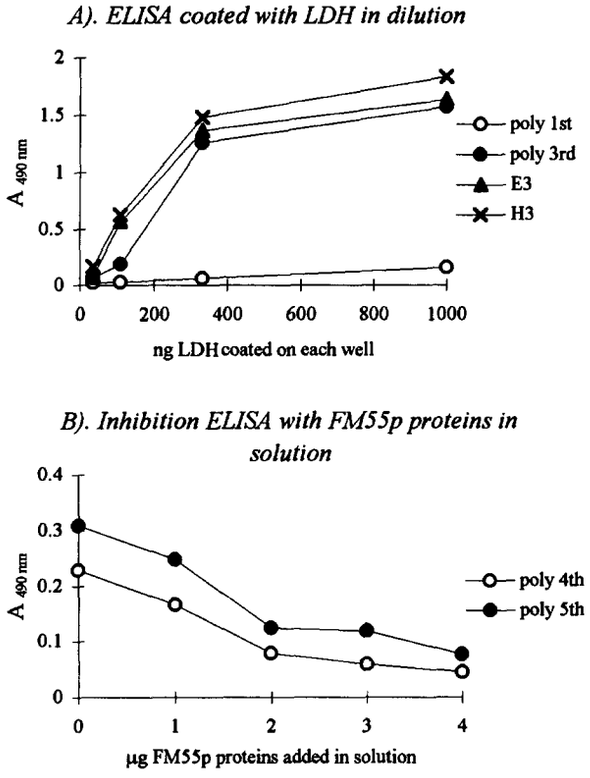

Fig. 3. (A) Phage (10^{10} cfu) in 100 µl 2% MPBS were added to wells coated with LDH in dilution, in order to test the specificity of the polyclonal phage (poly 1st and poly 3rd), and two clones randomly picked from the third round (clones E3 and H3). (B) Polyclonal phage (3×10^9 cfu) were pre-incubated for 20 min at room temperature with 0–4 µg of FM55p proteins in 100 µl 2% MPBS, before addition to wells coated with FM55p protein (1 µg/well).

ning against MIX+LDH proteins gave a 2.5×10^2 times higher cfu (Table 1), suggesting a specific binding of the phage antibodies towards LDH. Concordant results were obtained by comparing ELISA signals from wells coated with MIX proteins with signals from wells coated with MIX+LDH proteins (Fig. 2A). Hence, the signals against MIX+LDH proteins were 10-fold higher than the background signals from negative controls, whereas the signals against MIX proteins alone were equal to the background signals.

To analyse the relative number of positive clones reactive against LDH, 96 clones from each of the three rounds were analysed in ELISA assays. Clones with 2-fold higher signals than the average from unselected phage were considered positive. Already after two rounds of competitive selection > 80% of the picked clones were positive against LDH (Table 2). However, the mean signal (A_{490nm}) obtained from positive clones of this round (0.623) was lower than that from the third round (1.444), indicating that the reactivity of the individual clones increased from the second to the third round, and that binders with higher affinity had been selected. The specificity of polyclonal and monoclonal phage was verified by ELISA coated with LDH in a dilution series (Fig. 3A), and by immunoblotting (Fig. 4). Likewise, the specificity of the soluble scFv antibody fragments was verified by immunoblotting (Fig. 4). In order to ensure that binders could be identified, not only against LDH, but also against the other proteins in the protein mixture, the library was panned against each of the proteins individually, and clones recognising all six were isolated (data not shown).

The power of selection inhibition during the competitive panning was analysed by performing selections against an extract from a melanoma cell line (FM55p) with or without the same extract in competition. After the pannings, the binding of the polyclonal phage pools against FM55p proteins was compared in ELISA (Fig. 2B). The reactivity of the polyclonal phage in ELISA (A_{490nm}) towards FM55p proteins was 0.419 after four rounds, and 0.757 after five rounds of selection without competition. In contrast, the competitive selections resulted in signals that were equal to the background signals (0.107). Also, individual clones from the fifth round were picked and tested against FM55p proteins in ELISA, in order to evaluate further the value of selection inhibition. Following competitive selection, none of the clones picked were positive (2-fold above background), whereas in comparison, 67% of the clones picked after selection without competition were positive (Table 2). The specificity of the phage, which had been selected without competition, was verified by inhibition ELISA showing reduction of the signals following addition of increasing amounts of FM55p proteins to the wells (Fig. 3B). Thus, the competitive procedure impeded the reactivity towards FM55p proteins.

When aiming at subtraction between two components, the ideal situation is to have selection against all differences simultaneously with selection inhibition of all similarities. The dilemma remains, however, that attempts to improve the efficacy of selection inhibition by using higher stringency during the panning rounds will tend to decrease the diversity in the subtracted phage pool, because low-affinity binders are lost [33]. Consequently, a less stringent approach may be more suitable in some cases. Also, the possibility of isolating phage antibodies against most of the differences may be increased by sub-fractionation of the cell extracts before panning in order to increase the density of the individual antigen coated, or by using different coating conditions in parallel to cover as many proteins as possible.

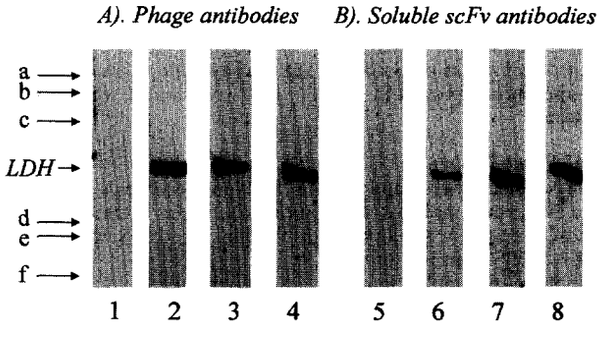

Fig. 4. In order to test the specificity against LDH, MIX proteins (a-f, 1 µg of each per lane) and LDH (1 µg per lane) were electrophoresed and electroblotted to nitrocellulose filters. Each lane was cut out and probed individually with phage from bacterial supernatants, or scFv antibody fragments obtained from periplasmatic purifications. (A) Immunoblots probed with polyclonal phage (2×10^{10} cfu per ml 2% MPBS) from the first round (lane 1) and third round (lane 2), or with phage (5×10^9 cfu per ml 2% MPBS) from the clones B5-2 (lane 3) and C5-1 (lane 4). (B) Immunoblots probed with polyclonal scFv fragments (1:3 in 2% MPBS) from the first round (lane 5) and third round (lane 6), or scFv fragments (1:10 in 2% MPBS) from clones B5-2 (lane 7) and D10-2 (lane 8).

Very recently, a robust phagemid library, with a diversity of 1.4×10^{10} scFv expressed on phage, has been developed [14]. In theory, such a library represents a source from which scFv antibody fragments with nanomolar affinities can be isolated against apparently any antigen. The chance of isolating cell population-specific antigens by competitive biopanning as described in this paper will increase once such libraries are available, because high affinity allows more stringent panning and washing procedures, and because cross-reactivity should be minimal. High-affinity libraries will also improve the probability of identifying binders against low-expressed antigens, as the high affinity compensates for a low antigen density. Hence, the use of a large naive antibody library and the subtractive panning strategy described is likely to be a fast and easy way to identify research reagents directed against biomarkers of cellular extracts or biological fluids. Such antibodies would be useful in mapping differences in gene expression between two populations of cells, like between old and young cells or between transformed and non-transformed cells, and thus in an understanding of their phenotypes.

Acknowledgements: We would like to thank Dr Greg Winter for kindly providing the antibody library. Dr Jesper Zeuthen for kindly providing the melanoma cell line, and Villy Jensen for providing the immunobeads. We are grateful to Dr Andrew Griffith for advice. This work was financially supported by The Danish Natural Science Research Council, The Institute of Experimental Clinical Research at the University of Aarhus, and Aage Bangs Foundation.

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