Selection of Phage-displayed Antibodies Specific for a Cytoskeletal Antigen by Competitive Elution with a Monoclonal Antibody

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A phage-display combinatorial library of V<sub>L</sub> and V<sub>H</sub> sequences of mouse antibodies was constructed, which contained 4.5x10<sup>6</sup> independent clones. From this library pools of phage were selected by up to four biopanning rounds on cytoskeletal preparations of ovarian carcinoma cells (OVCAR-3). Phage of these pools were then allowed to bind to a cytoskeleton preparation of bladder carcinoma cells (T24). The binding phage were challenged by a monoclonal antibody (mAb) directed against an epitope on cytokeratin 8. Displaced phage were rescued and screened for anti-cytokeratin immunoreactivity by ELISA, indirect immunofluorescence and Western blotting. About 50% of the phage selected by competition with the cytokeratin mAb reacted with the cytoskeletal preparations of T24 cells in ELISA. In contrast, in non-cytokeratin-containing cells, no reaction was observed. Immunofluorescence and Western blotting studies with a number of these clones showed reactivity against cytokeratin. We conclude that the phage-display competitive elution method can be used as a rapid technique to obtain immunoreactive phages, and eventually single-chain Fv (scFv) antibodies directed against defined epitopes, which were formerly characterized and validated by mAbs.

Keywords: phage display; scFv; competition elution; cytokeratins; bladder carcinoma cells

1. Introduction

During recent years considerable progress has been made in the construction and use of antibody phage-display libraries (Clackson et al., 1991; Burton et al., 1991; Marks et al., 1992a; Hoogenboom et al., 1992; Griffiths et al., 1993). These libraries consist of random combinations of the variable regions of immunoglobulin heavy and light chains, thus resulting in the expression of antibody fragments that are fused to one of the coat proteins of filamentous phage (Barbas et al., 1991). These fusion proteins displayed at the surface of filamentous phage are detected by affinity purification with immobilized antigen, and so specific scFvs† or Fabs can be obtained that bind to a certain antigen with reasonable affinity (K<sub>d</sub>, 10<sup>-6</sup> to 10<sup>-7</sup> M) (Marks et al., 1991; Hoogenboom & Winter, 1992). Molecular biological manipulation of these antibody fragments, for example by heavy–light chain shuffling or the introduction of point mutations, can increase the affinity of the antibody fragments up to a K<sub>d</sub> of 10<sup>-9</sup> M (Marks et al., 1992b). On the other hand, the use of larger repertoires will result in the isolation of antibodies with higher binding affinities. At present, completely artificial immune repertoires are available for the generation, selection and affinity maturation of antibody fragments (Nissim et al., 1994).

A considerable number of mouse mAbs have been demonstrated to be valuable in diagnosis and therapy (Larrick, 1989). Many of these mAbs are directed against well defined epitopes specific for the affected tissue. On the other hand, phage-display-derived scFvs offer a number of advantages over mouse mAbs, such as better clearance from blood, selection from human combinatorial libraries (no HAMA response) and relatively easy molecular manipulation (Hoogenboom et al., 1992). Clinical application of phage-derived antibodies will be accelerated if such antibodies can be directed against epitopes already characterized as valuable by mouse mAb studies. Therefore, in the present

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† Abbreviations used: scFv, single chain Fv fragment of an antibody; mAb, monoclonal antibody; HAMA, human anti-mouse antibody response; NCS, newborn calf serum; V<sub>H</sub> and V<sub>L</sub>, variable protein domains of an antibody; p.f.u., plaque forming units; PBS, phosphate-buffered saline; NPDM, non-fat dry milk; ELISA, enzyme-linked immunosorbent assay; PMSE, phenylmethylsulfonyl fluoride; g3p and g8p, products of M13 genes 3 and 8; PEG, polyethylene glycol; biopanning
study we have competed scFvs carrying phage with a mouse mAb. Cytoskeletal cytokeratins in bladder cancer cells were used as model system. Phage selected by competition elution were shown to react specifically with the cytoskeleton fraction of epithelial cells, but not with non-epithelial cells. These phage gave an immunofluorescence pattern typical for cytokeratins. We think that this method can be more widely applied, for instance, to select specific human scFvs by competing phage, from a human combinatorial library with mouse mAbs, or for the selection of scFvs directed against hormone receptors, by competition with the ligand.

2. Materials and methods

(a) Cell lines

OVCAR-3 human ovarian carcinoma cells were used for the primary selection of phage from a mouse combinatorial library. The characterization of this cell line has been described by Hamilton et al. (1983). In short, this cell line is an epithelial ovarian carcinoma cell line expressing androgen and estrogen receptors, cytokeratins 7, 8, 18, 19 and vimentin. The human bladder carcinoma cell line T24 was used for competition selection of the phage for cytokeratin 8. Culturing conditions, specific characteristics and the reaction of the T24 cell line with several anti-cytokeratin mAbs have been described previously (Schafmisl et al., 1989). In addition to the T24 cell line, we used two adenocarcinoma cell lines: NL-AC1 and NC1-H125 (Broers et al., 1986), as well as HUVEC cells, a human endothelial cell line (Bruggeman et al., 1988). The adenocarcinomas react with the RCK102 mAb which in these cell lines demonstrates the presence of cytokeratin 8. In contrast, HUVEC cells do not react with the RCK102 mAb, since these are known not to contain cytokeratins.

T24 cells were grown in MEM medium (Gibco, Paisley, UK) supplemented with 10% newborn calf serum (NCS), 1% glutamine, 1% Hepes and 1% non-essential amino acids (Flow, High Wycombe, UK); other cells (NL-AC1 and NC1-H125, HUVEC) were grown in RPMI-1640 medium (Gibco, Paisley, UK) supplemented with 10% NCS and 1% glutamine (Flow, High Wycombe, UK).

(b) mRNA isolation

Spleens from five non-immunized Black star mice were homogenized in 3 M LiCl/6 M urea and incubated at 4°C overnight. After centrifugation (28,000 revs/min, SW40, 30 minutes, 4°C) the pellet was resuspended in 10 mM Tris-HCl (pH 7.4) containing 0.1% SDS. After extraction with phenol/chloroform total RNA was precipitated and subsequently used for mRNA isolation using the poly(A)-tract system according to protocols supplied by the manufacturer (Promega, Madison, Wisconsin, USA).

(c) Generation of a mouse scFv phage display library and affinity selection of cytokeratin-specific clones

The scFv phage display library was prepared with the mouse scFv module/recombinant phage-display system (Marks et al., 1991) according to protocols supplied by the manufacturer (Pharmacia Biotech, Uppsala, Sweden). In short, 3 µg poly(A)+ RNA was used for random primed cDNA synthesis. The resulting single-stranded DNA was used for primary amplification of either VH and VL sequences using a mixture of VH or VL specific primers (Claekson et al., 1991). After purification of the specific PCR products, the VH or VL sequences were randomly assembled by a linker sequence (Ho et al., 1989). After a secondary amplification of the assembled scFvs using a mixture of VH specific primers containing a SfiI site at the 5’ end and a mixture of VL-specific primers with a NotI site at the 3’ end, the products were digested with SfiI and NotI (Pharmacia Biotech, Uppsala, Sweden) and ligated in pCANTAB5 (Pharmacia Biotech, Uppsala, Sweden). This vector enables the rescue of recombinant phages expressing a fusion protein of the g3p gene product and the assembled scFv protein at their surface. The ligation mixture was purified and electroporated into Escherichia coli TG1 cells at an efficiency of 4 x 10^8 transformants per µg plasmid. This procedure resulted in a library of 4.5 x 10^10 independent clones, comprising approximately 30% of the mouse immunoglobulin encoding repertoire. After phage rescue using M13K07 as a helper phage, a phage stock was prepared containing 2.5 x 10^13 p.f.u./ml, which was used for subsequent biopannings.

OVCAR-3 cells were grown to near 50% confluence and fixed by immersion in ice-cold methanol (5 s) and acetone (3 x 5 s). Then, coverslips were dried in air and stored at -20°C until use. Coverslips were incubated with phage (10^12 p.f.u./ml) of the described library for two hours at 37°C. After extensive washing with PBS/0.5% Tween-20 (10 x) and PBS (10 x), bound phage was released with a short incubation in 100 mM triethylamine and neutralized with 1 M Tris-HCl (pH 7.5). After washing the phage, the procedure was repeated four times (Marks et al., 1991). Stocks of different biopannings were stored at 4°C.

(d) The competitive elution method

For the competitive elution method, we took advantage of the specific cytokerin staining patterns revealed by immunohistologial staining with antibodies against cytokeratins. The RCK102 mAb (Sanbio B.V., Uden, Netherlands) is a broadly cross-reacting cytokeratin antibody of the IgG1 subclass, which recognizes cytokeratins 5 and 8,
and as a result stains virtually all epithelial tissue (Broers et al., 1986; Ramakers et al., 1987, 1990).

The competition assay was done on T24 bladder carcinoma cells. Cells were grown on coverslips and fixed by immersing (5 s) in cold methanol (−20°C) and then three short immersions in acetone. Blocking for background binding was performed with PBS containing 0.2% non-fat dry milk (NFFDM) (1 h/room temp). After washing with PBS, 10% phages were diluted in blocking buffer with 0.1% Triton X-100 and incubated for two hours at 37°C. After extensive washing (at least 10 times with PBS) cells were incubated with the competing antibody RCK102 (IgG1) for one hour at room temperature. The supernatant was removed and 10% was used for infection of E. coli strain TG1 (A550 = 0.6). Infection was achieved by incubation in a metabolic shaker (30 min, 37°C, 150 revs/min). Bacteria were plated out on TYE medium with ampicillin (100 µg/ml) and 2% (v/v) glucose (Miller et al., 1972), and single colonies were picked and grown in 2 x TY broth containing 100 µg/ml ampicillin and 2% (v/v) glucose (Miller et al., 1972). Once the bacteria reached A600 = 0.6, M13KO7 helper phage (2.5 x 109 p.f.u./ml) were added and incubated for 30 minutes at 37°C and 150 revs/min followed by 30 minutes at 250 revs/min. The bacterial cells were resuspended in 5 ml of 2 x TY medium containing 100 µg/ml ampicillin and 50 µg/ml kanamycin and incubated overnight (37°C, 250 revs/min) (Hoogenboom et al., 1992; Griffiths et al., 1993). Phage supernatant was used for the assays described below. If necessary phage was concentrated by precipitation with PEG (polyethylene glycol).

(c) ELISA

RCK102 competed phage were screened for binding on cytoskeletal preparations of cells by enzyme-linked immunosorbent assay (ELISA). Cells (2 x 10⁶ cells/well) were grown in 96-well plates and washed before fixation with PBS containing 0.1% Triton X-100, 1 mM PMSF (phenylmethanesulphonyl fluoride), 1 mM EDTA and 1 mM EGTA for 10 minutes at 0°C. Fixation was done by adding cold (−20°C) methanol/acetone (1/1, v/v), and blocking with PBS containing 2% NFFDM (1 h, 37°C). Selected phage (10⁸ phage/well) were added and incubated for two hours at 37°C. After five washes with PBS, bound phage were detected by incubation with a mouse mAb (IgG2A) directed against gp3 major coat protein of M13 (25 µg/ml tissue culture) (E. Meulemans et al., unpublished results) for one hour at 37°C, followed by an incubation with a peroxidase-labeled rabbit anti-mouse Ig antibody (Dako, Glostrup, DK) for one hour at 37°C. Slides were washed, absorbance substrate (1 mg/ml in 10 mM sodium citrate and 0.03% H₂O₂) added and the A₅₉₀ was measured, using a microphotometer (Pharmacia Biotech, Uppsala, Sweden).

(f) Indirect immunofluorescence

Binding of anti-cytokeratin scFv-bearing phage was also monitored by indirect immunofluorescence. T24 cells were grown, washed and fixed on coverslips as described before. Blocking was performed with PBS containing 4% normal rabbit serum for one hour at 37°C. Slides were incubated with phage (10⁸ p.f.u./ml) for one hour at 37°C. After extensive washing (3 x with PBS, 10 min each) phage were fixed with ethanol (10 min, −20°C) and incubated with the mouse anti-M13(gp3) antibody for one hour at 37°C. Binding was detected with fluorescein-conjugated rabbit anti-mouse antibody (Dako, Glostrup, Glostrup, DK).

(g) Western blotting

For the detection of phage binding to cytokeratins in Western blots, cytokeratin fractions were prepared from T24 cells (5 x 10⁶) by treating the cells with 0.5% Triton X-100/PBS solution supplemented with 0.4 mM PMSF 1 mM EDTA and 1 mM EGTA (5 min on ice). After centrifugation (10 min, 1500 g), pellets were fractionated on SDS-10% polyacrylamide gels (Laemmli, 1970) and electrophoretically transferred onto nitrocellulose membranes (Bers & Garfin, 1985). Membranes were blocked (1 h/room temp.) with 10% NFDM/0.5% Tween-20/PBS. Then, phages (10⁸ p.f.u./ml) were added and incubated overnight at 4°C under gentle shaking (Nissim et al., 1994). After extensive washing with 2% Tween-20/PBS, binding of the phage was detected with the mouse mAb directed against the phage gp3 protein (1 h/room temp.) followed by an incubation (1 h/room temp.) with peroxidase-conjugated rabbit anti-mouse mAb (Dako, Glostrup, Glostrup, DK). Peroxidase activity was detected using the electro-chemiluminescence (ECL) kit (Amersham, Little Shelford, Cambridge, UK).

3. Results

(a) Competition selection assay

The scFv phage-display library was constructed from spleen cells of non-immunized mice and was selected for binding to OVCAR-3 ovarian carcinoma cells. This pre-selection step comprised four consecutive rounds of biopannings. The number of phage bound to the cells was more or less the same for each biopanning (2.5 x 10⁶ eluted phage/2.5 x 10⁸ applied phage).

In order to select phage carrying scFvs against specific epitopes, a competition assay was carried out. The T24 bladder carcinoma cell line was chosen as an experimental model because cytokeratin expression is abundant and cells can easily be propagated. These cells were incubated with phage pre-selected by biopannings on OVCAR-3 cells. Phage bound to cells were competed with the cytokeratin mAb RCK102 and displaced phage were used for infection of bacteria. A 1/100 dilution of the RCK102 mAb (30 to 50 µg/ml tissue culture) achieved the best recovery of...
Figure 1. Results from the competitive elution, using phages from 3 different pre-selection biopanning rounds (2, 3, 4) showing the number of colonies obtained after competition of phage bound to T24 cells with RCK102. PBS was used as control.

Figure 2. Reactivity of the cytokeratin RCK102 and phage no. 27 selected by competitive elution with different cell types in an ELISA assay: 2 adenocarcinomas (NCI-H125 and NL-AC1), T24 bladder carcinoma cells and HUVEC endothelial cells. The results represent the average of 3 separate experiments.

Figure 3. Binding specificity in ELISA of the phage different pre-selection biopanning rounds replaced 2 cytokeratin preparations of T24 cells by competing the RCK102. The binding activity of the supernatant rescued phage was compared with those of the RCK.

(b) Characterization of phage selected by competition and specificity of selected clones

To verify whether the selected phage bound to cytokeratin filamentous structures and more specifically to cytokeratins, 60 colonies were picked and screened for binding characteristics in ELISA, immunofluorescence and Western blotting. Phage from selected colonies were rescued using the helper phage M13KO7.

(c) Screening of the clones on different cell types

Phage were screened for cytokeratin binding to a panel of cell lines. The phage isolated by competition on T24 cells were examined by ELISA using plates coated with the cytokeratin extracts of adenocarcinoma cell lines NCI-H125 and NL-AC1, and HUVEC endothelial cells. Phage positive on the T24 bladder carcinoma cells were also positive in ELISA on the cytokeratin preparations of NCI-H125 and NL-AC1, as these cell lines contain cytokeratins. In contrast, all phage were negative with the HUVEC endothelial cells, which do not contain cytokeratins (Figure 2).

(d) ELISA

Phage reactivity was measured by ELISA on cytokeratin preparations of T24 cells. The phage were visualized by an anti-gp8 mouse mAb, and binding affinity of the selected phage was compared with that of mAb RCK102. Phage were considered as “binders” when ELISA reactivity was > 50% of that of RCK102 binding reactivity. About 20% of the phage showed a strong reactivity in ELISA (Figure 3). HindIII and BamHI DNA restriction patterns of the selected phage showed that about 50% contained an insert corresponding to the size of a scFv fragment. The selected phage that gave no binding signal in ELISA did not contain the insert.

(c) Immunofluorescence

Binding of selected phage to the cytokeratin of T24 bladder carcinoma cells was screened by indirect immunofluorescence microscopy (Figure 4). Cytokeratins as visualized with RCK102 are visible as clearly defined filamentous structures, concentrating around the nucleus and venturing out into the cytoplasm of the cell, a pattern typical of these intermediate filament proteins in T24 cells (Figure 4D). By immunofluorescence assay the phage...
Figure 4. Immunofluorescence micrographs of the bladder carcinoma cell line T24 stained with the reactive phage (A, B, C) or with the RCK102 (D). Staining pattern of non-reactive phage are shown in E. Cytoskeletal preparations of HUVEC endothelial cells, which do not contain cytokeratins, were negative for reactive phage (F).

...gave a fibrogranular staining pattern (Figure 4A, B, C). The staining pattern of the phage resembles the pattern of RCK102: concentration of immuno-reactivity around the nucleus, spreading out into the cytoplasm of the cell. By adjusting the plane of focus, the dots appear to be aligned along fibre-like...
structures. However, phage that did not bind in ELISA did also not bind to the T24 cells in the immunofluorescence assay (Figure 4E). Not all the "binders", reactive in the ELISA, gave the intensity of staining in the immunofluorescence procedure. HUVEC endothelial cells, which do not contain keratin filaments, did not show a staining reaction after incubation with reactive phage (Figure 4F).

(f) Western blotting

Phage were applied to Western blots containing cytoskeleton preparations of T24. Phage had to be concentrated by precipitation with PEG to a minimal concentration of 10^{11} p.f.u./ml to provide a signal in Western blotting analyses.

Western blots incubated with the cytokeratin mAb RCK102 react with a 52 kDa protein responsible for cytokeratin 8. In addition, with the reaction of the 52 kDa cytokeratin band, incubation with phage results in a lot of bands, even if the phage could not bind. Incubation with phage carrying a scFv that appears to react with cytokeratins in ELISA and immunofluorescence assay showed a protein band with the same molecular mass (52 kDa) as the one reacting with the RCK102 mAb. Phage that did not react in ELISA, did not show a band at 52 kDa (Figure 5). Reactivity due to non-specific binding of phage to nitrocellulose, provided a serious problem. Not all the phage selected by the competitive elution method and defined as "good binders" by ELISA or by the immunofluorescence assay could detect cytokeratin 8 in the Western blots, which indicates a difference in binding properties between the selected phage.

4. Discussion

Selection of scFv's specific for certain cells or tissues from phage displays is time consuming because of extensive screening for properties such as species cross-reactivity, tissue/antigen specificity, and labelling efficiency. On the other hand, there are many mouse mAbs available that have been subjected to these series of tests and for which the epitopes are well characterized. Efforts to make scFv's derived from such mouse mAbs have often resulted in a considerable decrease in immunoreactivity (unpublished results). Considerable time and energy can be saved if, based on such mouse mAbs, scFv's derived from antibody phage display repertoires could be generated against well characterized antigens/epitopes. The competition assay presented offers an opportunity to select scFv's with predictable specificity. Our data demonstrate that competition between a mAbs and phage results in the release of phages that interact with the same antigen. In addition, it is demonstrated that whole cells can be used as a substrate for phage affinity selection.

Cytokeratin intermediate filaments were chosen as a model to test the validity of the competitive elution method. The advantages of this model can be summarized as follows: (1) cell lines with defined cytokeratin patterns, as well as cell lines that lack cytokeratins are available. (2) The specificity of immunoreactive fragments can be easily deduced from the morphological patterns that are typical of these filaments. (3) The biochemical properties of cytokeratins have been described extensively (Moll et al., 1988). In this model system the competition-selected phage recognized structures similar to cytokeratin filaments, as demonstrated by the same protein band revealed with RCK102 in Western blotting and by the specificity for epithelial cells revealed by ELISA. The data presented provide reasonable evidence, although no direct proof, that the selected (displaced) phage recognize the same epitope (or its flanking sequences) as the mAb.

The affinity of the selected phage depends strongly on the size of the combinatorial library from which they are derived (Griffiths et al., 1993; Nissam et al., 1994). The larger the library, the greater the chance of selection of strong binders. The library used in this study is small. Within the group of selected phage a wide variety of affinities was observed. These results show that a small number of phage clones have a high affinity towards cytokeratin.
indicating that the competition method also works for scFVs with high affinity. However, strong binders in ELISA sometimes failed to perform well in the other assays.

The fibrogranular staining pattern in indirect immunofluorescence assay may very well be due to the size of the phage, which may cause steric hindrance and reduce penetration into cells. These disadvantages can be overcome by application of scFVs, but sometimes at the cost of immunoreactivity (Nissim et al., 1994). On the other hand, the phage can be used for signal amplification by the use of mAbs directed against the coat protein.

We have tested the concept of an antibody phage-display competitive elution method on a relatively simple system with characteristics that made it easy to establish the feasibility of the method. The procedure can also be applied in other areas of research such as selection of human scFVs or generation of hormone receptor-binding antibodies. Well-characterized mouse mAbs can be used to compete with phage of human combinatorial libraries, selecting phage that recognize antigens already known to be indicators of particular malignancies. Antibodies to receptors may be selected by competing combinatorial libraries with the ligand. In this way producing potential agonists and antagonists, or phage that identify the receptor protein or its coding gene. In such investigations the method described here may prove to be efficient and fast.

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References

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