Phage displayed antibodies specific for a cytoskeletal antigen. Selection by competitive elution with a monoclonal antibody

Els V. Meulemans, Luc J. Nieland, Wiel H. Debie, Frans C. Ramaekers and Guillaume J. van Eys

Department of Molecular Cell Biology and Genetics, University of Limburg, Maastricht, The Netherlands

The display of repertoires of antibody fragments on the surface of filamentous phage offers a new way to produce immunoreagents with defined specificities. Here we report the selection of antibody fragments against the cytoskeletal fraction of T24 bladder cancer cells. To focus selection to a specific antigen, we eluted bound phage with a mouse monoclonal antibody directed against cytokeratin. Our initial studies proved that such a selection procedure with a library, carrying the mouse antibody fragment repertoire, resulted in phage specificity for the antigen against cytokeratin 8, recognized by the mouse monoclonal antibody. To facilitate detection of reactive clones, monoclonal antibodies against phage epitopes were developed. A human synthetic library (> 10^6 clones) was used for selection by competitive elution after binding to T24 cytoskeleton. About 50% of the phage reacted in ELISA with cytokeratins of T24 cells, while with noncytokeratins containing cells no reaction was observed. Immunofluorescence studies and Western blotting with a number of these clones showed reactivity against cytokeratin. We conclude that the competitive elution method can be used as a rapid technique to obtain immunoreactive phages, and eventually human single chain antibodies directed against defined epitopes which were formerly characterized and validated by mouse monoclonal antibodies. [Hum Antibod Hybridomas 1995; 6: 113-118]

Keywords: Phage display; scFv; competitive elution

Introduction
Antibody fragments (scFvs) have been generated and expressed in bacteria after selection by phage display technology. Repertoires of variable genes have been cloned into filamentous bacteriophage. The display of repertoires of antibody fragments on the surface of filamentous bacteriophage offers a way to select specific phages from the repertoire by binding to antigen. Such a selection can lead to scFvs with good affinities for the antigen, for example at least 10^-9 M for the hapten phOX. Molecular biological manipulation of these scFvs, for example by heavy-light chain shuffling or the introduction of point mutations, can increase the affinity of scFvs up to a Kd of 10^-9 M.

A considerable number of mouse monoclonal antibodies (MAbs) have been demonstrated to be valuable in diagnosis and therapy. Many of these MAbs are directed against well defined epitopes specific for the diseased tissue. On the other hand, phage display-derived scFvs offer a number of advantages over mouse MAbs, such as better clearance from the blood, selection from human combinatorial libraries (no HAMA response) and they can be manipulated relatively easily. In the present study we have tested the possibility to compete scFvs-carrying phage with a mouse MAb in order to obtain scFvs directed against known epitopes. Cytokeratins in bladder cancer cells were used as a model system. In this model, phages selected by competitive elution were demonstrated to react specifically with these cytokeratins of epithelial cells.

Materials and methods
Cell lines
The human bladder carcinoma cell line T24 was used for affinity selection of cytoskeleton specific clones. Characteristics and reactivity of T24 cells with several
anti-cytokeratin MAbS, have been described previously7. T24 cells were grown in MEM medium (Gibco, UK) supplemented with 10% new born calf serum (NCS), 1% glutamine, 1% HEPES, 1% essential amino acids (Flow, Wycombe, UK). Growth conditions and characteristics of the human lung cancer cell line NCI-H460 are described by Brower et al.4 The cytokeratin negative HUVEC endothelial cells are grown in RPMI-1640 medium supplemented with 10% NCS and 1% glutamine (Flow, Wycombe, UK).

**Monoclonal antibodies**

The RCK102 MAb 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 tissues10-12. RCK106 (IgG1) is monospecific for cytokeratin 18 in immunoblotting and recognizes columnar epithelial cells from digestive, respiratory and urogenital tracts, endocrine and exocrine tissues as well as a number of tumor cells11.

MAbs against phage coat epitopes were obtained by immunisation of Balb/C mice with filamentous phage (50 µg), purified by PEG6000 precipitation and killed by repeated freeze-thawing13. Two days after the second and final booster, spleen cells were fused with SP2/0Ag14 myeloma cells according to standard procedures4,13. Hybridoma supernatant was screened in ELISA on isolated phage and 3 clones were selected on basis of their specificity and high degree of reactivity (Table 1).

**Mouse scFv display library**

The mouse scFv-phage display library was prepared with the mouse scFv module recombinant phage display system14 according to protocols supplied by the manufacturer (Pharmacia, Biotech, Uppsala, Sweden). The construction of the library is described previously17.

**Human synthetic scFv library**

The human synthetic scFv library consisted of one human Vκ segment combined with rearranged VH gene segments in vitro from a bank of human VH gene segments15 and random nucleotide sequences encoding CDR3 with a length of 4-12 residues5. The construction of the library is described by Nissim et al.19.

**Competitive elution method**

The competitive elution assay was performed on T24 bladder carcinoma cells. Cytoskeletal preparations of T24 cells, obtained by Triton X-100 extraction, were incubated with the phage libraries, washed and then competed with the MAbs RCK102 or RCK106. Phage released from the preparations were collected and further screened. Identification of reactive phage was performed by ELISA and immunofluorescence microscopy. For the latter method, we took advantage of the specific cytokeratin staining patterns revealed by immunohistochemical staining with antibodies against cytokeratins. The methods are described elsewhere in detail17.

**Results**

**Applicability of anti-phage g8p MAbs**

Testing of anti-phage MAbs by Western blotting revealed that all three reacted specifically and two strongly with g8p-protein despite the fact that the sera of the immunized mice showed a strong reaction with both g8p and g3p coat proteins (Figure 1). One clone appeared to be of the IgM type, the other two clones were typed as IgG2a-kappa and IgG2b-kappa (Table 1).

The specificity of mouse anti-g8p MAbs was further tested in Western blotting for detection of scFv-phage Tell13, reactive with turkey egg white lysozyme14. Binding of this phage to its purified antigen could be visualized with the phage antibodies RL-ph2 and RL-ph3 (Figure 2), although a weak reaction was also seen in the negative control. For the cytoskeletal preparations, a high background staining was observed. For the specific binding of phage to other proteins was seen. In indirect immunofluorescence assays, the anti-g8p MAbs were used to detect binding scFv-carrying phage to the cytokeratin of T24 cells.

The two IgG anti-g8p MAbs can be used in ELISA and immunofluorescence assays while the IgM MAb is

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Figure 1: Western blot analysis of the polyclonal serum (PS) reactive with g8p and g3p coat proteins of the filamentous bacteriophage, and the three MAbs RL-ph 1, 2 and 3 reactive with the g8p protein of the filamentous phage.

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Table 1: Specificity of anti-phage gp8 MAbS in several immune assays

<table>
<thead>
<tr>
<th>Clone</th>
<th>Ig subclass</th>
<th>Western blotting</th>
<th>ELISA</th>
<th>Immunofluorescence</th>
<th>Western blotting</th>
</tr>
</thead>
<tbody>
<tr>
<td>RL-ph1</td>
<td>IgG2b, k</td>
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<td>IgM, k</td>
<td>+</td>
<td>0,575</td>
<td>+</td>
<td>+</td>
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</table>

For Western blot analyses, 70 μg M13K07 helper phage protein was separated by gel electrophoresis and transferred to PVDF membrane. For ELISA, optical density was measured at 490 nm and results compared with pre-immune sera. Columns 3 and 4 refer to results from tests performed on filamentous bacteriophage; columns 5 and 6 show results from tests performed on T24 cytoskeletal preparations.

Figure 2: Immunoblots of lysozyme incubated with scFv-phage Tef13 (6) and subsequently with the mouse anti-phage MAbS (RL-ph 2 and Riph-3) as secondary antibody

better suited in Western blotting analyses (Table 1). Further experiments will compare the reactivity and specificity of these MAbS with the available polyclonal phage antibody.

The competition selection assay

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.

A competitive selection procedure with a library carrying the mouse antibody repertoire, resulted in phage specific for the antigen recognized by the mouse MAb RCK102 directed against cytokeratin 8 17.

The human scFv synthetic library, was applied for binding to the cytoskeleton of T24 cells. The number of phage bound to the cells was similar for each biopanning (10^3 p.f.u. eluted / 10^12 p.f.u. applied). For the competitive elution, cells were incubated with phage presorbed by three rounds of biopanning. Then phage bound to cells were competed with RCK106 MAb and displaced phage were collected and used for infection. A 1:10 dilution of the RCK106 MAb achieved the best recovery of phage in this assay (Figure 3). For the 1:200 dilution form, more clones were obtained than the 1:100 dilution form which is probably due to the number of cells and the treatment of cells. Several incubation periods were tested. Already after 15 minutes, phage were found to be displaced by the MAb. The assay is dependent of the concentration of MAb used for competition and the incubation time for competitive elution.

Characterization of selected clones

Binding of selected mouse scFv phage to the cytoskeleton of T24 cells was checked by indirect immunofluorescence. The phage gave a fibrogranular staining pattern, the outline of which resembles the pattern of RCK102. Staining is concentrated around the nucleus, spreading out into the cytoplasm of the cell (Figure 4). HUVEC endothelial cells which do not contain keratin filaments, did not show staining with reactive phage. Western blots, containing cytoskeleton preparations of T24 cells, incubated with selected mouse scFv phage showed a protein band with the same molecular mass (52 KD) as the one reacting with the RCK102 MAb (Figure 5).
Figure 4: Cytokeratin8 stained with (A): RCK102 MAb and B: mouse scFv-phage #27 (in T24 cells with the indirect immunofluorescence assay). C: scFv-phage #27 shows no staining in cytokeratin negative HUVEC endothelial cells.

Of the human synthetic library, 25% of the selected RCK106 human scFv phage showed a reaction with the cytoskeleton of T24 in ELISA, but not with HUVEC endothelial cells which do not contain cytokeratin filaments (data not show). Binding of selected phage was observed in indirect immunofluorescence assay on the cytoskeleton of T24 cells and NCI-H460 cells. It revealed a similar fibrogranular staining pattern, as the one observed with the mouse library derived phage (Figure 4). Although, mouse MAb RCK106 showed a more refined and detailed cytoskeletal staining pattern, the dotted pattern resulting of the phage clearly resembles the outline of the cytoskeleton. No staining was found in HUVEC endothelial cells.

Discussion and conclusion

The phage display technique may facilitate the selection of antibody fragments of therapeutic value or research interest. In the past, affinity selection from 'single pot' combinatorial libraries using purified antigen produced scFvs with good binding affinities against foreign and self-antigens. We demonstrated that selection of scFv-phage for specific antigens can be facilitated by competitive elution with MAb directed against that antigen (see also Ref. 17). It was demonstrated that the MAbs are able to displace phage from antigens,

Figure 5: Immunoblots of the cytoskeleton of T24 bladder cancer cells, stained with nonbinding phage and reactive mouse scFv-phage #27, in comparison with the reaction of MAb RCK102. The arrowhead indicates the position of cytokeratin 8 (52kDa). The asterisk indicates the position of a cytokeratin 8 breakdown product.
resulting in the selection of phage reactive in ELISA, immunoblotting and immunofluorescence assays. The production of scFv-carrying phage directed against cytoskeletal antigens was used as a model system. A similar strategy has been used to isolate a peptide antagonist to the thrombin receptor using a phage display epitope library23.

Using competitive elution of the human synthetic library, we succeeded in selecting phage that bound cytokeratins. Reaction signal of the human scFv-phage appeared weaker than the one observed with phage derived from the mouse-combinatorial library. This may be due to the fact that only one Vl chain sequence has been used for the construction of this library23. Immunofluorescence assays on cytoskeletons using selected phage from both the mouse and human library showed the similar granular staining patterns. The data presented, demonstrate that: 1) scFvs, independent of source (human/mouse) can be selected by competitive elution with a mouse MAb and, 2) that from a library, with only
one type of V\textsubscript{a} and a limited type number of V\textsubscript{a}e, scFvs can be selected.

**Nomenclature**

- scFv: single chain Fv fragment
- MAb: monoclonal antibody
- g3p and g8p: products of the M13 gene 3 and gene 8
- CDR: complementarity determining region
- PEG: polyethylene glycol
- p.f.u.: plaque forming units

**Acknowledgments**

The authors thank Dr G. Winter (Cambridge, UK) for providing the human synthetic library.

**References**