Effective use of nitrocellulose-blotted antigens for phage display monoclonal antibody selection

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SUMMARY

The combinatorial phage display library approach to antibody repertoire cloning offers a powerful tool for the isolation of specific antibodies to defined target antigens. Panning strategy is often a very critical point for selecting antibody displayed on the surface of bacteriophages. Most selection strategies described to date have relied on the availability of purified and often recombinant antigen, providing the possibility to perform selections on a well defined antigen source. However, when the antigen is difficult to purify by means of laborious and time-consuming chromatography procedures, panning of phage antibody libraries has to be performed on complex antigen sources such as cell surfaces or tissue sections, or even by in vivo selection methods. This provides a series of technical and experimental complications. In the present work, we successfully generated a mouse monoclonal antibody fragment from a phage display library directed against protein E7 of HPV18 avoiding antigen purification as for immunizing mice as for antibody library selection. Our work demonstrates the feasibility of phage antibody selections on antigens transferred to a nitrocellulose membrane as solid support, using one-dimensional polyacrylamide gel electrophoresis system as the only practice to separate a given antigen present in bacterial crude cell lysate.

KEY WORDS: Phage display, Monoclonal antibody, Human papillomavirus (HPV18), Oncoprotein E7, Antibody selection

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INTRODUCTION

HPVs of the high-risk group are associated with squamous intraepithelial lesions with a high potential for progression to invasive squamous cell carcinoma (Zur Hausen, 2002). Expression of the early viral genes E6 and E7 is frequently dysregulated in HPV-related cervical carcinomas (Jeon *et al*., 1995a, 1995b).

These proteins exhibit the major oncogenic activity of the HPV DNA and many attempts have been made to study the biochemical functions and molecular interactions of these proteins. Procedures to purify the E7 oncoprotein have been developed by others (Fiedler *et al*., 2006), however, E7 proteins were found difficult to express and purify. Combinatorial antibody selection using molecular cloning techniques is a powerful technology for the generation of monoclonal antibodies of desired specificity (Barbas *et al*., 1993; Smith and Petrenko, 1997). Functional Fab fragments can be displayed on the surface of filamentous phage which provides a useful handle of selection with the appropriate binding antigen. Once selected, the expression of Fab fragments in *Escherichia coli* permits large-scale production, with low cost, enabling the use of such molecules in research and clinical application (Bugli *et al*., 2004).

Some authors successfully selected monoclonal phage antibodies against antigens blotted from 2D SDS-PAGE gels onto polyvinylidene floride (PVDF) membranes (Liu *et al*., 2002). We describe here the use of this approach for the generation of an HPV18-E7-specific mouse monoclonal antibody fragment exploiting a useful strategy that

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allowed mice immunization and subsequent antibodies selection without the specific viral antigen purification. To generate such antibody we developed a method which permits direct selection of phage antibody library against antigens blotted on nitrocellulose membranes from 1D SDS-PAGE gels.
This kind of approach could be useful in all that cases when the antigen results difficult to be purified by means of laborious and time-consuming chromatography procedures. Beside this, another important application of this approach is the possibility to immunize animals directly with a crude bacterial lysate expressing the desired antigen. The antibody fragment we selected applying this method is able to bind viral E7 protein in western blot analysis and it could be potentially used for direct detection of HPV oncogene expression in clinical samples.

MATERIALS AND METHODS

Cloning and expression of E7-HPV18
The E7 gene was amplified by polymerase chain reaction (PCR) from HPV18-containing HeLa cell line (ATCC: CCL-2, HPV18 positive) using specific primers.
The PCR product of E7 open reading frame was inserted into a suitable cloning vector and confirmed by colony-PCR, restriction enzyme analysis and sequencing. The E7 gene was than subcloned into pET-15b vector (Invitrogen) under the control of Lac-Z promoter. *E. coli* BL21(DE3) pLysS (Invitrogen) cells were transformed with the pET-HPV18-E7 expression vector and induced by isopropyl-β-D-thiogalactoside (IPTG) at 0·001 mol l^{-1} final concentration. Bacterial cell lysate from 50 ml of IPTG induced culture was prepared by using B-PER lysis buffer (Pierce) according to the manufacturer's protocols. Expressed E7 protein was detected by SDS-PAGE and by immunoblot with anti-His monoclonal antibody (Sigma).

Immunization of mice
Female Balb/c mice (6 to 8 weeks-old; 25 to 30 g) were housed in filter-top cages with free access to food and water. The mice were maintained in our Unit for Laboratory Animal Medicine according to protocols that had been approved by an institutional animal-use committee. Female Balb/c mice were injected subcutaneously (s.c.) with 0·5 mg total proteins from BL21 *E. coli* crude periplasmic extract in complete Freund's adjuvant (Sigma) derived from induced BL21 *E. coli* cells transformed with pET-HPV18-E7 vector and containing approximately 5 µg of E7 recombinant protein.
Boosts with the same amount of bacterial proteins and the same procedure were given on days 21 and 42. Animals were bled two weeks after each boost and sera from all bleedings were assayed for anti-E7 antibody by western blot analysis on viral E7 protein from HeLa cell lysate. Two weeks after the last boost, the animal with positive immunoblot at the highest serum dilution was sacrificed to harvest spleen cells.

Construction of phage antibody library
Total RNA was extracted from 10^6 spleen cells using RNeasy RNA extraction Kit (Qiagen) and first-strand cDNAs were synthetized using SUPERSCRIPT™ II (Invitrogen, Carlsbad, CA) with an oligo (dT) primer.
The cDNAs encoding the mouse Vh-Ch1 regions of the antibody heavy chains isotypes IgG1, IgG2a and IgG3 and the K light chains variable regions were then amplified by PCR using Pfu polymerase (Stratagene) and a panel of degenerate primers specific for VH and VK as described by Williamson, *et al.* (1996). Mouse antibody Fab library was constructed as described (Smith and Petrenko, 1997) using the pComb3 vector (Barbas *et al.*, 1993).
The light and heavy chain libraries were respectively composed of $2x10^6$ and $5x10^6$ independent clones. The final antibody library constructs were then electroporated into *E. coli* XL1-blue cells and bacteriophages displaying the Fabs library were rescued by the infection of the transformed cells with VCSM13 helper phage (Stratagene, La Jolla, CA).

E7 antigen preparation and Biopanning
pET-HPV18-E7 expression vector was used to transform fresh BL21 competent *E. coli* cells. Bacterial cells (50 ml) were grown until the optical density of 600 nm (OD600, induced with 0·001 mol l^{-1} IPTG for 4 h at 25°C and lysed in 1 ml of B-Per buffer (Stratagene). Un-induced pET-HPV18-E7 transformed BL21 cells were grown

in parallel in the same conditions. After centrifugation at 15 000 g for 30 min, bacterial lysate was microfiltered (0·22 m pore-size) and dialyzed using Slide-A-Lyzer Dialysis Cassette (Pierce) against PBS. After dialysis bacterial extract was analyzed by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS- PAGE) in Tris Glycine electrophoresis buffer, under non-reducing condition.

Proteins from un-transformed, induced and un-induced cell cultures were transferred on nitrocellulose membrane (Biorad) and visualized by Ponceau Red staining. Expression of E7 protein was tested out with anti-His monoclonal antibody (Sigma).

The band corresponding to HPV18-E7 protein of approximately 14 kDa present in the induced cells culture extract and absent in the un-induced and un-transformed cells samples, was cut away from the filter with a scalpel and put in a 2 ml microcentrifuge tube. The antigen was than blocked with 1% BSA in PBS for 2 h at 37°C. 250 l of phages library (10^{12} cfu ml^{-1}) were incubated with blotted E7 antigen at 37°C for 2 h. After extensive washing by vortexing with 500 l of 0·5% Tween 20 in PBS (PBST), bound phages were eluted with 200 l 0.1 mol l^{-1} glycine-HCl (pH 2·2) by vigorous vortexing and immediately neutralized with 12 l of 2 mol l^{-1} tris-HCl pH 9·0. The eluted phages were amplified by infecting XL1-blue *E. coli* cells followed by super-infection with VCSM 13 helper phages (Stratagene). The amplified phages were then subjected to another round of panning.

Four rounds of panning were conducted and the stringency of selection was increased with each round by increasing the number of washes. After the final round of panning, phage-bound Fab fragments were converted into soluble Fabs by vector modification as described by Burioni *et al.* (1998). XL1- blue *E. coli* cells were transformed with the modified vector and 25 single randomly picked colonies were grown and induced by isopropyl- -D-thiogalactoside (IPTG). Cell lysate of each clone was screened by immunoblot for anti-E7 immunoreactivity.

Western blot analysis

HPV16 E7 expressing CaSki cell lines and HPV18 E7 expressing HeLa cervical carcinoma cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Euro-Clone), 10% fetal bovine serum (FBS, Euro-Clone). Cells were lysed by freeze-thawing in PBS containing 1% Triton-X, 0·001 mol l^{-1} dithiothreitol (DTT), 0·2 mM PMSF and 1x Protease Inhibitor Cocktail (Sigma). Cell debris was removed by centrifugation. Yield of total proteins obtained in the supernatant was determined by the Bio-Rad protein assay kit I (Bio-Rad, Hercules, CA, US).

Cell lysates were denatured at 90°C for 5 min and separated on SDS-PAGE 15% with Tris-Glycine running buffer under reducing conditions. Subsequently proteins were transferred to nitrocellulose membranes (Bio-Rad) using Mini Trans-Blot Cell (Bio-Rad) according to the manufacturer's instruction. After blocking the membranes overnight in 1% BSA to reduce non-specific binding, crude BL21 cell lysate containing the anti-E7 monoclonal antibody was added to the membrane and incubated for 2 h at RT. After three washes with PBS 0·05% Tween 20, the AP-conjugated goat anti-mouse IgG Fab-specific (Sigma) secondary antibody, was incubated with the membranes for 1 h. After repeating the wash step, bound antibodies were visualized using BCP-NBT substrates (Sigma) according to the manufacturer's instructions.

Antibody expression and purification

XL1-Blue cells were transformed with the pComb-HPV18-E7 expression vector and induced by isopropyl-β-D-thiogalactoside (IPTG). Purification of Fab fragments was obtained by immunoaffinity chromatography. Gamma Bind G Sepharose (Pharmacia Biothec), bound to G protein and conjugated with polyclonal goat anti mouse IgG Fab-specific (Sigma) was used for the preparation of the chromatography column. Bacterial cell lysate from 2 L of IPTG induced culture was prepared by three ultrasound cycles of three min each. After centrifugation at 15000 g for 30 min and filtration, sample was applied to the column. After extensive washing with PBS, Fab was recovered with 10 ml of elution buffer (0·1 mol l^{-1} glycine, pH 2·5) and neutralized with Tris 1 mol l^{-1} pH 9·0. Antibody purity was analyzed using coomassie staining of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. After purification, the antibody preparation was dialyzed against PBS.

RESULTS

HPV18 E7 protein expression and mouse immunization

Conditions for E7 protein expression were first optimized by small scale induction and it was found that 4 h induction at 25°C with 0·001 mol l^{-1} IPTG turned out to be the optimal conditions. The E7 protein expression was than visualized on SDS-PAGE together with the untransformed and un-induced BL21 cell lysate. Cell lysate from 50 ml induced *E. coli* transformed with pET-E7 expression vector was prepared for mice immunization. The amount of E7 protein in the lysate, estimated by SDS-PAGE, was approximately 50 µg ml^{-1} (Figure 1).

Anti-His tag monoclonal antibody was used to confirm the expression of the recombinant protein (data not shown). After three immunizations with 250 µg total proteins of crude cell lysate containing approximately 5 µg of recombinant E7 protein, mice produced a strong immune response against the antigen resulting in high titres of circulating anti-HPV18 E7 antibody. Sera were screened for reactivity with E7 native antigen by western blot on HeLa cell lysate. A positive band of 14 kDa was visualized using sera dilutions ranging from 1/500 after the first immunization up to 1/5000 after the third boost. We selected the mouse showing the strongest response against the viral protein to prepare splenocytes for the generation of the antibody library displayed on M13 bacteriophage.

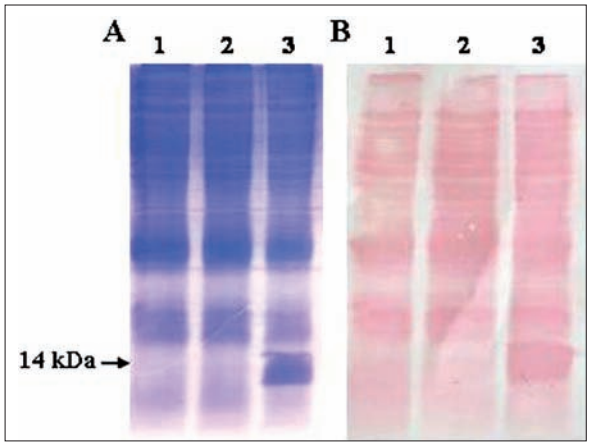

FIGURE 1 - *SDS-PAGE of BL21 E. coli crude cell lysates. Lane 1: untransformed cell lysate induced with IPTG, Lane 2: transformed and uninduced cell lysate, Lane 3: transformed and induced cell lysate of E. coli expressing HPV18 E7 recombinant protein. A. Coomassie blue staining. B. Ponceau red staining after blotting on nitrocellulose membrane.*

Library construction and antibody generation

Mouse antibody repertoire was cloned into the pCombIII phagemid vector (Barbas *et al*., 1993) to obtain a phage display antibody library of $3x10^6$ independent clones. After four rounds of phage selection against the antigen transferred on nitrocellulose filter, 25 single clones were expressed as soluble fab fragments in XL1-Blue *E. coli* cells and assayed for their ability to bind the native antigen from HeLa cell lysate by immunoblot.

Five clones resulted reactive against native oncoprotein E7 from HeLa lysate. All five phagemids carrying the light and heavy chains of antibodies fragments were extracted and the variable regions were sequenced. This approach led to the identification of identical nucleotide sequences corresponding to a unique monoclonal fab fragment that we will refer to as E7-5. Both the nucleotide and the amino acid sequences were analysed by comparison to GenBank sequences. The mouse monoclonal antibody fragment was purified by immunoaffinity chromatography. The final yield of purified E7-5 was 2 mg l^{-1}. Under non-reducing condition, purified E7-5 runs as a single band of about 50 kDa in SDS-PAGE (Figure 2), with a small amount of unassembled chains running around 25 kDa. Free recombinant E7 antigen was

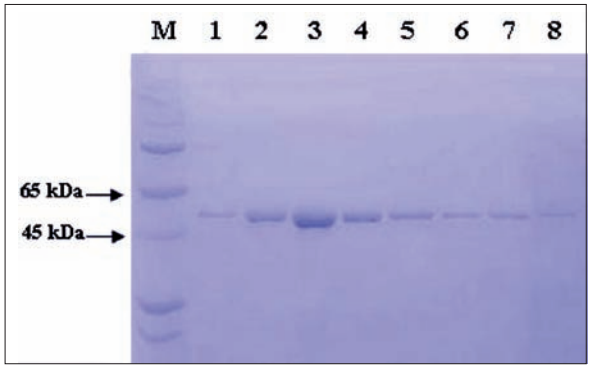

FIGURE 2 - *Coomassie blue staining of purified recombinant E7-5 monoclonal fab fragment produced in XL-1Blue E. coli cells. From lane 1 to lane 8: elution fractions of affinity purified antibody run on polyacrylamide gel under undenaturing conditions.*

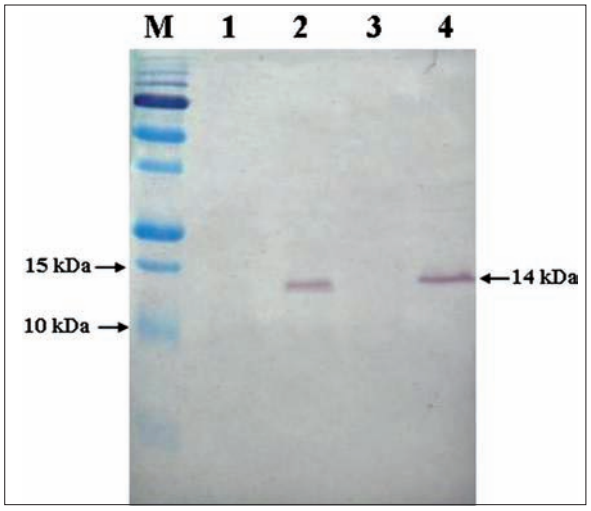

FIGURE 3 - *Western blot analysis of the established HPV18 E7 monoclonal fab fragment. Lane 1: CaSki (HPV16-positive) cell lysate, Lane 2: HeLa (HPV18-positive) cell lysate. Lane 3: untrasformed BL21 cell lysate. The antibody detected recombinant HPV18 E7-His expressed in BL21 E. coli cell lysate (Lane 4).*

used to compete binding of E7-5 monoclonal antibody to the same antigen bound to solid support in order to evaluate the relative binding affinity (data not shown). The Fab fragment displayed a high-affinity binding to E7 protein.

Characterization of antibody fragment specificity
Western blots were performed with HeLa cell lines (transformed with HPV18) and human cell lines CaSki (transformed with HPV16) in order to check reactivity and cross-reactivity against the viral antigens. The fab fragment demonstrated specific binding to a protein of the expected size of 14 kDa in HeLa cells. No cross-reactivity to the HPV16 E7 protein in CaSki cells or to other cellular proteins was obtained (Figure 3). No reactivity was observed in HeLa or CaSki cells when an irrelevant mouse recombinant antibody fragment was used. *E. coli* expressing HPV18-E7 was used as positive controls for the technique.

DISCUSSION

The possibility of obtaining a specific monoclonal antibody directed against a certain antigen without the need to purify that antigen represents a very useful strategy to save time and money, especially when obtaining good quantities of an antigen or even obtaining the purified protein is problematic. Expression of the small viral protein E7 of HPV18 in BL21 *E. coli* bacterial cells enabled the identification by SDS-PAGE of a clear band of the expected molecular weight. Cloning the E7 gene in fusion with a His-tag epitope gave us the possibility to use a commercial monoclonal antibody directed against the epitope to confirm the expression of the recombinant antigen. Crude bacterial lysate containing HPV18 E7 protein used to immunize mice induced a strong immune response against the viral antigen. Our original and effective selection strategy of the antibodies phage display library enabled us to perform four consecutive rounds of panning exploiting a prokaryotic fusion protein, well separated on SDS-PAGE and just transferred on filter support, as the specific antigen.
The monoclonal antibody fragment selected with this approach is able to bind, in immunoblot, HPV18 E7 protein expressed in HeLa cells with the potential to be a valuable tool in cervical cytology. However, we were unable to detect HPV18 E7 protein by the method of indirect immunofluorescence.
This may be because the SDS-PAGE of E7 protein caused the unfolding of the protein and, therefore the antibody we selected recognized a linear epitope of the viral protein, probably this is not present in sufficient amounts on entire HeLa cells to allow its detection by this method. Moreover, the selected antibody did not cross-react with E7 of HPV16, demonstrating that a high degree of linear sequence homology does not necessarily confer antigenic cross-reactivity.
The demonstrated genotype specificity of our Fab fragment should permit the establishment of clinically applicable assays for HPV18 E7 protein recognition. Obviously, antibodies for such applications must discriminate oncogenic E7 from the corresponding protein of low-risk HPVs (e.g. HPV6 and HPV11) and other viral and cellular proteins.
Cross reactivity against other HPVs antigens is still under investigation. Our Fab fragment showed the ability to recognize the E7 antigen with high affinity in his linear conformation, confirming our supposition that antigens from a crude lysate run in a denaturing SDS-PAGE and blotted on a nitrocellulose filter would be a good

mean to screen monoclonal antibodies. Antibodies selected with this methodology could be anyway competent in recognizing conformational epitopes (Bugli *et al*., 2008). As our data show, the use of a phage display selection process such as the one described here, will allow the isolation of valuable monoclonal antibodies directed against various antigens that appear hard or hardly possible to purify.

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