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ORIGINAL ARTICLE

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Serological cloning of cancer/testis antigens expressed in prostate cancer using cDNA phage surface display

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Abstract Serological cloning of tumor-associated antigens (TAAs) using patient autoantibodies and tumor cDNA expression libraries (SEREX) has identified a wide array of tumor proteins eliciting B-cell responses in patients. However, alternative cloning strategies with the possibility of high throughput analysis of patient sera and tumor libraries may be of interest. We explored the pJuFo phage surface display system, allowing display of recombinant tumor proteins on the surface of M13 filamentous phage, for cloning of TAAs in prostate cancer (PC). Control experiments established that after a few rounds of selection on immobilized specific IgG, a high degree of enrichment of seroreactive clones was achieved. With an increasing number of selection rounds, a higher yield of positive clones was offset by an apparent loss of diversity in the repertoire of selected clones. Using autologous patient serum IgG in a combined biopanning and immunoscreening approach, we identified 13 different TAAs. Three of these (NY-ESO-1, Lage-1, and Xage-1) were known members of the cancer/testis family of TAAs, and one other protein had previously been isolated by SEREX in cancer types other than PC. Specific IgG responses against NY-ESO-1 were found in sera from 4/20 patients with hormone refractory PC, against Lage-1 in 3/20, and Xage-1 in 1/20. No reactivity against the remaining proteins was detected in other PC patients, and none of the TAAs reacted with serum from healthy subjects. The results demonstrate that phage surface display combined with postselection immunoscreening is suitable for cloning a diverse repertoire of TAAs from tumor tissue cDNA libraries. Furthermore, candidate TAAs for vaccine development of PC were identified.

Keywords Serological cloning · Cancer/testis antigens · Prostate cancer

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Introduction

In Western countries, prostate cancer (PC) is the most frequent cancer in males and a leading cause of death from cancer [22, 30]. Though potentially curable in early stages, treatment for metastatic PC relies on hormonal intervention by androgen deprivation [30]. However, nearly all of these patients eventually develop disease progression, and no therapy has yet demonstrated a survival benefit in patients with hormone refractory PC (HRPC). Hence, continued interest exists in development of novel therapeutic approaches, including immunotherapy and biological agents targeting specific molecular alterations in the tumor cell, such as signal transduction pathways or molecules involving control of cell cycle progression.

Ideally, development of immunotherapy requires knowledge of tumor-associated antigens (TAA) that fulfill a number of criteria, including predominant expression in tumors, capability of inducing immune responses, and reliability as targets for effector functions of the immune system, in particular cytotoxic T cells [21]. In PC, much work has focused on proteins associated with prostate epithelial differentiation, such as prostate-specific antigen (PSA) or prostate-specific membrane antigen (PSMA) [24, 26]. In other types of cancer, such as malignant melanoma, T-cell based approaches have yielded a broader spectrum of TAAs with potential for vaccine development [39], but similar success has so far not been reported for PC.

SEREX (serological identification of tumor antigens by expression cloning) represents an approach well suited for identification of immunogenic proteins expressed

in tumors [27]. A large number of proteins have been identified by SEREX in nearly all cancer types analyzed, several of which are candidates for vaccine development [23]. The cancer/testis (C/T) antigens appear particularly interesting, as these proteins are expressed in cancer and in testicles, but not, or at a much lower level, in other normal tissues. Furthermore, patient antibodies have been identified against proteins involved in cancer-related molecular aberrations, including mutations (p53), chromosomal amplification/overexpression (HER-2/*neu*), or chromosomal translocations (SSX-2) [5, 23]. Such findings demonstrate the potential of serological cloning strategies in understanding cancer cell biology. We and others have used SEREX to identify TAAs in PC [6, 42]. However, conventional SEREX technology in our hands has proven labor-intensive, and automation, which would be required for a rapid analysis of a larger number of patient sera and tumor libraries, is difficult to achieve. Furthermore, as SEREX relies on the expression of recombinant proteins in the λ phage, the system has inherent limitations. The most important ones are the limited number of clones screenable with reasonable effort and the production of proteins under reducing conditions, which may bias the spectrum of identifiable proteins.

Phage surface display systems have been developed that allow presentation of cDNA encoded polypeptides on the surface of a bacteriophage, such as the filamentous phage M13 [2, 8, 11]. Phage surface display technology potentially overcomes some limitations of SEREX, and therefore, we explored the fitness of phage display using M13 phage to selectively clone TAAs from PC tissue cDNA libraries.

Materials and methods

Biopsy samples and sera

The regional ethics committee, Oslo, Norway, approved the study. Sera from 20 patients with HRPC and 11 male blood donors were stored in aliquots at –30°C. After written informed consent, serum was withdrawn and a lymph node metastasis surgically removed from HRPC-patient MD2. The biopsy sample was immediately frozen in liquid nitrogen. Six mouse antihuman NY-ESO-1 monoclonal antibodies (mAb) were kindly provided by Dr G. Spagnoli, Basel, Switzerland [32].

cDNA library construction and biopanning

Total RNA was isolated from the MD2 biopsy sample, mRNA purified, and cDNA synthesized with EcoRI and XhoI restriction sites in the 5' and 3' ends as described [6]. cDNA was directionally cloned into the pJuFo vector and transformed into *Escherichia coli* XL-1 Blue MRF' (Stratagene, LaJolla, CA) resulting in a library of 2×10^7 independent clones. A phage surface expression library was generated by VCSM13 helper phage superinfection (Stratagene) [2]. IgG from 0.25-ml serum or hybridoma supernatants was purified using ProteinG-Sepharose 4 Fast Flow (Amersham-Pharmacia Biotech, Uppsala, Sweden), and wells of 96-well MaxiSorp plates (Nunc, Roskilde, Denmark) coated overnight (ON) at 4°C with 100-µl IgG (20–500 µg/ml) in phosphate-buffered saline (PBS) pH 7.4. Wells were blocked with blocking buffer (BB; 5% [w/vol] milk powder in Tris-buffered saline pH 7.5 [TBS]/0.1% Tween 20). After washing in wash buffer (WB; TBS/0.1% Tween 20), 100-µl phage library (10^{11} colony-forming units [cfu]) was added and incubated for 2 h at 37°C. Wells were washed 15 times with WB, bound phages eluted with 100-µl 0.1-M glycine/HCl pH 2.2, transferred to a suitable tube, and neutralized with 6-µl 2-M unbuffered Tris base. Eluted phages were used to infect XL-1 cells, amplified, and harvested [2]. Phagemid DNA was isolated from bacterial pellets and analyzed for insert size distribution by agarose gel electrophoresis.

Colony immunoscreening

XL-1 cells were infected with phage, incubated 15 min at RT, and plated on Luria-Bertani (LB) agar plates (tetracycline [10 µg/ml] and ampicillin [100 µg/ml]). After incubation at 37°C and appearance of single colonies, plates were overlaid by Hybond-C filters (Amersham), and filters transferred to Super broth (SB) agar plates (antibiotics as above) containing 1-mM isopropyl-β-D-thiogalactoside (IPTG). After protein induction for 16 h at 37°C, filters were processed for immunoscreening [29], blocked 1 h in BB and washed with WB. Filters were incubated with patient IgG, preabsorbed against *E. coli* proteins at 1:200 in dilution buffer (DB; 1% [w/vol] milk powder in TBS/0.1% Tween 20) for 24 h at 4°C, followed by washing. Horseradish peroxidase (HRP) conjugated goat antihuman IgG (Sigma Chemical, St. Louis, MO) was used as secondary antibody 1:3,000 in DB buffer for 6 h at RT followed by washing and visualization by the ECL + Plus chemiluminiscence system (Amersham). After stripping, filters were probed with secondary antibody for exclusion of Igγ-chain harboring clones. Positive clones were subjected to secondary screening for confirmation. For analysis of IgG antibodies against isolated clones in other patients and healthy subjects, approximately 100 phage-infected bacteria were spotted onto separate areas 0.5 cm in width on NC filters overlying SB agar plates as above, incubated until visible colonies appeared, and processed as above.

Isolation, production, and analysis of individual phage clones

Single colonies were grown ON in SB (antibiotics as above) at 37°C. Phagemid DNA was isolated and sequenced as described [6]. For phage ELISA, clones were grown in 1-ml SB (antibiotics as above) to OD_{600} of 0.1, followed by infection with Hyperphage (PROGEN, Heidelberg, Germany). After incubation 15 min at RT, 100-ml SB with 1-mM IPTG was added (antibiotics as above) and shaken for 2 h at 37°C, followed by addition of kanamycin (100 µg/ml) and incubation for 12–36 h at 30°C. Phages were harvested as above and dissolved in TBS.

Production of recombinant proteins

Hexahistidine-tagged proteins were produced using the pQE3 vector system (QIAGEN, Hilden, Germany). For pJuFo inserts in reading frame 0 or −1 relative to c-Fos, cDNAs were cloned into pQE30 and pQE31 using appropriate restriction sites. Inserts with a +1 frame were amplified by PCR introducing one additional base in the sequence of the EcoRI adaptor used for cDNA synthesis and inserted into pQE30. Full length NY-ESO-1 cDNA was amplified from the MD2 library using primers containing BamHI and HindIII sites and cloned into pQE30. cDNA (NM_032306) encoding a 26.5-kDa protein was cloned into pQE31 as an irrelevant control protein (p352). Proteins were produced in *E. coli* M15 cells and purified under denaturing conditions as described by the manufacturer, dialyzed against PBS or water, and analyzed for homogeneity by SDS/polyacrylamide gel electrophoresis (PAGE).

ELISA and Western blotting with phages and proteins

For phage ELISA, MaxiSorp plates were coated ON at 4°C with 100-µl (10 µg/ml) rabbit antihuman IgG (DAKO, Glostrup, Denmark), blocked with BB, and washed. One hundred microliters of serum diluted 1:100 or more in DB was added, and IgG allowed to bind for 1 h at 37°C. After washing, 100-µl phage suspension (10^{10} cfu) was added, incubated for 2 h at 37°C, and washed again. Bound phages were detected with 100-µl HRP-conjugated goat anti-M13/pVIII (Amersham) at 1:5,000 in DB containing 500 µg/ml normal serum IgG, followed by incubation 1 h at 37°C, washing, and HRP enzymatic reaction using OPD tablets (DAKO). Absorbance at 492 nm was read on a Multiskan EX photometer (Labsystems Oy, Helsinki, Finland). Washes consisted of three rinses with PBS/0.1% Tween 20. ELISA with sera containing human and mouse IgG were performed similarly with plates coated with goat antimouse IgG (Sigma) and irrelevant mouse IgG added to the anti M13 antibody.

For ELISA using recombinant protein, 50-µl protein (10 µg/ml) in PBS was coated ON at 4°C. After blocking, 50-µl serum diluted 1:100 or more in DB was added and incubated 2 h at 37°C. HRP-conjugated goat antihuman IgG (Sigma) 1:5,000 in DB was used as secondary antibody.

In Western blotting, 1-µg protein was applied per lane. After blotting, filters were processed as described for colony immunoscreening using human serum diluted 1:100 in DB.

Polymerase chain reaction (PCR)

Primers differentiating between NY-ESO-1 and NY-ESO-1sv (retaining the second intron of the NY-ESO-1 gene) were used in PCR [38].

Results

Library construction and characterization

The cDNA phage display library pJuFoMD2 was constructed from an HRPC biopsy sample using the pJuFo vector. The pJuFo system allows display of fusion proteins linked via a Jun-Fos leucine zipper to the pIII protein of M13 and has been applied successfully to selectively enrich IgE-binding clones using sera of allergic patients [2]. Furthermore, pJuFo has been associated with better display and higher enrichment factors compared with other M13 phage display systems available for cDNA cloning [8].

Theoretically, biopanning of tumor cDNA phage display libraries on immobilized patient serum presents several challenges [15]. Most importantly, patient sera contain tumor-associated antibodies with unknown specificities, affinities, and concentrations in a polyclonal background of cancer unrelated IgG. These background IgG molecules may interfere with selection. cDNA-encoded proteins may further affect the life cycle of phage or *E. coli* differently, or may be presented at different efficiencies, resulting in bias of selected phages. To address these questions, experiments were set up to test different concentrations of a model tumor-associated antibody in a background of normal polyclonal IgG. The pJuFoMD2 library was shown to contain the C/T antigen NY-ESO-1 (Fig. 1a, Lane 1). Therefore, a mixture of 6 anti-NY-ESO-1 monoclonal antibodies was diluted at 10^{-1} to 10^{-4} molar ratios in IgG (100 µg/ml) from healthy blood donors. Four rounds of biopanning were performed with each test serum, and enrichment was monitored by restriction analysis and semiquantitative PCR on pools of selected phagemids, or by sequencing of individual clones (Fig. 1a). After round 2, enrichment of fully spliced NY-ESO-1 cDNA was seen for sera supplemented with 10% and 1% anti-NY-ESO-1 antibody. This was the only isoform of NY-ESO-1 also detected in the unselected library. A shift toward selection of phages containing a longer NY-ESO-1 cDNA fragment was seen after 4 rounds, representing a splice variant retaining the second intron of the NY-ESO-1 gene (NY-ESO-1sv) and encoding a truncated NY-ESO-1 protein. With test sera containing less than 1% specific antibody, NY-ESO-1 clones were

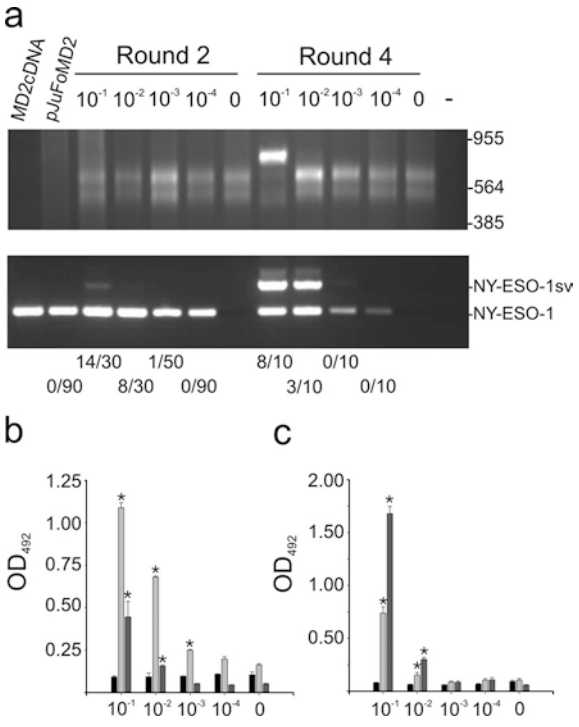

Fig. 1 **a** Biopanning with anti-NY-ESO-1 antibodies diluted in IgG from healthy human individuals. *Top panel:* ethidium bromide stained agarose gel of EcoRI/XhoI digests of pJuFoMD2 library *(lane 2 from left)*, selected phagemids after round 2 *(lanes 3–7)* and round 4 *(lanes 8–12)*. Empty lanes in 1 and 13. *Lower panel:* PCR for NY-ESO-1 cDNA in MD2 tumor cDNA (10 ng, positive control, *lane 1*), pJuFoMD2 library (50 ng, *lane 2*), selected phagemids (50 ng) after round 2 *(lanes 3–7)* and round 4 *(lanes 8–12)*. PCR without template *(lane 13)*. Figures above *top panel* denote molar fraction of NY-ESO-1-specific antibodies, figures below *lower panel* denote fraction of NY-ESO-1-encoding clones. Size markers are given to the right. **b** ELISA with sera in **a** and phages displaying NY-ESO-1 *(dark gray)* or NY-ESO-1sv *(light gray)* and empty pJuFo phages *(black)*. *Vertical lines* denote standard error for 4 experiments. Reactivity significantly different from empty pJuFo phages and normal human IgG are highlighted by * ($p < 0.05$, Student's *t*-test). **c** ELISA with sera in **a** and recombinant NY-ESO-1 *(dark gray)* or NY-ESO-1sv *(light gray)* proteins and negative control protein p352 *(black)*

detectable by PCR in pools of phages from round 2 and by analysis of isolated phages (1 of 50 phages selected on 0.1% anti-NY-ESO-1 test serum). However, with diluted test sera, NY-ESO-1 clones appeared to be lost at four rounds of selection. For reasons not analyzed, at concentrations of anti-NY-ESO-1 below 1% and in wells coated with normal IgG only, most sequenced clones contained Igγ-chain cDNA.

The artificial anti-NY-ESO-1 sera were tested in ELISA using phage and purified recombinant NY-ESO-1 or NY-ESO-1sv proteins (Fig. 1c). NY-ESO-1 and NY-ESO-1sv phages were specifically recognized by sera down to 1% and 0.1% specific antibody, respectively. Signals appeared stronger against NY-ESO-1sv phages than phages harboring NY-ESO-1, possibly due to better presentation of the shorter NY-ESO-1sv protein.

Together, these data demonstrate that the biopanning procedure described allows specific enrichment of immunodominant cDNA-encoded proteins. The data further suggest that less immunodominant proteins or proteins that are less efficiently processed during the phage / *E. coli* life cycle may be lost with increasing rounds of selection.

Biopanning with autologous serum IgG

Biopanning was performed using the pJuFoMD2 library and autologous patient IgG immobilized at two different concentrations (500 and 20 μg/ml). Two coating concentrations were chosen because altering target antibody density theoretically allows for enrichment of phages with different binding affinities [15]. Enrichment during four rounds of selection was monitored by restriction analysis of pools of phagemids, together with PCR and sequencing of individual clones after each round (Fig. 2a). Using wells coated with the higher IgG concentration, a dominant single 800-bp cDNA fragment appeared in restriction analysis after three and four rounds of selection, corresponding to NY-ESO-1sv. After three rounds, analysis of 36 randomly isolated clones revealed that 10 and 22 contained NY-ESO-1 and NY-ESO-1sv cDNAs, respectively. Of 19 clones isolated after four rounds, NY-ESO-1sv cDNA was found in 18 and NY-ESO-1 cDNA in 1. With the lower IgG coating concentration, a heterogeneous restriction pattern was seen after three and four rounds of selection, but sequencing of 20 random clones from round 4 revealed 6 NY-ESO-1sv and 1 NY-ESO-1 clones. The other clones harbored cDNAs from several different transcripts, including Igγ-chain cDNAs in 5 clones.

Serum IgG from patient MD2 specifically reacted with NY-ESO-1 and NY-ESO-1sv phages as well as recombinant NY-ESO-1 and NY-ESO-1sv proteins at high serum dilutions (Fig. 2b). Compared with results obtained with murine mAbs, the reactivity of MD2 serum appeared superior, most likely reflecting higher affinities of anti-NY-ESO-1 antibodies present in the patient serum.

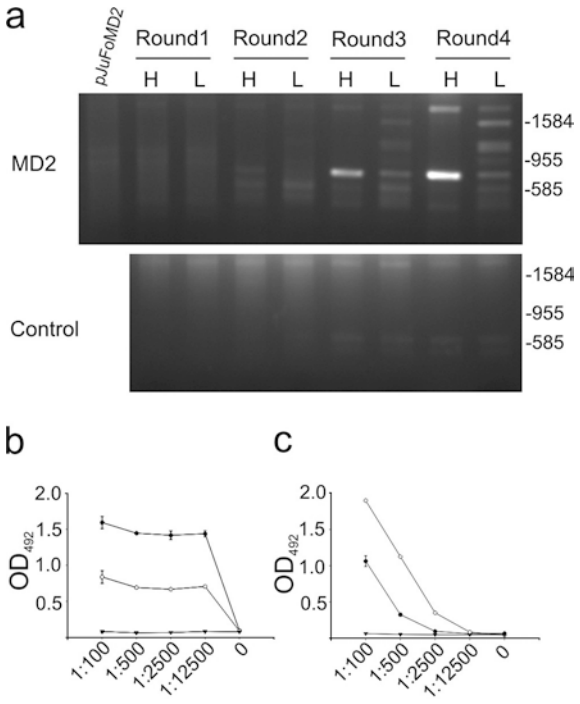

Fig. 2 **a** Biopanning with autologous patient MD2 serum. *Top panel:* Ethidium bromide stained agarose gel of EcoRI/XhoI digests of pJuFoMD2 library *(lane 1 from left)*, selected phagemids after rounds 1–4 *(lanes 2–9)*. *H* and *L* denote experiments coating with high and low IgG concentrations, respectively. *Lower panel:* as above, using IgG from healthy male blood donor. Size markers are indicated to the right. **b** Titration of serum MD2 in ELISA using phages encoding NY-ESO-1 *(open circle)*, NY-ESO-1sv *(solid circle)*, or pJuFo empty phages *(solid triangle)*. *Vertical lines* denote standard error of 4 experiments. **c** As in **b**, using recombinant proteins NY-ESO-1 *(open circle)*, NY-ESO-1sv *(solid circle)*, or p352 control protein *(solid triangle)*

Together these data indicate that NY-ESO-1-encoded proteins were the specifically and dominantly enriched immunogens expressed in the tumor.

To analyze for enrichment of less dominant, yet specific, cDNA-encoded proteins, approximately 1,000 phage clones from selection rounds 2 (high IgG coating) and 4 (low IgG coating) were subjected to immunoscreening with MD2 serum. After excluding clones expressing the Igγ-chain, positive clones were confirmed by secondary screening and sequenced. Reactivity of serum from a healthy individual was excluded (data not shown).

The sequencing data of the seroreactive clones are summarized in Table 1. NY-ESO-1 and NY-ESO-1sv phages were preferentially enriched with both high and low IgG coating concentrations. Moreover, other C/T antigens were detected (i.e., Lage-1 and Xage-1), encoded by two different phages each. Four clones harbored peflin cDNA. Three clones encoded a hypothetical protein with a nudix/MutT motif characteristic of hydrolases reacting on nucleoside diphosphate groups linked to other (X) residues. Known proteins encoded by single isolates were glyoxalase II (GlyII), glyceraldehyde-3-phosphate dehydrogenase (GAPDH),

Table 1 Sequence analysis of positive clones from immunoscreening following biopanning with high and low IgG concentration. Identity of cDNA inserts of immunoreactive phages. *H.p.* hypothetical protein. Accession numbers of databank entry in italics

Sequence identity	Number of clones isolated	5' nucleotide[a]	Reading frame[b]
High IgG concentration			
NY-ESO-1 *XM_049359*	23	307/340	0
NY-ESO-1sv *AJ275978*	44	171	0
Lage-1 *AJ223041*	2	31/88	0
Xage-1 *NM_133431*	2	510/542	−1
GlyII *NM_005326*	1	1,009	0
GAPDH *NM_002046*	1	366	−1
C1q*β* *P02746*	1	415	+1
H.p. with nudix/MutT motif*BC18644*	2	397/454	0
Low IgG concentration			
NY-ESO-1 *XM_049359*	30	307	0
NY-ESO-1sv *AJ275978*	31	171/180	0
Peflin *XM_034479*	4	187	−1
H.p. with nudix/MutT motif *BC18644*	1	397	0
Nuclear autoantigen sp100*NM_003113*[c]	1		
NADUOx *NM_004548*	1	23	0
Human EST *BF110350*[d]	1	27	
H.p. HSCARG *AF225419*	1	274	+1
H.p. MGC12943 *NM_032317*	1	529	−1

[a]First 5' nucleotide in databank entry matching pJuFo clone
[b]Reading frame of databank entry relative to pJuFo pelBpIII sequence
[c]Clone with internal deletion, start of insert and reading frame not known
[d]No predicted reading frame

nuclear autoantigen sp100, complement factor C1q *β*-polypeptide (C1q*β*), and NADH ubiquinone oxidoreductase PDSW subunit (NADUOx). Finally, three different clones were identified as hypothetical proteins or as an EST with no predicted open reading frame. Several clones contained inserts out of frame relative to the c-Fos linker of the pJuFo vector, likely representing a natural way to optimize the production of viable phage by down-regulating expression of the pIII fusion protein [9]. Three of the identified sequences (Lage-1, NY-ESO-1, and GAPDH) were found in the public part of the SEREX database (www.licr.org/SEREX.html). Finally, 16 randomly isolated seronegative clones were also sequenced, with 6 unrelated clones derived from the mitochondrial cytochrome *c* oxidase II gene and others containing cDNA inserts starting 3' to the open reading frames of the corresponding proteins (data not shown).

Several clones isolated by immunoscreening were tested further in phage ELISA and Western blotting of the corresponding recombinant proteins (Fig. 3). NY-ESO-1sv, NY-ESO-1, Lage-1, and Xage-1 showed reactivity in ELISA, whereas no reactivity was seen for other phage clones (Fig. 3a). However, the recombinant proteins encoded by additional clones were specifically recognized by autologous serum IgG in Western blotting

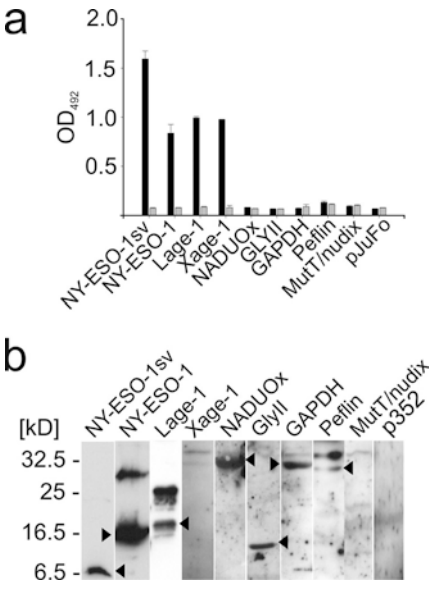

Fig. 3 a ELISA reactivity of individual phage clones with autologous serum MD2 *(black bars)* and normal serum *(gray bars)* diluted 1:100. *Vertical lines* denote standard error of 2 experiments. **b** Western blotting of recombinant proteins encoded by selected phages using MD2 serum diluted 1:100. Size markers are indicated to the left. Lanes 1–3 (from *left*) are exposed for a shorter time than lanes 4–10. *Top band* in lanes 4, 7, 8, and 9 represents background *E. coli* protein. *Arrows* indicate reactive proteins. Parallel filters hybridized to one normal serum diluted 1:100 did not show similar reactivity (data not shown)

(peflin, GLYII, GAPDH, and NADUOx; Fig. 3b). As suggested by others, the difficulty in using certain phage clones as antigen in ELISA most likely results from poor presentation of cDNA-encoded protein on the phage surface and the low purity of phages precipitated with polyethylene glycol [4]. Antibody epitopes may further be conformation-dependent, explaining the lack of reactivity of certain proteins in Western blotting following SDS/PAGE.

To analyze for IgG antibodies against the selected TAAs in sera from healthy subjects and other patients with HRPC, colony immunoscreening was chosen (Fig. 4). Colonies from each selected clone were grown in circular areas allowing each clone to be visualized as a dot on NC filters. None of the 10 healthy control sera reacted to any of the 13 TAAs. Among the 20 HRPC patients, 4 displayed IgG reactivity toward NY-ESO-1, 3 toward Lage-1, and 1 toward Xage-1. No reactivity was found in patient sera for the other isolated TAAs, indicating that serological reactions to these antigens may be rare in HRPC patients. Reactivity toward NY-ESO-1, Lage-1, and Xage-1 was confirmed using phage ELISA (data not shown).

Discussion

The present paper describes the use of cDNA phage display for cloning of TAAs inducing serological responses in the tumor host.

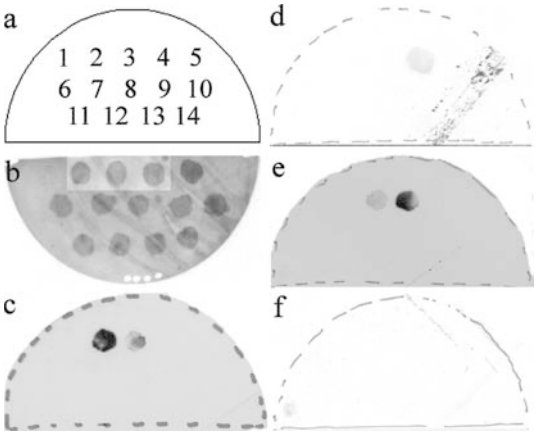

Fig. 4a–f Colony immunoscreening of spotted bacterial colonies infected with isolated TAA-encoding phages using sera from HRPC patients and healthy subjects. **a** *Spots* as numbered: pJuFo empty vector (1), NY-ESO-1 (2), Lage-1 (3), Xage-1 (4), MutT/nudix (5), GAPDH (6), GlyII (7), C1qβ (8), peflin (9), nuclear autoantigen sp100 (10), NADUOx (11), human EST BF110350 (12), H.p. HSCARG (13), H.p. MGC12943 (14). **b** Patient MD2, dots 2, 3, and 4 incubated with serum diluted 1:2,000; the remaining, 1:200. **c–e** HRPC patients, sera diluted 1:200. **f** Healthy control serum diluted 1:200

SEREX has been used successfully for identification of immunogenic proteins expressed in cancer [23]. Despite the large number of TAAs identified, only selected patients have been analyzed for each tumor entity, and the biological and clinical impact of the identified proteins and immune responses are poorly understood in most cases. Alternative methods, possibly allowing detection of complementary repertoires of TAAs and allowing high throughput automated analysis of a larger number of patients would seem to be of interest in characterizing and understanding the immune response of the tumor host, both during the natural course of cancer and during therapy [8].

Adaptation of cDNA phage display to cancer serology has not been widely used. Hufton et al. investigated the pJuFo display system for serological identification of TAAs in colon cancer, but the results were disappointing [7]. More recently, using a cell line–derived cDNA pVI phage display library and sera from patients undergoing tumor vaccine treatment, serological TAAs were identified in colon cancer patients [35]. By pVI display, Sioud et al. identified putative IgG-binding TAAs in breast cancer [34], but their experiments lack appropriate controls to exclude false positives due to binding of human IgG to *E. coli* proteins. The successful use of fresh tumor cDNA libraries has not been reported. Furthermore, loss of immunoreactive clones during multiple rounds of biopanning is not addressed. The results of the present study demonstrate the ability of biopanning to enrich for TAAs from tumor cDNA libraries under certain experimental conditions. However, enrichment may be associated with loss of positive clones and bias may result from nonimmunological factors such as inefficient protein presentation and delayed growth of phage-infected bacteria. To balance enrichment versus desired diversity, immunoscreening of phage populations after a few rounds of selection proved advantageous. As expected, patient IgG specifically recognized only 5–10% of these complex repertoires, but a broad range of seroreactive proteins could be identified reproducibly. Although postselection immunoscreening of clones is labor intensive, these steps are amenable to automation for high throughput analysis. A combined automated approach has been described recently for identification of allergens using the pJuFo display system [13]. Using robotic colony picking and spotting, we are currently performing immunoscreening of selected phage repertories, and repeated probing of filters with randomly sequenced inserts of positive phages is used for identification of reiterative clones. Using the same robot technology, a larger number of sera can be probed to selected TAAs and analyzed quantitatively, facilitating identification of proteins with frequent but tumor-restricted immune responses. Such TAAs potentially represent the most interesting candidates for vaccine development.

To evaluate the relative potential in identifying TAAs and differences in methodological design, between available cDNA phage display systems and SEREX, direct comparison using standardized libraries and sera may be worthwhile. Serum from patient MD2 has been used for SEREX analysis of a cDNA library from the HRPC cell line DU145, with two different proteins identified [6]. One of these, No55, was tested in semi-quantitative PCR on pools of phages and shown to be enriched during biopanning in the present study, but is not identified among the limited number of clones analyzed by immunoscreening (data not shown). Also, three of the identified TAAs in our study are present in the SEREX database, further suggesting that the methods may yield at least partially overlapping results. Recently, systems for display of cDNA-encoded proteins on the surface of lytic phages, such as λ and T7, have been developed, with the potential of expressing a higher copy number of the encoded protein on each phage [3, 19, 31]. Experimental evidence suggests that highly multivalent display may be advantageous in biopanning on complex targets, such as human serum IgG [9, 31].

The present study in PC identified several C/T antigens: NY-ESO-1, Lage-1, and Xage-1. NY-ESO-1 and Lage-1 have been extensively characterized as TAAs in other cancer types, and clinical trials based on NY-ESO-1 are being performed [1, 10, 14]. Expression of NY-ESO-1 and Lage-1 has been shown by RT-PCR in smaller series of PC patients [1, 14]. The present data reveal that NY-ESO-1 and Lage-1 are immunogenic in a subset of HRPC patients. Splice variants of Xage-1 are expressed in different cancer types [16, 40, 41], but to our knowledge, Xage-1 has not been identified by SEREX previously. Antibodies against the L552S splice variant were found in pleural effusions of lung cancer patients [40]. The partial cDNA clones recognized by patient MD2 in the present study are identical to the

carboxyterminal part of the L552S sequence, containing one of the antibody-binding epitopes. However, both Xage-1 clones identified in our study are out of frame relative to the pIII protein. Therefore, immunogenicity in MD2 may be related to the L552S epitopes present on the selected phages by infidelities in translation in *E. coli* or may be the result of immunization against out-of-frame products expressed in the MD2 tumor. The exact epitopes recognized by MD2 serum will thus need further characterization.

The demonstration of three C/T antigens in the present study warrants a comprehensive expression analysis of this family of TAAs in PC. As in other cancer types, this would constitute an important step toward development of a polyvalent C/T antigen-based vaccine in PC. Furthermore, the recent finding that SEREX-defined proteins may increase the potency of immune responses against other antigens, offers encouragement to the idea of combining C/T antigens with other molecules, such as PSA or PSMA, antigens already explored for vaccine therapy of PC [18].

Apart from C/T antigens, other immunogens were identified by the described phage display strategy. IgG responses against these TAAs were absent in other PC patients, and based on published expression patterns or tissue origins of the respective UNI-Gene cluster entries, these proteins appear to be broadly expressed in human tissues. However, immunogenicity in the context of PC indicates a possible pathogenetic relevance. Interestingly, several of the proteins may be involved in cell survival or detoxification processes. Peflin, a calcium-binding protein with unknown function, is known to dimerize with apoptosis-linked gene 2 (ALG-2) which is involved in execution of apoptosis in response to various signals [12]. Publicly available gene expression data (http://www.ncbi.nlm.nih.gov/SAGE/) for a decanucleotide (CTCTGCTCGG) predicted to be specific for peflin mRNA suggest about a threefold overexpression in PC compared with normal prostate epithelium. Thus, peflin may be involved in deregulation of apoptosis in PC. GLYII, together with glyoxalase I (GLYI), is a key enzyme involved in detoxification of methylglyoxal [37]. Overexpression of GLYI and GLYII has been described in human cancer, including PC, and has been linked to resistance to antitumor agents [25]. Furthermore, inhibitors of GLYI exhibit preclinical antitumor activities [33]. The predicted protein with a nudix/MutT motif is a putative member of the nudix hydrolase family, believed to eliminate toxic nucleotide derivatives from the cell and regulate levels of signaling nucleotides [17].

Through BLAST searches, all sequences identified in the present study could be mapped to their corresponding chromosomal loci. Three of the sequences mapped to 16p13 (GLYII, NADUOx, and hypothetical protein HSCARG). Loci on 16p have been linked to inherited susceptibility of PC and aberrations of 16p13, including amplifications, have been described in sporadic cases [20, 36]. In this respect, the reported association of GLYI expression phenotypes with risk of PC is noteworthy, as it suggests that genetic variation in methylglyoxal detoxification may be linked to an increased risk of PC [28]. The GLYII gene on chromosome 16p13 may therefore represent such a susceptibility gene.

Together, the identification of proteins possibly involved in apoptosis and detoxification suggests that serological cloning strategies may give insights into the survival and resistance mechanisms of PC. A better understanding of mechanisms governing the remarkable resistance of PC to most known systemic treatment options, including chemotherapy, will aid the development of novel treatment strategies.

In summary, serological cloning using cDNA phage display is a valid tool for identification of TAAs and may well constitute an adjunct to the widely used SEREX approach. The competitive nature of the selection and amplification steps involved necessitates measures to balance specific enrichment and diversity within the repertoire of identified TAAs. To this end, limited rounds of selection followed by postselection immunoscreening of phage clones proved valuable. Several of the described steps are amenable to automation and may allow more efficient screening of tumor libraries and patient sera.

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