

IDENTIFICATION OF TUMOR-ASSOCIATED ANTIGENS BY SCREENING PHAGE-DISPLAYED HUMAN cDNA LIBRARIES WITH SERA FROM TUMOR PATIENTS

Olga MINENKOVA^{1*}, Andrea PUCCI¹, Emiliano PAVONI¹, Amedeo DE TOMASSI¹, Paola FORTUGNO¹, Nicola GARGANO¹, Maurizio CIANFRIGLIA², Stefano BARCA², Sabino DE PLACIDO³, Angelo MARTIGNETTI³, Franco FELICI¹, Riccardo CORTESE⁴ and Paolo MONACI⁴

¹Kenton Labs, c/o Sigma Tau, Pomezia (Roma), Italy

²Laboratorio di Immunologia, Reparto Immunologia dei Tumori, Istituto Superiore di Sanità, Roma, Italy

³Dipartimento di Endocrinologia ed Oncologia Molecolare e Clinica, Università di Napoli Federico II, Napoli, Italy

⁴Istituto di Ricerche di Biologia Molecolare P. Angeletti, Pomezia (Roma), Italy

Screening cDNA libraries from solid human tumors with sera of autologous patients (SEREX) has proven to be a powerful approach to identifying tumor antigens recognized by the humoral arm of the immune system. In many cases, application of this methodology has led to the discovery of novel tumor antigens as unknown gene products. We tried to improve the potency of the SEREX approach by combining it with phage-display technology. We designed a new lambda vector to express protein fragments as N-terminal fusions to the D capsid protein and generated high-complexity cDNA libraries from human breast carcinoma cell lines and solid tumors. Screening these phage-displayed libraries required limited amounts of sera from patients and efficiently identified several tumor antigens specifically reacting with sera from breast cancer patients.

© 2003 Wiley-Liss, Inc.

Key words: breast cancer; immune response; phage display

Tumor-associated antigens recognized by humoral effectors of the immune system are an attractive target for diagnostic and therapeutic approaches to human cancer. A considerable number of this type of antigen have been identified over recent years through screening expression cDNA libraries from human solid tumors with sera of the autologous patients (SEREX, serologic identification of antigens by recombinant expression cloning).^{1–4} This type of screening of a cDNA expression library by conventional methods requires the preparation of a large number of membrane filters blotted with bacteriophage plaques that are then searched with a specific probe. In the case of the SEREX experiments, the screening is performed using sera from cancer patients, which are usually available in very limited quantity. The second limitation is that such immunoscreening procedure does not allow selection of antigens that are recognized by sera from different patients.

We tried to overcome these limitations and to simplify the screening procedure by performing affinity selection of cDNA libraries in very small volumes by combining the SEREX approach with that of phage-display technology. Phage-display technology is based on the insertion of foreign nucleotide sequences into genes encoding for various coat proteins of filamentous phage, resulting in a heterogeneous mixture of phages, each displaying the different peptide sequence encoded by a corresponding insert. A physical link between a displayed fusion protein and DNA encoded for it make this phage target selectable.

Phage-display technology was introduced in 1985 by Smith⁵ and has been widely used since for generating and screening peptide libraries to identify ligands for various kinds of receptor molecules.^{6–8} However, significantly less striking progress has been reported on the use of filamentous phage as display vector for constructing cDNA libraries. In most cases, foreign peptides are displayed on the filamentous phage capsid as N-terminal fusions to the major (pVIII) or the minor (pIII) coat protein. Large protein domains fused to pVIII disturb (with some rare exceptions) the process of protein transport across the inner bacterial membrane

and assembly of phage particles.^{9–11} pIII is less sensitive to the size of protein inserts but has the much lower number of copies than pVIII, which markedly reduces the avidity contribution in the ligand binding. This seriously limits the selection efficiency of ligands for receptors only available at low concentration or present in complex mixtures (as in the case of biologic fluids, such as serum). Moreover, several observations have confirmed that selecting known ligands for a target of interest from genomic or cDNA libraries, displayed as pIII-fusion, have often failed.¹² cDNA libraries based on pJufO vector¹³ or fused to the carboxy terminus of another filamentous phage minor protein pVI¹⁴ have been successfully screened with different antibodies. Sioud and Hansen reported the use of filamentous phage M13 pVI-based display system for cancer gene discovery by screening cDNA repertoires from breast cancer cell lines with serum antibodies from cancer patients.¹⁵ However, all these filamentous phage-based libraries display only those recombinant proteins able to pass through the inner bacterial membrane still maintaining their correct folding in the oxidizing environment of the periplasmic space.

Using lambda phage as a display vector overcomes many of the limitations of filamentous phage systems. Indeed, the lambda capsid is assembled in the reducing milieu of the cytoplasm and does not depend on the ability of fusion proteins being translocated across the membrane. Protein D (pD) is one of the head proteins that form the protruding trimeric structure essential for the stability of the capsid. Polypeptides and even large multimeric proteins can be expressed as amino-¹⁶ and carboxy-¹⁷ terminal fusions to the capsid protein pD without interfering with lambda phage production. Recently, the successful construction and screening of several cDNA lambda-displayed libraries was reported.^{18–20} In all these

Abbreviations: aa, amino acids; Ag, antigen; Ap, ampicillin; CFA, complete Freund's adjuvant; EST, expressed sequence tags; FACS, fluorescence-activated cell sorting; i.p., intraperitoneally; i.v., intravenously; GST, glutathione S-transferase; LB, Luria broth; PEG, polyethylene glycol; SEREX, serologic identification of antigens by recombinant expression cloning; TAA, tumor-associated antigen.

Grant sponsor: MIUR-URST "P.N.R. per le Tecnologie in Oncologia (Tema 6)".

*Correspondence to: Kenton Labs c/o Sigma Tau, Via Pontina km 30.4, 00040 Pomezia (Roma), Italy. Fax: +39-06-91629012. E-mail: minenkova@kenton.it

Received 16 October 2002; Revised 12 February 2003; Accepted 4 April 2003

DOI 10.1002/ijc.11269

cases, recombinant proteins encoded by the cDNA were expressed as fusion to the carboxy terminus of pD. Taken together, these experiments demonstrate that complex repertoires from natural sources displayed on lambda phage capsid can be efficiently exploited for antigen discovery. An alternative approach based on the use of T7 phage display was developed and applied.^{21–23} However, lambda display system, as compared to T7 bacteriophage, has the ability to display proteins with the highest density.²²

In this article, we report the successful construction of high-complexity lambda-displayed cDNA libraries from a human breast carcinoma cell line and from a human breast carcinoma. We screened these libraries using a limited amount of sera and driving the selection toward the identification of cDNA-encoded tumor-associated antigens recognized by antibodies from different tumor patients.

MATERIAL AND METHODS

Bacterium and phage strains

For phage plating and amplification we used *Escherichia coli* strain BB4 (*supF58 supE44 hsdR514 galK2 galT22 trpR55 metB1tonA ΔlacU169 F'[proAB⁺lacI^qlacZΔM15Tn10(ter^r)*). For construction of lambda vector phage *λ*Dam15imm21nin5 was used.¹⁶

Human sera and breast tumors

Negative human sera used in our study (samples N1–N42) were collected from healthy volunteers from institute staff (27 men, 15 women, aged 22–46 years), from healthy donors from “Servizio Ematologia e Emotrasfusione” of “Università Cattolica del Sacro Cuore”, Roma (samples N46–N50, N52, N57–N59, N61, N64, N70, N73, N74 from 12 men, 2 women, aged 20–55 years) and from Institut Jacques Boy, Reims (samples N80–N82, N89, N92–N96, N102, N105–N109, N111, N113, N115, N117, N119, 50% men, 50% women, aged 24–58). Human sera from breast cancer patients B1–B80 (100% women of aged 32–77 years) and specimens of solid tumors were obtained from the Division of Medical Oncology, Federico II University, Naples, Italy.

Construction of plasmids and lambda vector

The plasmid pNS3785¹⁶ was amplified by inverse PCR with oligos 5'-TTTATCTAGACCCAGCCCTAGGAAGCTTCTCCT-GAGTAGGACAAATCC-3' bringing *Xba*I site (underlined) for subsequent cloning in lambda phage and 5'-GGGTCTAGATA-AAACGAAAGGCCAGTCTTTC-3' bringing *Xba*I. In inverse PCR we used a mix of *Taq* polymerase with *Pfu* DNA polymerase to increase fidelity of DNA synthesis. Twenty-five cycles of amplification (30 sec at 95°C, 30 sec at 55°C, 20 min at 72°C) were done. Self-ligation of the PCR product previously digested with *Xba*I endonuclease resulted in plasmid pKM3. The lambda pD gene was amplified by PCR from plasmid pNS3785 using primers 5'-CCGCCTTCCATGGGTACTAGTTTAAATGCGGCCGCAC-GAGCAAAGAAACCTTTAC-3' and 5'-AGCTTCCTAGGGC-TGGGTCTAG-3' containing restriction sites *Nco*I, *Spe*I, *Not*I and *Eco*RI, respectively (underlined). PCR product was purified, digested with *Nco*I and *Eco*RI restriction sites and recombined into *Nco*I and *Eco*RI sites of pKM3, resulting in plasmid pKM4 bringing unique *Spe*I and *Not*I restriction sites at the 5'-end of gpD. Plasmid was digested with *Xba*I enzyme and cloned in *Xba*I site of lambda phage *λ*Dam15imm21nin5¹⁶ generating *λ*KM4. For amplification, the resulting bacteriophage can be plated in top agar to form typical phage plaques on the bacterial lawn or alternatively on LB-agar plate with ampicillin to form Ap-resistant lysogenic colonies, slowly (about 24 hr) growing at 32°C. The last method of plating we used to get lysogenic bacteria for large-scale preparation of bacteriophage.

The plasmid pGEX-SN was constructed by cloning the oligonucleotide duplex K108 5'-GATCCTTACTAGTTTATAGCGG-GCCGCGGG-3' and K109 5'-AATTCCCGCGGCCGCTAC TA-AACTAGTAAG-3' into *Bam*HI and *Eco*RI sites of plasmid

pGEX.²⁴ Resulting plasmid contains *Spe*I, *Not*I sites in the same reading frame, as *λ*KM4.

The plasmid pKM4-6H was constructed by cloning the oligo duplex K106 5'-GACCGCGTTTGCCGGAACGGCAATCAG-CATCGTTACCACCACCACCACCACCTAATAGG-3' and K107 5'-AATTCCTATTAGTGGTGGTGGTGGTGGTGAACGATGCT-GATTGCCGTTCCGGCAAACGCG-3' into *Rsr*II and *Eco*RI restriction sites of the plasmid pKM4. Protein D encoded by pKM4-6H has a tail of histidine residues.

The sequence of lambda KM4 vector is available on request.

Construction of *λ*NS3 phage

The NS3 domain encoded DNA fragment was amplified by PCR using primers K66 5'-GCACTAGTGGCCGCAACCAAAGC-CATCCCCATTGAAGC-3' and K67 5'-GGAGGCTCGAGCGGC-CGCTGGCACATGTGTTACAGTCGATCACTGAGT-3' containing *Spe*I and *Not*I restriction sites (underlined) and cloned in *λ*KM4.

To demonstrate the lambda clone stability, we performed an additional round of amplification of the *λ*-NS3 phage. Approximately 20,000–30,000 phages were plated in NZY top agar from the pool of phage after first amplification to get confluent growth of the plaques. After ON incubation amplified phage was eluted and again plated on a Petri dish to get numerous single plaques. Plaques were transferred to nitrocellulose membrane and tested in immunoscreening assay with one of HCV-positive serum (data not shown). As negative control, we used a filter with plaques formed by vector phage. One hundred percent of *λ*-NS3 plaques were positive in this assay.

Cloning fused GST and pD proteins

To produce fusion proteins, each clone was amplified by PCR with oligonucleotide primers: K84 5'-CGATTAAATAAGGAG-GAATAAACCC-3' e K86 5'-CTCTCATCCGCCAAACAGCC-3'. The resulting fragments were purified by QIAquick PCR purification kit (Qiagen,), digested with *Spe*I and *Not*I restriction sites and cloned in plasmid pKM4-6H to express recombinant protein D possessing a tail of 6 histidine residues, or in vector pGEX-SN to generate fusion with GST. The corresponding recombinant proteins were prepared and purified according to the standard protocol.²⁵

cDNA libraries construction

mRNA was isolated from 10⁷ MCF-7 cells (T1 library) or from 0.1 g solid tumor specimen (T4 library) by QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, UK) following the manufacturer's instructions. Double-strand cDNA was synthesised from 5 µg of the poly(A)⁺ RNA using TimeSaver cDNA Synthesis Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Random tagged priming was performed as described earlier.¹⁸ From 500 ng of cDNA, the first-strand cDNA copy was synthesised with the tagged random primer 5'-GCGGCCGCTGG(N)₉-3'; the second strand with primer 5'-GGCCGCCAAC(N)₉-3'. The final cDNA product was amplified using oligos, bringing *Spe*I with 3 reading frames or *Not*I sites to facilitate cloning in lambda vector *λ*KM4 (5'-GCACTAGTGGCCGCAAC-3', 5'-GCAC-TAGTCGGCCGCAAC-3', 5'-GCACCTAGTCGGCCGCAAC-3' and 5'-GGAGGCTCGAGCGGCCGCTGG-3'). PCR products were purified through QIAquick columns (Qiagen, Hilden, Germany) and filtrated through Microcon 100 (Amicon, Bedford, MA) to eliminate small inserts, digested with *Spe*I, *Not*I restriction enzymes and after phenol extraction again filtrated through Microcon 100.

Vector *λ*KM4 was digested with *Spe*I/*Not*I and dephosphorylated; for each library were performed 8 ligation mixes, each one containing 0.5 µg of vector and about 3 ng of insert. After overnight incubation at 4°C, the ligation mixtures were packaged *in vitro* by a lambda packaging kit (Ready-To-Go™ Lambda Packaging Kit, Amersham Pharmacia Biotech) and plated in top-agar on 100 (15 cm) NZY plates. After overnight incubation,

phage was eluted from plates with SM buffer, purified, concentrated and stored at -80°C in SM buffer, 7% DMSO.

The complexity of the 2 libraries calculated as total independent clones with insert was 10^8 for library T1 and 3.6×10^7 for library T4.

Affinity selection

Falcon plates (6 cm, Falcon 1007, Becton-Dickinson Labware, Franklin Lakes, NJ) were coated overnight at 4°C with 3 ml of 1 $\mu\text{g}/\text{ml}$ Protein A (Pierce, # 21184, Rockford, IL) in 50 mM NaHCO_3 , pH 9.6. After discarding the coating solution, plates were incubated with 10 ml of blocking solution (5% dry nonfat milk in PBS $\times 1$, 0.05% Tween 20) for 2 hr at 37°C . Ten microliters of human serum were preincubated for 30 min at 37°C with gentle agitation with 10 μl BB4 bacterial extract, 10 μl 1M MgSO_4 in 1 ml of blocking solution. About 10^{10} phage particles of lambda library were added to the serum solution and further incubated for 1 hr at 37°C with gentle agitation. Incubation mixtures were placed on the Protein A coated plate and left for 30 min at RT with agitation. The plate was washed several times with 10 ml of washing solution (1 $\times$ PBS, 1% Triton, 10 mM MgSO_4). The bound phages were recovered by infection of BB4 cells added directly to the plate (600 μl per plate). Ten ml of molten NZY-Top Agar (48°C – 50°C) were added to infected cells and poured immediately on NZY plates (15 cm). The next day, phages were harvested from the incubation plate by agitation with 15 ml of SM buffer for 4 hr at 4°C . Phages were purified by PEG and NaCl precipitation and stored in 1/10 of initial volume of SM with 0.05% NaN_3 at 4°C .

Nucleotide sequences of cDNA inserts were controlled in different available databases (Non-Redundant Genbank CDS, Non-Redundant Database of Genbank Est Division, Non-Redundant Genbank+Embl+Ddbj+Pdb Sequences).

Immunoscreening

Phage plaques from bacterial lawn were blotted onto a dry nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) for 1 hr at 4°C . Filters were blocked for 1 hr at RT in blocking buffer (5% dry nonfat milk in PBS $\times 1$, 0.05% Tween 20). Twenty microliters of human serum were preincubated with 20 μl BB4 bacterial extract, $10^9/\text{ml}$ wild-type lambda phage in 4 ml of blocking buffer. After discarding the blocking solution, filters were incubated with serum solution for 2 hr at RT with agitation. Filters were washed several times with PBS $\times 1$, 0.05% Tween20 and incubated with 1:5,000 secondary antibody alkaline phosphatase-conjugated anti-human IgG (Sigma, St. Louis, MO) in the blocking buffer for 1 hr at RT. Then the filters were washed as above and rinsed briefly with substrate buffer (100 mM Tris-HCl, pH 9.6, 100 mM NaCl, 5 mM MgCl_2). Each filter was incubated with 10 ml of substrate buffer containing 330 mg/ml nitro blue tetrazolium, 165 mg/ml 5-bromo-4-chloro-3-indolylphosphate. Reaction was stopped with pouring water.

Bacterial extract of BB4 cells was prepared as described earlier.²⁶

Individual phage clones positive in immunoscreening assay were isolated and then grown on the lawn of bacteria on plates of 15 cm by picking in arrayed order. To make the procedure more robust and reproducible, we used a Genesys Tecan Robotic Station to pick phage on the plate. Using this instrument, we analysed up to 384 individual clones on the membrane of 11 cm $\times$ 7.5 cm, or simultaneously tested a lower number of clones repeatedly picked on the same plate with a set of positive and negative sera, cutting the membrane before incubation with sera (Fig. 5a). All of the described procedures were optimised to consume small amounts of serum.

Large-scale preparation of lambda phage (from lysogenic cells)

The BB4 cells were grown up to $\text{OD}_{600} = 1.0$ in LB containing 0.2% of maltose with agitation, the cells were spun down by centrifugation and cell pellet was resuspended in buffer SM up to

$\text{OD}_{600} = 0.2$. The 100 μl of cells were infected by lambda with low phage multiplicity, preincubated for 20 min at RT, plated on LA with Ap and incubated for 18–20 hr at 32°C . The next day, a single colony was incubated in 10 ml of LB with Ap overnight at 32°C with agitation. Five hundred milliliters of fresh LB with Ap and 10 mM MgSO_4 were inoculated with 5 ml of overnight culture in a big flask at 32°C up to $\text{OD}_{600} = 0.6$ with vigorous agitation. The flask was incubated for 15 min in a water bath at 45°C , then incubated with agitation at 37°C for a further 3 hr. Ten milliliters of chloroform were added to the culture to complete cell lysis and incubated in a shaker for an additional 15 min at 37°C . The phage from lysed culture was purified according to standard procedure.²⁵

Phage lysates for the ELISA test were prepared using an analogous procedure from lysogenic cells but without adding chloroform. After NaCl and PEG precipitation, bacteriophage pellet was resuspended in 1/10 of the starting volume of SM buffer with NaN_3 (0.05%) and stored at 4°C .

Lambda ELISA

ELISA using phage lysates and human sera was performed as following. Multiwell plates (Immunoplate Maxisorb, Nunc, Naperville, IL) were coated overnight at 4°C with 100 $\mu\text{l}/\text{well}$ of polyclonal antilambda antibodies in a concentration of 0.7 $\mu\text{g}/\text{ml}$ in 50 mM NaHCO_3 , pH 9.6. After discarding coating solution, plates were incubated with 250 $\mu\text{l}/\text{well}$ of blocking solution (5% dry nonfat milk in PBS $\times 1$, 0.05% Tween 20). Plates were washed twice with washing buffer (PBS $\times 1$, 0.05% Tween 20). A 100 μl mix of blocking buffer with phage lysate (1:1) was added to each well and incubated 1 hr at 37°C . A 1 μl human serum was incubated for 30 min at room temperature with 10^9 plaque forming units of phage λKM4 , 1 μl rabbit serum, 1 μl BB4 bacterial extract, 1 μl FBS in 100 μl of blocking buffer. Plates washed after incubation with phage lysate were incubated with a solution of serum for 60 min at 37°C . Plates were then washed and a 1:20,000 dilution of goat anti-human HRP-conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA) secondary antibody in blocking buffer (1:40 rabbit serum in blocking solution) was added. After 30 min incubation, plates were washed and peroxidase activity was detected with 100 μl of TMB liquid substrate system (Sigma). After 15 min of developing, reaction was stopped by adding 25 μl 2M H_2SO_4 .

The plates were read by an automated ELISA reader (Lab-systems Multiskan Bichromatic, Helsinki, Finland) and the results were expressed as $A = A_{450\text{nm}} - A_{620\text{nm}}$. For each serum, binding of antibodies to the tested phage, GST- or pD-fusion protein and to the corresponding control molecules (wt lambda, GST and pD, respectively) was measured. ELISA data reported in display items are average values from 2 independent assays. They were considered statistically significant if they were greater than the arbitrarily chosen 0.20 cutoff value and differed more than 3σ from the background signal observed for the wt phage. Standard deviation (σ) of the difference between the mean ELISA data for the tested phage or fusion protein p and the wt molecule was calculated as $\sigma = [(\sigma_p^2 + \sigma_w^2)/2]^{1/2}$, where σ_p and σ_w are the standard deviation of the ELISA data for the tested phage or fusion protein and the corresponding wt molecule, respectively.

Immunization

Recombinant proteins fused with protein D: D1–52, D4–11, D4–19 were used for immunization. Balb/c mice were immunized according to previously described protocol²⁷ with minor modifications. Briefly, the 25 μg dose of the antigen was resuspended in CFA and administrated intraperitoneally (i.p.) 4 times at a weekly interval. An equal amount of the antigen (25 μg) was resuspended in PBS (100 μl) and administered to animals both i.p. and intravenously (i.v.) at days 2 and 1 before the sacrifice. Blood samples were collected from animals by tail vein puncture after an accurate anaesthesia.

Rabbits were immunized by 3 subcutaneous injections with 200 µg doses of the antigen pD or D4-11 resuspended in CFA, IFA or PBS at 2 week intervals.

Flow cytometry

MCF7 cells were harvested by trypsinization washed by serum-free medium (SFM) and then fixed with 4% paraformaldehyde for 5 min at room temperature. After an extensive washing with SMF, the cells were treated by FACS permeabilized solution (Becton Dickinson, Mountain View, CA) for 10 min at RT, then washed twice with 10% fetal bovine serum in PBS and incubated for an additional 60 min at 4°C with 50 µl of 1:1,000 mice sera. This serum concentration was identified (after a calibration experiment) as the most suitable for flow cytometry analysis. To determine the specificity of the antibody reaction, the cell suspension was incubated for 30 min at 4°C with FITC conjugated F(ab')₂ goat anti-mouse IgG, washed with PBS-FCS and the cell pellet was resuspended and maintained in ice until the analysis with a FAC-Scan cytometer (Becton Dickinson) equipped with a 15 mW argon ion laser emitting light at a fixed wavelength of 488 nm.

Western blot analysis

Protein extract from about 5×10^7 p.f.u. of λNS3 or wt phage purified by PEG precipitation was fractionated by SDS/PAGE, transferred onto a nitrocellulose membrane and probed with rabbit anti-pD serum (dilution 1:1,000) or serum from HCV-positive patient C39 (dilution 1:150).

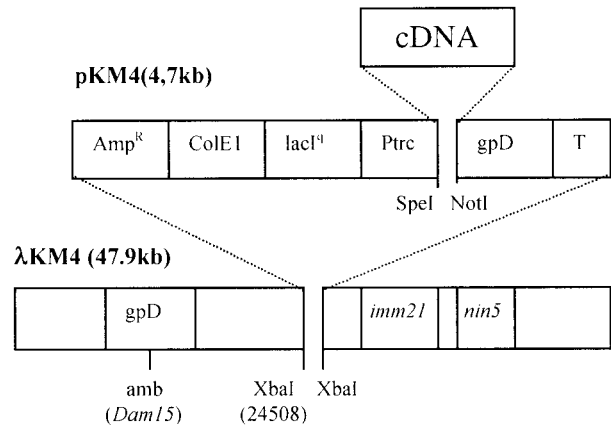
Protein extract from 10^5 breast carcinoma MCF7, promyelocyte HL-60 cells were fractionated by SDS/PAGE, blotted onto a nitrocellulose membrane and probed with anti T4-11 rabbit serum (1:200 dilution), anti T4-11 rabbit serum affinity purified on T4-11 peptide exposed on filamentous phage (1:50 dilution) and anti-actin antibodies (Sigma, A2066) (1:200 dilution).

RESULTS

Construction of vector λKM4

In a library where cDNA sequences are nondirectionally cloned as carboxy terminal protein fusions, less than 17% (1/6) of the proteins expressed are expected to be in the correct frame. Thus, screening or affinity selection of such cDNA libraries using complex receptor mixtures (as in the case of human sera) very often identifies clones expressing nonnatural peptide sequences. These sequences derive from the translation of out-of-frame or reverse-orientation cDNA inserts that are efficiently expressed and displayed on the phage. To minimize the display of out-of-frame peptides and to enrich the library with authentic translation product, we have designed a vector for the display of protein sequences as fusions to the amino terminus of pD. Phage clones display a given protein fragment on the phage surface only when the correct reading frame of the insert matches that of pD. Similarly, sequence frames that include a nonsuppressed stop codon will not be displayed.

Unique *SpeI* and *NotI* restriction sites were introduced in the 5' region of *gpD* in the plasmid pNS3785.¹⁶ Cloning cDNA inserts into these 2 sites will express the corresponding cDNA-encoded protein fragments as fusion to the amino terminus of pD. In addition, a unique *XbaI* site was introduced downstream of the *gpD* expression cassette, composed of the *P_{trc}* promoter, *gpD*, terminator of transcription. The resulting plasmid pKM4 was *XbaI*-linearized and inserted into the *XbaI* (24508) site of λ*Dam15imm21nin5*¹⁶ genome to generate phage λKM4 (Fig. 1). This phage contains the genomic copy of *gpD* harbouring an amber mutation (*Dam15*), bears *imm21* and *nin5* mutations in the right arm of lambda and has length of about 47.9 kb, thus accepting inserts up to 3 kb of length without disturbing the lambda packaging. Phage particles grown on suppressor bacterial strain BB4 display on their capsids a chimeric array of wild-type pD (encoded by the *gpD* lambda genomic gene) and recombinant protein D (encoded by the *gpD* plasmid gene).



Phage cloning sites (λD protein)

M G T S F K C G R T S ...
 TAA ACC ATG GGT ACT AGT TTT AAA TGC GGC CGC ACG AGX ...
 NcoI *SpeI* *NotI*

FIGURE 1 – Genetic map of λKM4 with cDNA insert.

Expression of N-terminal fusion proteins on the lambda surface

We tested the display efficiency of large polypeptides on lambda surface by expressing the 237 bp DNA fragment encoding the region between aa 1378–1457 of hepatitis C virus (HCV)²⁸ polypeptide as N-terminal fusion to the pD protein using the λKM4 vector. This HCV fragment corresponds to a domain of the nonstructural viral protein NS3, well characterised in our laboratory for its high specific reactivity with sera from HCV-infected patients. After *in vitro* packaging, recombinant phage was plated as lysogenic cells on Ap plate, and phage lysate was prepared from a single colony resistant for Ap, as described in Material and Methods. The reactivity of purified phage was tested in ELISA using several HCV-positive human sera possessing anti-NS3 protein antibodies and HCV-negative sera as negative control (Fig. 2a). Phage lysate specifically reacted with positive sera, indicating that NS3 domain was efficiently displayed on the phage surface. The lambda clone stably maintained NS3 domain expression after repeated rounds of amplification (see Material and Methods).

The number of the fusion proteins displayed on the phage capsid varied with the length and the amino acid composition of the foreign sequence. When the foreign sequence is expressed as C-terminal fusion to the D protein, the percentage of fusion protein incorporated in the capsid can reach up to 90% of the total D protein content.²² A similar situation is detected when the foreign sequence is expressed as N-terminal fusion to D protein. In the case of λNS3, Western blot analysis revealed a high D fusion/wt ratio on the phage capsid (Fig. 2b).

Construction of cDNA libraries from human breast cancer cell line MCF7 and from human breast tumor

We purified mRNA from breast cancer cell line MCF7 and from frozen specimen of a breast solid tumor as described in Material and Methods. Double-stranded cDNA was synthesised according to standard procedure and elongated by tagged random primer amplification, according to procedure developed earlier.¹⁸ The elongation products were then reamplified by PCR using a set of primers incorporating *SpeI* and *NotI* restriction sites. The ampli-

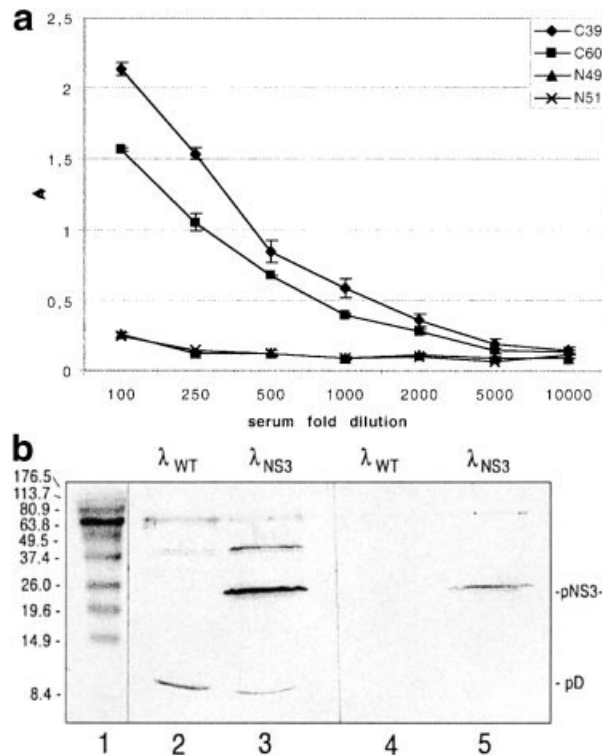


FIGURE 2 – (a) ELISA reactivity of λ NS3 phage with HCV-positive (C39, C60) and -negative (N49, N51) human sera. Tested dilutions of sera are indicated on the abscissa. Results are expressed as $A = A_{450nm} - A_{620nm}$. Data reported are the average value from 2 independent assays. (b) Fusion/wt D protein ratio on the λ NS3 phage capsid. Protein extract from λ NS3 phage particles was fractionated by SDS/PAGE, transferred onto a nitrocellulose membrane and probed with anti-pD rabbit serum (lanes 2, 3) or serum from an HCV-positive patient C39 (lanes 4, 5). The protein size marker is included (lane 1). Bands corresponding to wt protein D (pD; approximately 11 kDa) and to NS3-D fusion protein (pNS3-D; approximately 20 kDa) are indicated. Additional bands correspond to bacterial proteins detected by polyclonal serum.

fication products were *SpeI*, *NotI* digested and cloned into the corresponding sites in λ KM4 vector. *In vitro* packaged libraries yielded 1×10^8 and 3.6×10^7 independent clones for MCF7 (T1) and breast carcinoma (T4) library, respectively. About 40 clones from each library were randomly chosen and showed to have inserts with sizes ranging from 100–600 bp (T1 library) and 80–300 bp (T4 library).

Selection of tumor-associated antigens

In our current study, we used a collection of sera from 75 breast cancer patients (indicated as B1–B75 or positive sera) and 70 sera from healthy donors (indicated as N1–N119 or negative sera).

The screening strategy was aimed at identifying cDNA products that specifically or preferentially reacted with multiple positive sera. It typically consisted of a couple of rounds of affinity selection (biopanning) performed with a single or a mixture of positive sera. Phage pool thus generated was analysed for its reactivity with sera used in the panning step and then immunoscreened for identifying positive clones. The sequence of these clones was determined and their reactivity with a panel of positive and negative sera was evaluated.

To illustrate the selection strategy, we detail 2 representative selection protocols (Fig. 3). Phage library T1 was panned twice on 10 positive sera (B9, B11, B13, B14, B15, B16, B17, B18, B19, B20) to generate phage pools p9^{II}, p11^{II}, p13^{II}, p14^{II}, p15^{II}, p16^{II},

p17^{II}, p18^{II}, p19^{II}, p20^{II}). Some of these pools demonstrated an increased reactivity with the corresponding serum in ELISA (p9^{II}, p13^{II}, p15^{II}, p19^{II}, p20^{II}; Fig. 4a). Individual clones were isolated by immunoscreening these pools with the same serum used for the selection.

A different selection strategy was implemented to single out clones recognized by multiple positive sera. Phage pool p13^{II} (derived from 2 rounds of panning on serum B13) was further panned on a mixture of 8 sera that did not include serum B13 (mix Δ B13, composed by sera B11, B14, B15, B16, B17, B18, B19 and B20) (Fig. 3b). In ELISA, the pool of phage p13^{III} thus displayed strong reactivity with sera mix Δ B13 (Fig. 4b).

Individual phage clones positive in immunoscreening assay were isolated and then grown on the lawn of bacteria on plates of 15 cm by picking in arrayed order. Then the plaques were transferred to nitrocellulose membrane and probed with a number of positive sera and negative sera (details in Material and Methods) (Fig. 5a).

Sequence analysis of selected cDNA clones

The nucleotide sequences of 16 clones that exhibited multiple specific or preferential reactivity with sera from breast tumor patients as compared to sera of healthy donors were determined (Table I).

Sixteen different sequences were determined. By aligning on various databases, these sequences could be classified into 7 groups: (i) Known breast cancer antigens (T1–2, T1–8). The clone T1–2 is a fragment of intestinal mucin (MUC3). The mucins are glycoproteins secreted by epithelial cells and their carcinomas. Membrane-bound mucin 3 is normally expressed in the mucosa of the duodenum. Immunoreactive epitopes of MUC3 were expressed in 26% of ductal carcinoma *in situ* (DCIS) of the breast.²⁹ The clone T1–8 represents a newly defined cross-reactive epitope of RBP1 (retinoblastoma binding protein 1) different from RBP1 peptide (250–256), recently identified by screening the MCF7 cDNA expression library with serum of breast cancer patients.³⁰

(ii) Known tumor antigens described for tumor types other than breast cancer (T1–2, T1–17). The sequence of clone T1–17 corresponds to the beta isoform of type II DNA topoisomerase (TOPIIb). TOPIIb was previously described as tumor antigen for malignant mesothelioma³¹ when the SEREX technique was applied to cDNA expression library of the correspondent cell line. The precise role of TOPIIb is not clear yet.³² However, TOPII can co-immunoprecipitate with p53 and, moreover, type II topoisomerases are overexpressed in the majority of hepatocarcinomas.³³ We identified 25 aa fragments of the TOPII that also elicited an immune response in the breast cancer patients. MUC3 (clone T1–2) has certain prognostic significance for several types of carcinoma different from breast cancer. Aberrant expression of MUC3 gene have been found during the neoplastic transformation of stomach mucosa³⁴ and in mucinous type of bronchioloalveolar carcinoma.³⁵

(iii) Autoantigens (T1–6, T1–101). Clones T1–6 and T1–101 contain sequences for Golgin p245 and Lupus La autoantigen, respectively. Both clones are autoantigens whose expression is not related to neoplastic disease. Golgin p245 is one of the 5 known Golgi autoantigens recognized by sera of patients with various systemic rheumatic disease, such as Sjogren's syndrome (SS).³⁶ La antigen is recognized by certain lupus erythematosus autoantibody (SLE) and SS antibodies.³⁷

(iv) Known proteins, not described as tumor-associated antigens (T1–52, T1–10 and T1–35). The sequence of clone T1–52 corresponds to the nuclear localization motif of topoisomerase I binding protein, *topors*.³⁸ *Topors* has not been reported as a tumor-associated antigen, nevertheless nuclear localization signals are fairly common among tumor antigens identified by SEREX. The screening of cDNA expression libraries derived from renal-cell carcinoma revealed several sequences of that type,³⁹ probably because of the particular features of nuclear targeting sequences⁴⁰ that

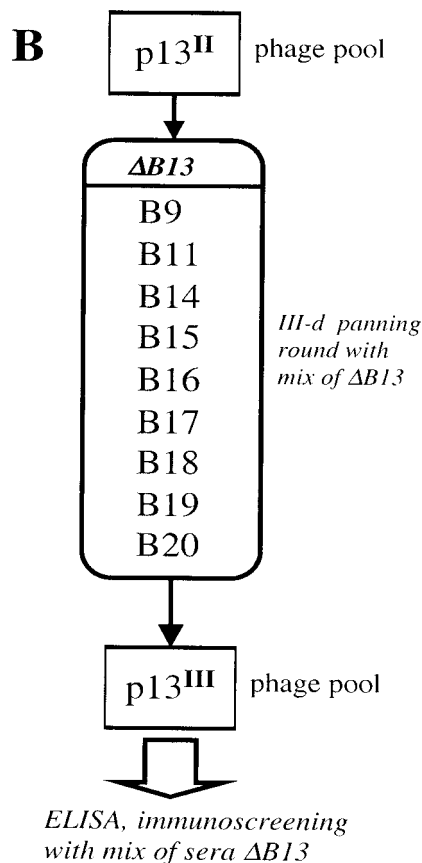
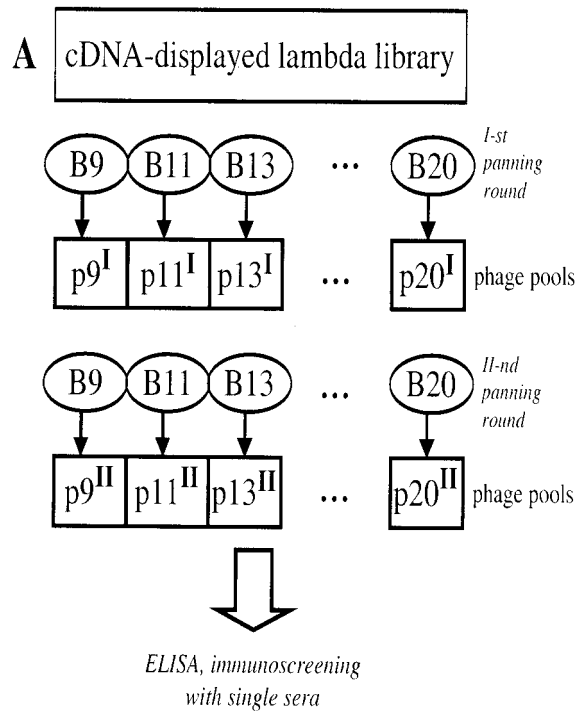


FIGURE 3 – Scheme of the selection strategies leading to the TAA identification.

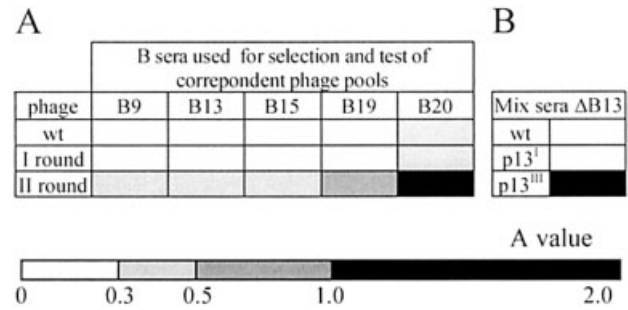


FIGURE 4 – ELISA reactivity of phage pools. (a) Reactivity of phage pools derived from the second round of panning of the T1 library using sera from breast cancer patients. Reactivity of pools remaining negative after the second round of selection is not shown. (b) Reactivity of phage pool p13^{III} derived from the third round of panning using a mix of sera applying the second selection strategy. Data reported are the average value from 2 independent assays, expressed as $A = A_{450nm} - A_{620nm}$ according to the color scale included at the bottom of the picture.

contain a high proportion of highly immunogenic positively charged amino acids. Ribosomal protein S3a (clone T1–10) and nuclear matrix protein p84 (clone T1–35) have been isolated in the screening with breast cancer patient sera. However, immunoreactivity of T1–35 does not appear to be cancer related. Protein S3a is not known as autoantigen or tumor antigen; its further characterization is in progress.

(v) Unknown or putative proteins were found in databases as human DNA sequences. For clone T1–55 was found 100% homology with hypothetical human protein (CAB66672) or with putative human protein pc326 (AAF67474).

(vi) Sequences not found in databases (T1–12, T1–32, T4–19).

(vii) Mimotopes (T4–2, T1–74, T4–11). This last group is composed of short out-of-frame sequences that encode peptides not existing in natural proteins (T4–2, T1–74, T4–11). In spite of the low frequency of short sequences in the library, these peptides were enriched through panning steps. Some of them show a very specific reactivity for sera from tumor-bearing patients, suggesting they mimic tumor-specific epitopes (mimotopes). Clone T1–39, a chimeric fusion of 2 different DNA fragments, could also mimic a tumor-specific epitope.

Immunoreactivity of selected clones

Phage lysates were prepared from all selected clones as described in Material and Methods and tested in ELISA with a collection of negative and, successively, positive sera. We empirically observed a good relation between the reactivity of a given clone with a serum detected in the immunoscreening assay and that measured by ELISA (Fig. 5b). Differences in the intensity of immunoscreening signals between stripes can be ascribed to the fact that development of each strip is variable, lasting until the signal from lambda wt phage is detectable.

Reactivity data for the clones tested are presented in Table II.

Clone T1–35 reacted with both positive and negative sera with a comparable frequency. In contrast, phage clone T1–52 detected antibodies with high specificity in 17% of the positive sera. Similarly, phage clones T1–74, T4–2, T4–11, T1–12, T4–19, T1–32 and T1–39 showed a quite remarkable specific reactivity with positive sera. For example, clone T4–2 scored more than 60% of the sera from breast cancer patients, whilst it did not detect antibodies in any of the sera from healthy donors tested.

Clones with a specific reactivity to positive sera were further characterized by expressing their cDNA sequence as C-terminal fusion to GST or as N-terminal fusion with lambda D protein. The fusion proteins thus generated were tested in ELISA for their reactivity with the same sera. Sequences T1–52, T1–32, T4–2,

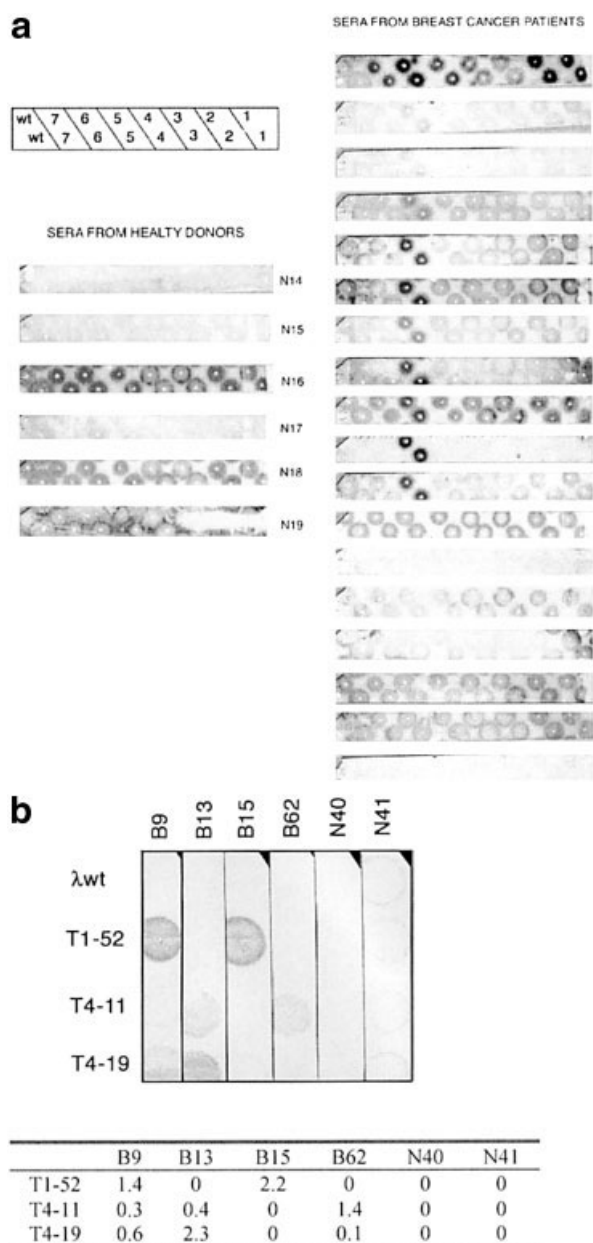


FIGURE 5 – (a) Immunoreactivity of phage clones. The present picture illustrates the analysis of positive clones isolated by immunoscreening. Phages derived from panning library T4 on autologous serum B76 were analyzed by immunoscreening using the same B76 serum. Positive clones were distributed as an ordered array of plaques on a bacterial lawn. Multiple replicas of the same ordered array were generated, transferred to nitrocellulose membrane and individually screened for their reactivity with positive and negative sera. All clones were scored positive by serum B76 used for selection and immunoscreening. Position 6, according to the scheme included in the picture, hosted clone T4-2. (b) An ordered array of plaques of 4 phage clones (λ wt, T1-52, T4-11 and T4-19) was generated on a bacterial lawn and transferred to nitrocellulose strips, as described in Material and Methods. Each strip was probed with a 1:200 dilution of serum B9, B13, B15, B62, N40 or N41 (upper panel). Purified phage particles from the same 4 clones were tested for their reactivity with a 1:100 serum dilution of the same sera (lower panel). ELISA data are expressed as the difference between the average value of the tested phage and that of wild-type γ .

T4-11, T4-19 and T1-52 retained most of their specific reactivity with positive sera. In contrast, T1-12, T1-39 failed to react with positive sera when expressed in a different protein context, indicating that the phage contributed critical elements for the binding of specific antibodies from breast cancer patients in terms of flanking sequences or folding.

Specificity of immune response elicited by clones T1-52, T4-11, T4-19

We wanted to demonstrate that the selected proteins detect antibodies directed against tumor-associated antigens. To this end, we immunized mice and rabbits to elicit antibody response to some of the identified protein fragments.

Mice were immunized with protein D fusions D1-52, D4-11 and D4-19. Immunogenicity of these fusions was tested in ELISA. The same peptide sequences were expressed in a different protein context (to exclude the reactivity against protein D) and used as target antigen. In the case of D1-52, the sera were assayed with GST fusion protein (GST1-52), while for D4-11 and D4-19 the corresponding peptide sequences were cloned as fusion with pVIII (major coat protein of filamentous phage) in phagemid pC89.⁴¹ Both D1-52 and D4-11 proved to be efficient immunogens (data not shown). Anti D1-52 and anti-D4-11 mouse sera were tested for their capacity to specifically recognize tumor cells. Antibody binding to breast carcinoma MCF7 cells was detected by FACS analysis. Both sera specifically bound to MCF7 cells, as indicated by fluorescent shift (Fig. 6). In contrast, no variation in the distribution of fluorescent-labeled cells was detected when pre-immune mouse sera were tested. No significant differences in fluorescent shift were observed when human ovary tumor SKOV3 or other cell lines were tested (data not shown).

Rabbits were immunized with D4-11 protein, and the anti-D4-11 serum thus generated was affinity purified on filamentous phage displaying T4-11 peptide. These affinity-purified anti-T4-11 antibodies recognize a 49 kDa protein band on MCF7 extract, which is absent in non-breast cancer cell lines HL-60 (Fig. 7).

DISCUSSION

The well-established method for identifying specific tumor antigens from cDNA libraries by screening with sera of autologous patients known as SEREX offered the possibility to enlarge the list of proteins recognized by the immune system of tumor-bearing patients and possibly involved in the tumor-forming process.

In our study, we describe a variant of the SEREX method that can considerably improve the efficiency by which relevant TAA are identified. This new approach implies generating lambda-displayed cDNA libraries, which are then affinity selected using patients' sera.

A first crucial benefit is that this method requires very small quantities of patient serum, as the library affinity selected before direct screening is enriched for positive clones and has a lower complexity. A second, major advantage is that it efficiently screens libraries with a much higher complexity than those currently used. Direct screening of a library as large as technology of recombinant DNA can generate is not feasible. By contrast, phage-display libraries with a complexity 10–100 times higher than those traditionally used in SEREX experiments can be effectively surveyed, thus increasing the probability of identifying low represented or downregulated⁴² antigens.

A third relevant feature is the possibility of performing successive cycles of selection with different combinations, or mixtures of patient sera this system offers, which facilitates the identification of cross-reactive tumor antigens, the principal objective of our research.

In our study, we have identified several breast cancer-associated antigens: these protein fragments exhibit a significant specific

TABLE I—THE AMINO ACID SEQUENCES OF SELECTED CLONES REPORTED IN SINGLE LETTER CODES¹

T1-2	ITPTNTMTSMRTTTYWPTATMMEPPSSTVSTTGRGQTTFPSSSTATFNTKHP
T1-6	LEELQKKYQQKLEQEENPGNDNVTIMELQTLAQKTTLISDSKLKEQEFREQIHNLEDRLKKYEKNVYATTVGTPYKGGNL YHTDVSLFGEP
T1-8	TRQLEEVKLKDQEMPLTEVKSEPEENIDSNSSESEREIEELKSPRGRRIARD
T1-10	TAVVLEKPLGTRQVLKLNELMELHGEHSSSGKATGDETGAVERADGMT
T1-12	MRYTTATKTYELMLDATTQ
T1-17	LVEELDKVESQEREDVLAGMSGKSSFQRSEGDFLLRSL
T1-32	LHAFPLHSTTLYYTTP
T1-35	QIRQIECDSEDMKMRKQLLVAWQDQEGVHATPENLINALNKSGLSDLAES
T1-55	TTDEWSSIASSSRGIGSHCKSEGQEESEFVPQSSVQPPPEGDSETKAPESSSEDVTKYQEGVSAENPVENHINITT
T1-39	ALVREVMRVIDRAQAFVDEIFGGDDAHNLNQHS
T1-52	VAGQRYQSRSGHDQKNHRKHHGKKRMSKRSTSLSSPRNG
T1-74	REAKQLHHQPHSIELIQS
T1-101	FVEISEDKTKIRRSPPSKPLPEVTDEYKNDVKNRSVYIKGFPT
T4-2	SEVYKPTLLYS
T4-11	TVGFTLDFTVDPP
T4-19	LYRTTLTYT
M G T S G R P T S G R T S K	
ATGGGTACTAGTGGCCGCCAAC [cDNA insert] CCAGCGGCCGCACGAGCAA	
<i>SpeI</i> <i>NotI</i>	

¹gpD sequences flanking the cDNA inserts are reported in the lower panel, where restriction sites engineered to facilitate cloning also are indicated.

TABLE II—CHARACTERIZATION OF CDNA SEQUENCES IDENTIFIED

Clones	Sera used for selection	Identity	Classification	Frequency reactivity					
				Phage clones			pD ¹ - or GST ² - fusion protein		
				Positive sera	Negative sera	<i>P</i>	Positive sera	Negative sera	<i>P</i>
T1-8	<i>B20, B20/B20</i>	Retinoblastoma binding protein 1	Breast cancer Ag	1/10	n.d.		1/13 ¹	0/15 ¹	
T1-2	<i>B12, B12/B12</i>	Intestinal mucin	Tumor antigen	1/20	0/9		1/31 ²	n.d.	
T1-17	<i>B20, B20/B20</i>	DNA-topoisomerase II beta	Malignant mesothelioma Ag	1/10	n.d.		2/16 ²	0/15 ²	
T1-6	<i>B20, B20/B20</i>	Golgin p245	Auto-Ag	1/10	n.d.		n.d.	n.d.	
T1-101	<i>B9, B9, mix Δ B9/B3</i>	Human protein lupus La	Auto-Ag	2/20	n.d.		n.d.	n.d.	
T1-10	<i>B19, B19/B19</i>	Ribosomal protein S3a	Unknown as tumour Ag	1/10	n.d.		n.d.	n.d.	
T1-35	<i>B19, B19/B19</i>	Nuclear matrix protein p84	Unknown as tumour Ag	4/10	14/21		n.d.	n.d.	
T1-55	<i>B18, B18/B18</i>	Hypothetical protein (CAB66672)	Unknown as tumour Ag	1/20	0/9		n.d.	n.d.	
T1-52	<i>B9, B9/B9</i>	Topoisomerase I-binding protein	Unknown as tumour Ag	7/41	0/20	0.0495	13/72 ¹	2/24 ¹	0.2560
T1-74	<i>B13, B13, mix Δ B13/mixΔB13</i>	AB007891 (out of CDS)		29/72	2/27	0.0017	21/72 ²	4/32 ²	0.0664
T4-2	<i>B76, B76/B76</i>	BC022845 (reverse orientation)		11/18	0/17	0.0001	9/28 ¹	1/31 ¹	0.0031
T4-11	<i>B13/B13</i>	M17733 (alternative frame)		4/21	0/26	0.0200	8/70 ¹	0/30 ¹	0.0536
T1-12	<i>B13, B13, mix Δ B13/mix ΔB13</i>	Not found		23/72	0/31	0.0004	Not reactive ^{1,2}	n.d.	
T4-19	<i>B13/B13</i>	Not found		5/20	0/26	0.0069	15/74 ¹	0/70 ¹	0.0001
T1-32	<i>B13, B13, mix ΔB13/mixΔB13</i>	Not found		17/72	0/31	0.0031	10/72 ²	1/31 ²	0.1080
T1-39	<i>B19, B19/B19</i>	NADH dehydr.sub 5+mitoch. DNA		11/34	0/26	0.0013	Not reactive ^{1,2}	n.d.	

Binding of the indicated phage clones, pD-(¹) or GST-(²) fusion proteins, to positive and negative human sera was detected by ELISA and analyzed as described in Material and Methods. The *p*-value is the probability that the observed frequency distributions of reactivities of the positive and negative sera are statistically the same according to the χ^2 test. Sera used for panning or immunoscreening are indicated in italic or underlined, respectively. Mix ΔB9 indicates the mixture of sera B1–B10 in which serum B9 was omitted. Similarly, mix ΔB13 refers to the mixture of sera B9, B11, B14–B20 in which serum B13 was excluded.

reactivity with breast cancer patient sera compared to sera from healthy donors. Their analysis shows the presence of a class of antigens with a nuclear localization in the cell: topoisomerase-II-beta (TOPII) and topoisomerase I binding protein (*topors*). This result is related to possible tissue necrosis associated with tumor growth. The immunogenicity of these nuclear proteins may be

either the result of tumor-specific posttranscriptional protein modification or a consequence of particular structural features, such as high positive charge of domains interacting with DNA. This is the first time to our knowledge that *topors* is identified as a breast cancer-associated antigen. The clone T1-52, as phage or pD-fusion protein, detects with high frequency antibodies specifically

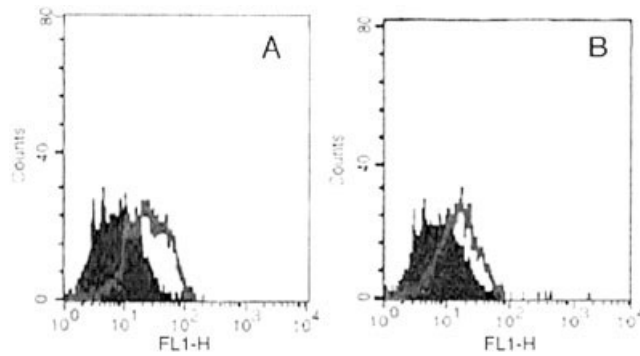


FIGURE 6 – Staining of MCF7 with mice sera. Immunoreactivity of 1:1,000 dilution of the mice sera anti-D4-11 (a) and anti-D1-52 (b) with MCF7 permeabilized cells is represented by the fluorescence profile in bold. Staining of MCF7 permeabilized cells with sera from pre-immune animals is represented by the shaded graph. Similar negative patterns of reactivity were observed with immune sera to living/intact cells or with permeabilized cells from a different source.

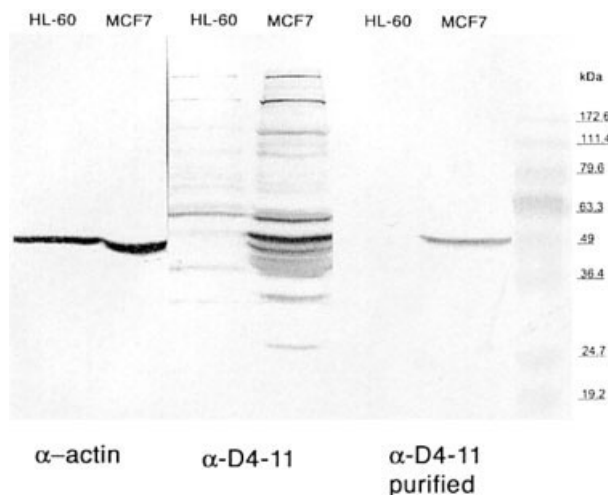


FIGURE 7 – Anti-T4-11 antibodies recognize a tumor-associated protein. Protein extracts from breast carcinoma MCF7, promyelocyte HL-60 cells were analyzed by western blotting using anti-D4-11 rabbit serum (1:200 dilution), anti D4-11 rabbit serum affinity purified on T4-11 peptide exposed on filamentous phage (1:50 dilution) and anti-actin antibodies (as positive control).

present in the serum of breast cancer patients, indicating its possible use as a serologic marker for breast cancer. Furthermore, immunization of mice using T1-52 elicited antibodies that specifically targeted breast carcinoma cell line MCF7.

Another group of antigens identified in this work share a serologic profile associated with breast cancer. These clones have quite short sequences not found in databases (T1-32, T4-19) or found only in the human EST database but not having a natural reading frame (T1-74, T4-2, T4-11). Several antigens with an alternative reading frame, such as NY-ESO-1,⁴³ RU2AS⁴⁴ and probably NY-BR-87⁴² have been described as tumor antigens for melanoma, human kidney tumor and breast cancer, respectively. Similarly, the immunogenicity of T4-2, T4-11 and T4-19 might result from the expression of an alternative reading frame in tumor cells, which occurs after abnormal splicing or chromosome rearrangement. Alternatively, these short peptide sequences may mimic tumor antigens of nonpeptidic nature, such as posttranscriptional protein modifications, not present in the library expressed in bacteria. Indeed, clones T4-2 and T4-19 display sequence homology, have

a similar pattern of reactivity with positive sera and cross-compete with each other (data not shown). These data suggest that the 2 clones mimic the same epitope, since it is very unlikely that alternative expression of 2 different proteins elicit the same immune response. Most of these short sequences contain numerous aromatic amino acids that are typically present in peptides mimicking carbohydrate structures.^{45,46} Affinity-purified anti-T4-11 antibodies bind to a 49 kDa protein in breast carcinoma MCF7 extract that is not detected in promyelocytic cells HL-60. The availability of such anti-T4-11 antibodies might allow the identification of the tumor antigen corresponding to this clone.

In most cases, the presence of antibodies against tumor-associated antigens is detected only in a small proportion of patients (10–30%) with tumors overexpressing the corresponding antigen.¹ Thus, it is not surprising that only a fraction of the sequences identified are true tumor-specific antigens. The list of the selected clones also includes antigens that react uniquely with serum from a single breast cancer patient that has been used in the selection protocol (idiosyncratic reactivity). In addition, antigens whose serologic profile is not restricted to cancer patients are also present in the list. The immune system is constantly in contact with an enormous number of antigens and can synthesize a vast repertoire of antibodies. Human proteins or protein fragments that are not always properly folded when expressed in bacteria fall in this class. They can be detected by the immune system simply because of their sequence or structural homology to different antigens. This class also includes antigens recognized by the natural autoantibody repertoire. Indeed, SEREX experiments demonstrated that normal human sera from individuals without obvious disease probed on a cDNA expression library prepared from cytogenetically normal meningioma revealed different natural autoantigens.⁴⁷ The biologic significance and function of natural autoantibodies are not clear, but their presence interferes with the identification of tumor-associated antigens.

Negative sera used in our study was derived from healthy donors of both sexes and from a population younger than the positive breast cancer patients population composed exclusively from women (see Material and Methods). For this reason, some of the selected antigens' sera reactivity could possibly correlate with sex or age of tested donors. Conversely, where a correlation exists it could also reflect individual humoral history of humans protected routinely from neoplasms due to humoral immunity mechanism.⁴⁸

In this work, we have designed a new-concept vector for the display of cDNA-encoded protein fragments as fusions to the amino terminus of pD. The size of cloned DNA fragments in our libraries was adjusted to an average of 200–300 base pairs, which is a reasonably sufficient size to encode for a protein domain. As 3 of 64 codons in the genetic code correspond to a translation stop, the vast majority of out-of-frame sequences of the above length will contain at least one in-frame stop codon. Thus these inserts will not be expressed as D fusions and will not be displayed on the phage surface. In these cases, phage capsid will contain only wt pD protein encoded by lambda. Usually cDNA libraries are generated as C-terminal fusions. When such a library is panned on a serum, the presence of a complex receptor mixture gives to out-of-frame short sequences a high chance to be enriched. This is because short peptides are more efficiently displayed on the phage and have a lower biologic bias than longer proteins. The N-terminal display system clearly reduced the occurrence of these artificial peptides compared to a C-terminal fusion library displayed on lambda.¹⁸ Nevertheless, a tiny fraction of contaminant short inserts (or rare out-of-frame long inserts not containing any stop codons) are expected to be present in the library and still act as a source of random peptide sequences not existing in natural proteins. Recently this new vector was successfully used to identify the immunodominant epitopes of *T. gondii* antigens involved in the human B-cell response against this parasite: a novel cDNA-dis-

played library from *T. gondii* tachyzoites was screened using a panel of sera of pregnant women infected with this parasite.⁴⁹

When our present work was submitted for publication, our attention was drawn by a recent study also using a phage-display approach for the analysis of the humoral response in tumor patients. This work⁵⁰ revealed a panel of colorectal cancer tumor antigens using an M13 pVI-based display system. All these results together with aforementioned studies of Sioud *et al.*^{15,23} confirm

the validity of a phage-display-based approach to tumor antigens discovery.

ACKNOWLEDGEMENTS

We thank Prof. P.P. di Fiore for providing us part of the collection of sera from breast cancer patients. We also thank Mr. L. Bruno and Dr. G. Borriello for excellent technical assistance and Ms. J. Clench for the linguistic revision of the text.

REFERENCES

- Sahin U, Tureci O, Pfreundschuh M. Serological identification of human tumor antigens. *Curr Opin Immunol* 1997;9:709–16.
- Tureci O, Sahin U, Neumann F, Pfreundschuh M. Exploitation of the antibody repertoire of cancer patients for identification of human tumor antigens. *Hybridoma* 1999;18:23–8.
- Pfreundschuh M. Exploitation of the B cell repertoire for identification of human tumor antigens. *Cancer Chem Pharm* 2000;46:S3–S7.
- Chen Y-T. Cancer vaccine: identification of human tumor antigens by SEREX. *Review. Cancer J Sci Am* 2000;3:S208–17.
- Smith GP. Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* 1985;228:1315–7.
- Rodi DJ, Makowski L. Phage-display technology—finding a needle in a vast molecular haystack. *Curr Opin Biotechnol* 1999;10:87–93.
- Zwick MB, Shen J, Scott JK. Phage-displayed peptide libraries. *Curr Opin Biotechnol* 1998;9:427–39.
- Deroo S, Muller CP. Antigenic and immunogenic phage displayed mimotopes as substitute antigens: applications and limitations. *Comb Chem High Through Screen* 2001;4:75–110.
- Iannolo G, Minenkova O, Petruzzelli R, Cesareni G. Modifying filamentous phage capsid: limits in the size of the major capsid protein. *J Mol Biol* 1995;248:835–44.
- Iannolo G, Minenkova O, Gonfloni S, Castagnoli L, Cesareni G. Construction, exploitation and evolution of a new peptide library displayed at high density by fusion to the major coat protein of filamentous phage. *Biol Chem* 1997;378:517–21.
- Malik P, Terry TD, Gowda LR, Petukhov DA, Perham RN. Role of capsid structure and membrane protein processing in determining the size and copy number of peptides displayed on the major coat protein of filamentous bacteriophage. *J Mol Biol* 1996;260:9–21.
- Castagnoli L, Zucconi A, Quondam M, Rossi M, Vaccaro P, Panni S, Paoluzi S, Santonic E, Dente L, Cesareni G. Alternative bacteriophage display systems. *Comb Chem High Through Screen* 2001;4:121–33.
- Cramer R, Kodzius R. The powerful combination of phage surface display of cDNA libraries and high throughput screening. *Comb Chem High Through Screen* 2001;4:145–55.
- Jespersen LS, Messens JH, De Keyser A, Eeckhout D, Van den Brande I, Gansemans YG, Lauwereys MJ, Vlasik GP, Stanssens PE. Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene VI. *Biotechnology* 1995;13:378–82.
- Sioud M, Hansen MH. Profiling the immune response in patients with breast cancer by phage-displayed cDNA libraries. *Eur J Immunol* 2001;31:716–25.
- Sternberg N, Hoess RH. Display of peptides and proteins on the surface of bacteriophage λ . *Proc Natl Acad Sci USA* 1985;92:1609–13.
- Mikawa YG, Maruyama IN, Brenner S. Surface display of proteins on bacteriophage λ heads. *J Mol Biol* 1986;262:21–30.
- Santini C, Brennan D, Mennuni C, Hoess RH, Nicosia A, Cortese R, Luzzago A. Efficient display of an HCV cDNA expression library as C-terminal fusion to the capsid protein D of bacteriophage lambda. *J Mol Biol* 1998;282:125–35.
- Santi E, Capone S, Mennuni C, Lahm A, Tramontano A, Luzzago A, Nicosia A. Bacteriophage lambda display of complex cDNA libraries: a new approach to functional genomics. *J Mol Biol* 2000;296:497–508.
- Niwa M, Maruyama H, Fujimoto T, Dohi T, Maruyama IN. Affinity selection of cDNA libraries by phage display. *Gene* 2000;256:229–36.
- Rosenberg A, Griffin K, Studier FW, McCormic M, Berg J, Novy R, Mierendorf R. T7 select phage display system: a powerful new protein display system based on bacteriophage T7. *Novations* 1996;6:1–6.
- Zucconi A, Dente L, Santorico E, Castagnoli L, Cesareni G. Selection of ligands by panning of domain libraries displayed on phage lambda reveals new potential partners of synaptojanin 1. *J Mol Biol* 2001;307:1329–39.
- Hansen MH, Ostenstad B, Sioud M. Identification of immunogenic antigens using a phage-displayed cDNA library from an invasive ductal breast carcinoma tumor. *Int J Oncol* 2001;19:1303–9.
- Smith DB, Johnson KS. Single step purification of polypeptides expressed in *Escherichia coli* as fusion with glutathione S-transferase. *Gene* 1988;67:31–40.
- Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989.
- Minenkova O, Gargano N, De Tomassi A, Bellintani F, Pucci A, Fortugno P, Fuscaldi E, Pessi A, Rapicetta M, Miceli M, Iudicone P, Cortese R, et al. ADAM-HCV, a new-concept diagnostic assay for antibodies to hepatitis C virus in serum. *Eur J Biochem* 2001;268:4758–68.
- Cianfriglia M, Mariani M, Armellini D, Massone A, Lafata M, Presentini R, Antoni G. Methods for high frequency production of soluble antigen-specific hybridomas; specificities and affinities of the monoclonal antibodies obtained. *Meth Enzymol* 1986;121:193–210.
- Alter HJ. To C or not to C: these are the questions. *Blood* 1995;85:1681–95.
- Diaz LK, Wiley EL, Morrow M. Expression of epithelial mucins Muc1, Muc2, and Muc3 in ductal carcinoma in situ of the breast. *Breast J* 2001;7:40–5.
- Cao J-n, Gao T-w, Giuliano AE, Irie RF. Recognition of an epitope of a breast cancer antigen by human antibody. *Breast Cancer Res Treat* 1999;53:279–90.
- Robinson C, Callow M, Stevenson S, Scott B, Robinson BWS, Lake RA. Serological responses in patients with malignant mesothelioma. Evidence for both public and private specificities. *Am J Respir Cell Mol Biol* 2000;22:550–6.
- Austin CA, Marsh KL. Eucaryotic DNA topoisomerase II beta. *Bioassays* 1998;20:215–26.
- Yuwen H, Hsia CC, Nakashima Y, Evangelista A, Tabor E. Binding of wild-type p53 by topoisomerase II and overexpression of topoisomerase II in human hepatocellular carcinoma. *Biochem Biophys Res Commun* 1997;234:194–7.
- Wang R, Fang D, Liu W, Luo Y. Aberrant expression of MUC2 and MUC3 genes in gastric carcinoma and its significance. *Chin Med J* 2000;113:502–7.
- Copin MC, Buisine MP, Leteurtre E, Marquette CH, Porte H, Aubert JP, Gosselin B, Porchet N. Mucinous bronchioloalveolar carcinomas display a specific pattern of mucin gene expression among primary lung adenocarcinomas. *Hum Pathol* 2001;32:274–81.
- Fritzler MJ, Lung CC, Hamel JC, Griffith KJ, Chan EK. Molecular characterization of Golgin-245, a novel Golgi complex protein containing a granin signature. *J Biol Chem* 1995;270:31262–8.
- Reichlin M. Antibodies to Ro and La. *Review. Ann Med Interne (Paris)* 1998;149:17–24.
- Haluska P Jr, Saleem A, Rasheed Z, Ahmed F, Su EW, Liu LF, Rubin EH. Interaction between human topoisomerase I and a novel RING finger/arginine-serine protein. *Nucleic Acids Res* 1999;27:2538–44.
- Scanlan MJ, Gordan JD, Williamson B, Stockert E, Bander NH, Jongeneel V, Gure AO, Jager D, Jager E, Knuth A, Chen YT, Old LJ. Antigens recognized by autologous antibody in patients with renal-cell carcinoma. *Int J Cancer* 1999;83:456–64.
- Dingwall C, Laskey RA. Nuclear targeting sequences—a consensus? *Trends Biochem Sci* 1991;16:478–81.
- Felici F, Castagnoli L, Mussacchio A, Jappelli R, Cesareni G. Selection of antibodies ligands from a large library of oligopeptides expressed on a multivalent exposition vector. *J Mol Biol* 1991;222:301–10.
- Scanlan MJ, Gout I, Gordon CM, Williamson B, Stockert E, Gure AO, Jager D, Chen Y-T, Mackay A, O'Hare MJ, Old LJ. Humoral immunity to human breast cancer: antigen definition and quantitative analysis of mRNA expression. *Cancer Immunol* 2001;1:4.
- Wang RF, Johnston SL, Zeng G, Topalian SL, Schwartzentruber DJ, Rosenberg SA. A breast and melanoma-shared tumor antigen: T cell responses to antigenic peptides translated from different open reading frames. *J Immunol* 1998;161:3598–606.
- Van Den Eynde BJ, Gaugler B, Probst-Kepper M, Michaux L, De-

- vuyst O, Lorge F, Weynants P, Boon T. A new antigen recognized by cytolytic T lymphocytes on a human kidney tumor results from reverse strand transcription. *J Exp Med* 1999;190:1793–800.
45. Hoess R, Brinkmann U, Handel T, Pastan I. Identification of a peptide which binds to the carbohydrate-specific monoclonal antibody B3. *Gene* 1993;128:43–9.
46. Phalipon A, Folgori A, Arondel J, Sgaramella G, Fortugno P, Cortese R, Sansonetti PJ, Felici F. Induction of anti-carbohydrate antibodies by phage library-selected peptide mimics. *Eur J Immunol* 1997;27:2620–5.
47. Comtesse N, Heckel D, Maldener E, Glass B, Meese E. Probing the human natural autoantibody repertoire using an immunoscreening approach. *Clin Exp Immunol* 2000;121:430–6.
48. Houghton AN, Taormina MC, Ikeda H, Watanabe T, Oettgen HF, Old LJ. Serological survey of normal humans for natural antibody to cell surface antigens of melanoma. *Proc Natl Acad Sci USA* 1980;77:4260–4.
49. Beghetto E, Pucci A, Minenkova O, Spadoni A, Bruno L, Buffolano W, Soldati D, Felici F, Gargano N. Identification of a human immunodominant B-cell epitope within the GRA1 antigen of *Toxoplasma gondii* by phage display of cDNA libraries. *Int J Parasitol* 2001;31:1659–68.
50. Somers VA, Brandwijk RJ, Joosten B, Moerkerk PT, Arends J-W, Menheere P, Pieterse WO, Claessen A, Scheper RJ, Hoogenboom HR, Hufton SE. A panel of candidate tumor antigens in colorectal cancer revealed by the serological selection of a phage displayed cDNA expression library. *J Immunol* 2002;169:2772–80.