Published in final edited form as:

J Immunol Methods. 2006 May 30; 312(1-2): 79–93.

Cloning of B cell lymphoma-associated antigens using modified phage-displayed expression cDNA library and immunized patient sera

Soung-Chul Cha^a, Larry W. Kwak^{a*}, Pier Adelchi Ruffini^b, Hong Qin^a, Sattva Neelapu^a, and Arya Biragyn^c

^aDepartment of Lymphoma and Myeloma, Division of Cancer Medicine, Center for Cancer Immunology Research, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 0903, Houston, TX 77030, United States

^bDivisione di Oncologia Medica Falck, Ospedale Niguarda Ca' Granda, Milan, Italy

^cLaboratory of Immunology, Gerontology Research Center, National Institute on Aging, Baltimore, MD 21224, United States

Abstract

Active immunization of follicular lymphoma patients with idiotypic vaccines elicits antigen-specific antibody responses, T-cell responses, and antitumor effects. We hypothesized that these vaccinated patients could generate tumor-specific immune responses, not only against idiotypic, but also against other tumor-associated antigens (TAA) by a mechanism of epitope spreading. To identify potential antigens, a phage surface expressed cDNA library derived from primary tumor cells was screened with sera from idiotypicvaccinated patients. Consistent with our hypothesis, we identified two immunogenic peptides (FL-aa-7 and 18), unrelated to idiotypic, which were recognized by postvaccine sera but not by prevaccine or normal human sera. These peptide sequences derived from the 5'-untranslated regions of the human GTPase, IMAP family member 7 gene (FL-aa-7) and an alternative reading frame of U1-snRNP 70 (FL-aa-18), respectively, suggesting that epitope spreading had occurred.

Keywords

Phage display; Tumor antigen discovery; B cell lymphoma; Epitope spreading

1. Introduction

It is becoming apparent that active immunotherapy is an effective strategy to fight cancer by inducing the immune system of patients to recognize malignant cells as foreign. Particularly, the importance of CD8⁺ and CD4⁺ T cells in the inhibition of tumor growth and eradication of cancer cells has been emphasized (Rosenberg, 2001; Wang, 2001). Tumor-associated antigens (TAA) are defined as a product of genes preferentially expressed in malignant cells, such as cancer testis antigens (CT) (Jager et al., 1998), or antigens derived from mutated (Brandle et al., 1996; Mandruzzato et al., 1997), improperly processed and regulated genes (Kao et al., 2001). At present, a number of TAAs have been identified, although methods for identifying clinically relevant, rejection antigens are needed. Although TAAs as self-tumor

*Corresponding author. Tel.: +1 713 563 3415; fax: +1 713 563 4625. E-mail address: lkwak@mdanderson.org (L.W. Kwak).

antigens are often weakly immunogenic in hosts, low levels of specific immune responses, including antibody against the antigen, can be detected in many patients with cancer.

This assumption has been successfully utilized in SEREX (serological identification of recombinantly expressed genes) (Sahin et al., 1995) to isolate a number of human TAAs. Antigens such as tyrosinase (Brichard et al., 1993; Sahin et al., 1995), MAGE, and NY-ESO-1 (Chen et al., 1997; Jager et al., 1998) were originally cloned using SEREX. However, the majority of antigens have been cloned from solid tumors, while almost no TAAs were reported to date for hematopoietic malignancies. B cell lymphoma-specific antigens are not defined, except for immunoglobulin, idiotype (Id), which is being successfully used for immunotherapy of patients with follicular lymphoma (Bendandi et al., 1999). It is presumed that CT antigens would not be good markers for B cell malignancies, such as non-Hodgkin's lymphomas and myeloma (Xie et al., 2003), since only a small proportion of B cells express CT antigens (Huang et al., 2002). Furthermore, unlike solid tumors, antigen cloning from B cell malignancies is hampered by a number of technical problems, such as relatively lower numbers of malignant cells per follicular lymphoma biopsy and significantly smaller yields of mRNA. B cell malignancies express immunoglobulin genes; thus, screening sera from patients is hampered by false positive reactions. Moreover, SEREX is a laborious and time-consuming procedure, which requires extensive repeated screenings to eliminate false positive clones or numerous autoantigens. In addition, the method requires substantial amounts of patients' autologous serum, which may often be in a short supply. Therefore, to overcome these weaknesses and to simplify and streamline the screening method, we modified the approach by expressing a cDNA library on the surface of T7 phage. Unlike the filamentous M13 phage system, proteins displayed on the surface of T7 do not need to be secreted to the bacterial periplasmic space, and the time for the phage replication is significantly shorter. It usually required 1 to 2 h to detect lysis in bacterial liquid cultures, and 3 h to observe plaques on plates. Thus, it is possible to perform multiple rounds of panning in a regular work day. In addition, the method requires a few milliliters of sera.

Here, we present a simple and rapid strategy specifically adapted for the cloning of TAA from human B cell lymphomas using a surface-displayed, T7 phage approach. A cDNA library of the genes from a lymphoma was expressed on the surface of T7 phage and screened with IgG-derived from sera of the patient. Importantly, we selected sera from lymphoma patients who had a positive clinical response to Id-vaccinations (designated postvaccine sera), hypothesizing that these sera would allow us to clone clinically relevant antigens, associated with tumor eradication. Both SEREX and a phage surface display technique resulted in preferential cloning of autoantigens, despite using the most stringent screening procedures. For example, cDNA for human hypothetical protein MGC9515 (named SMG, BC036263) was repeatedly cloned. However, the outcome of the screening was drastically improved when we used these autoantigens to block "nonspecific" binding. As a result, we have cloned a number of unidentified clones, which were specifically recognized by postvaccine, but not prevaccine, IgG, suggesting that they may participate in tumor clearance.

2. Materials and methods

2.1. Reagents

"Panning" (screening) was performed with rabbit anti-human IgG (DakoCytomation, Glostrup, Denmark) and goat anti-human IgG antibody-coated magnetic beads (Biosource, Camarillo, CA). Peroxidase-conjugated goat anti-human IgG, Fc was used for the ELISA (Jackson Immuno Research Lab, Bar Harbor, ME; Caltag, San Francisco, CA). Four peptides, FL-aa-7 peptide: N-QKQQLKQPPWRKPENSTCSREGLVRA-C, FL-aa-18 peptide: N-GQRQPRHVHGV-C, CDR3 peptide: N-YYCVLGVTERWFDPW-C, and influenza peptide: N-GILGFVFTL-C were synthesized by the Fmoc/tBu protection protocol and purified using

reverse phase HPLC. The peptides were characterized using MALDI-TOF mass spectrometry and analytical HPLC. (Global Peptide Services, Fort Collins, CO).

2.2. Serum samples and preparation of IgG

Following informed consent, sera were taken from patients who had a positive clinical response in the ongoing idiotype vaccine trial for follicular B cell lymphoma at the National Cancer Institute (Bendandi et al., 1999). Pre-vaccine and post-vaccine sera from patients or serum from healthy donors were diluted 1:10 and pre-absorbed by repeated passing on a Sepharose 4B column coupled with the lysate from BLT5615 *E. coli* infected with T7 phage. The pre-absorbed sera were further diluted to a final dilution of 1:100, and serum IgG were purified using protein A affinity chromatography. Protein A-bound IgGs were eluted with 0.1 M glycine-HCl buffer and then neutralized with Tris base. The final protein concentration was adjusted to 100 µg/mL.

2.3. RNA isolation and construction of cDNA expression libraries

Primary lymph node biopsies from patient with previously untreated follicular lymphoma, confirmed by the Laboratory of Pathology, National Cancer Institute, were cryopreserved as single-cell suspensions. For the study, only IgM-expressing cells from follicular lymphoma patients were used. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient separation. Normal B cells were depleted from samples using goat anti-human IgG magnetic beads (Biosource, Camarillo, CA). Total RNA was extracted using standard methodology according to the manufacturer's recommendation (FastRNA Kit, Bio101, Carlsbad, CA), and mRNA was purified using the FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, CA). A cDNA library was constructed from 1 to 2 µg mRNA following manufacturer's instructions for the cDNA synthesis kit (Stratagene, La Jolla, CA), with some modifications. Briefly, the first-strand cDNA synthesis was generated using an oligo(dT) primer that contained an internal *Xho*I and *Hind*III restriction sites. We have used mixtures of following *Eco*RI adapters to allow translation of each cDNA in all three reading frames: adapter-*Eco*RI-1: 5' AATTTCGGCAGCAGG-3' and 5'-CCTCGTGCCG-3'; adapter-*Eco*RI-2: 5'-AATTTCGGGCA CGA-3' and 5'-TCGTGCCCG-3'; and adapter-*Eco*RI-3: 5'-AATT-CGGGCACGACG-3' and 5'-CGTCGTGCCCCG-3'. All three *Eco*RI-adapters were ligated to the generated cDNA, digested with *Hind*III and cloned into the T7Select 10-3b vector (Novagen, Madison, WI) packaged into phage particles, and transfected BLT5615 *E. coli*. The average efficiency of the library was 1.7×10^6 recombinants.

2.4. Preparation of recombinant T7 SEREX-defined antigens phage

Initially we used T7 phage for the screening of cDNA clones found using the SEREX technique. The cDNA from twenty two SEREX-enriched, follicular lymphoma sera-specific were re-cloned using a mixture of PCR forward primers to allow the translation of each cDNA in all three reading frames: PR-BKT7-1; 5'-ACCGAATTCGAGCAGGTCGACACTAGTGGATTCAAAGTATTC, PR-BKT7-2; 5'-ACCGAATTCGGAGCAGGTCGACACTAGTGGATTCAAAGTATTC, PR-BKT7-3; 5'-ACCGAATTCGCAGGTCGACACTAGTGGATTCAAAGTATTC; and a single reverse primer PR-BKT7-R1; 3'-TTTTTTGA GCTCTCATGAAGATC TCG-TTCGAATT. The PCR fragments were purified from a 1.2% agarose gel, digested with *Eco*RI and *Hind*III and re-cloned into *Eco*RI and *Hind*III T7 Select 10-3b vector (Novagen, Madison, WI). DNA was packaged by using a T7 packaging extract (Novagen, Madison, WI). Phage titers were determined using plaque assay.

2.5. Preparation of blocking reagents for immunoscreening

Control phage used as a blocking reagent was killed with UV irradiation. Briefly, 25 µL of phage at 1×10^3 pfu/mL were UV exposed five times with 120,000 µJ/cm² in the Stratalinker

UV cross-linker (Stratagene, La Jolla, CA), as described by others (Prezzi et al., 1996). FO-blocking solution was prepared by treatment with formaldehyde (37%) at 37 °C as described (Tolpin et al., 1980). Briefly, a control SMG-1/MGC9515 T7 phage culture was centrifuged at 8000 $\times g$ for 10 min and the supernatant was collected. Phage particles were concentrated to 3×10^{11} pfu/mL using the Ultralab system (Pall, Ann Arbor, MI). Formaldehyde at 37% (Sigma) was diluted 1:1000 with PBS and added to an equal volume of phage. The mixture was incubated at 37 °C for 72 h and then dialyzed in PBS at 4 °C for 32 h.

3. Immunoscreening of the cDNA expression library

3.1. The first biopanning strategy

Ninety-six-well, flat-bottom microtiter plates (Immunoplate Maxisorp; Nunc, Roskilde, Denmark) were coated with 200 μ L purified postvaccine IgG (10 μ g/mL) in coating buffer (50 mM NaHCO₃ pH 9.6) overnight at 4 °C. The wells were blocked with 200 μ L blocking solution (3% BSA/0.1% Tween-20, 0.02% NaN₃/PBS) for 3 h at room temperature. Then, wells were incubated with a phage library (approximately 1×10^9 pfu) for 16 h at 4 °C. Unbound phage was removed by washing 5 times with wash buffer (1.5 M NaCl/0.05% Triton X-100) and 5 times with PBS. The IgG-bound phage was eluted with elution buffer (1% SDS/PBS) and infected BLT5615 *E. coli*. Infected bacteria left to grow in suspension for 3 h at 37 °C. The bacterial supernatant (phage lysate) was again used in the screening (panning) four more times. To ensure preferential selection of clones specific for postvaccine IgG, the selected phage after the fifth round of panning with postvaccine IgG was depleted from non-specific binders by passing through columns coated with IgG from normal Ab serum and prevaccine serum.

3.2. The second panning strategy

Goat anti-human IgG antibody-coated magnetic beads (Biosource, Camarillo, CA) were incubated with post-vaccine patient serum (1:100 dilution) at a concentration of 10 μ g/mL in PBS/0.1% Tween-20 for 12 h at 4 °C. After extensive washes with the same buffer, the magnetic beads were preincubated with 10 μ L of phage library (about 1×10^{11} pfu/mL) together with a 100-fold excess of control UV-killed T7 phage in PBS/0.5% Tween-20 for 4 h at 4 °C. Unbound phage was removed by extensive washes with PBS/0.5% Tween-20. The bound phage was infected a log-phase *E. coli* culture (20 mL). The panning was again repeated for three more times.

3.3. The third panning strategy

Sera from 5 follicular lymphoma patients were pooled and were adsorbed by repeated passage through columns of Sepharose 6B column coupled with the lysate from BLT5615 *E. coli* infected with T7 phage as described above. Prior to screening, 1 mL of post-vaccine sera (1:100 diluted in 4% dry milk/PBS, PBS-M) was incubated with 200 μ L FO-blocking solution (10% 3×10^{11} pfu/mL of formaldehyde-inactivated T7 phage/4% dry milk in PBS) for 16 h at 4 °C. The immunotube (Nunc) was coated with rabbit anti-human IgG (Dako) at a concentration of 10 μ g/mL in coating buffer (0.1 M sodium hydrogen carbonate, pH 9.6) for 16 h at 4 °C, and washed twice with PBS-T (PBS, and 0.1% Tween 20 [w/v]) and twice with PBS. Then, the tube was blocked for 2 h at room temperature with 2% PBS-M, and incubated with phage library ($\sim 3 \times 10^{10}$ pfu in 500 μ L of PBS) in 500 μ L of 4% PBS-M and 1 mL complex of pooled sera (1:100 diluted in 4% PBS-M). Unbound phages were removed by washing for 20 times with PBST and 20 times with PBS. The retained phages were eluted with elution buffer (1% SDS/PBS) and *E. coli* strain BLT5615 was infected. The panning was again repeated three more times.

3.4. ELISA

Ninety-six-well plates (Immunoplate Maxisorp; Nunc) were coated for overnight at 4 °C with the synthetic peptide, 10 µg/mL in 50 mM NaHCO₃, pH 9.6. After several washes with ELISA wash buffer (1.5 M aCl/0.05% Triton X-100), wells were blocked with PBS containing 5% bovine serum (PBS-BS) for 1 h at 37 °C. Prevacine and postvaccine sera (1:100 in PBS-BS/0.1% Tween 20) from follicular lymphoma patients were added to each well and incubated for 1 h at room temperature. The bound antibodies were detected with peroxidase-conjugated goat anti-human IgG, Fc (Jackson Immuno Research Lab, Bar Harbor, ME; Caltag, San Francisco, CA) and the ABTS peroxidase substrate (KPL, Gaithersburg, MD) at an absorbance of 405 nm.

3.5. Binding and inhibition assays

For the binding assay, 96-well, flat-bottom microtiter plates (Immunoplate Maxisorp; Nunc) were coated overnight at 4 °C with 200 µL rabbit anti-human IgG (Dako; 10 µg/mL) in coating buffer, and blocked with 200 µL 2% PBS-M for 1 h at room temperature. The wells were washed three times with PBST and three times with PBS. Then, 200 µL FO-blocking solution (10% 3 × 10¹¹ pfu/mL of formaldehyde-inactivated T7 phage/4% dry milk in PBS) with the phage clone (50 µL, 1 × 10⁸ pfu) and 2 µL prevaccine or postvaccine serum (1:100 diluted) were preincubated for 16 h at 4 °C. The mixture was transferred to anti-human IgG coated plates and further incubated overnight at 4 °C. Then, unbound phages were removed by washing 20 times with PBST and 20 times with PBS, and the remaining phages were eluted with elution buffer (1% SDS/PBS) and infected *E. coli* strain BLT5615 in log-phase growth. Infected bacterial stock was plated on LB agar plates containing 100 µg/mL of ampicillin and incubated for 3 h at 37 °C. To determine bound phage titer, plaque numbers were counted.

The peptide inhibition assay, the titrated amounts of peptides (0.1 – 100 µg) were preincubated with the phage clones and patient serum (diluted 1:50) for 16 h at 4 °C and then added to the rabbit anti-human IgG coated plates, described above, and incubated overnight at 4 °C. Unbound phage were removed by 5 washes with 200 µL of ELISA wash buffer and with PBS each. The bound phage were eluted with elution buffer and were used to infect BLT5615 cells, as described above.

3.6. Semi-quantitative RT-PCR for FL-aa-7 and 18 mRNA

Cryopreserved cells from the lymph node biopsy were enriched for tumor cells by depleting T cells with CD3 microbeads over a magnetic column (Miltenyi Biotec, Auburn, CA) and B cells were isolated using the B Cell Isolation Kit (Miltenyi Biotec, Gladbach, Germany) according to the manufacturer's protocol. Control B cells were isolated from normal human PBMCs using the same method. The purity of the isolated B cells was greater than 95%, as determined by FACS staining with anti-CD20 and CD3 (BD Biosciences, San Diego, CA). Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad CA) and used for cDNA synthesis. PCR primer pairs were designed to amplify 5'-untranslated regions of the human GTPase, IMAP family member 7 gene (FL-aa-7, forward 5'-AAAAGCAACAGCTCAAGCAGCCT CCTT, and reverse 5'-CCATTCCCGGGATGCTTTTT GACAGTTCTT) or U1-70K gene (FL-aa-18, forward 5'-CTGACGGCCCTGACGGTCCAGAGGAA, and reverse 5'-TCACTCCGGCGCAGCCTCCATCAAA TACC), respectively. PCR was amplified from about 2 µg cDNA using a touchdown condition in (GenAmp PCR system 2700, AB Applied Biosystem) (Hold [95 °C 10 min] 10 cycles [94 °C 45 s; 62 °C 45 s; 72 °C 45 s; reducing the annealing temperature by 1 °C each cycle] 22 cycles [94 °C 45 s; 57 °C 45 s; 72 °C 45 s;] and hold [72 °C 7 min; 4 °C]). PCR samples were separated in 1% agarose gel in TAE buffer with 0.5 mg/mL ethidium bromide and the image was analyzed using 1-D analysis software (Bio-Rad Laboratories, Hercules, CA). PCR data were normalized against control amplification for

β -actin cDNA using following primers (β -actin forward 5'-TGACGGGGTCACCCACACTGTGCCCATCTA and β -actin reverse : 5'-CTAGAAGCATTGCGGTGGA CGATGGAGGG).

4. Results

4.1. Limited cDNA lymphoma library expressed on T7 phage surface

We first generated a cDNA library expressed in λ phage (SEREX) from a follicular B cell lymphoma biopsy and screened it with autologous sera from patients. Surprisingly, the screening resulted in selection of too many clones (more than 100), and screening required relatively large amounts of patient sera, thus, limiting further the feasibility of this strategy. To simplify the strategy, we tested whether patient sera (IgG) would recognize these antigens, if expressed on the surface of T7 phage. The SEREX-generated gene fragments were recloned to C-terminus of the 10B capsid protein of T7 bacteriophage to be expressed in all three reading frames for display on the phage surface. The resulting phage expressing a limited cDNA library was repeatedly screened (panned) in 96-well plates coated with 10 μ g/mL IgG from the same patient (see schema of panning, Fig. 1). Screening was monitored by PCR analysis of twenty randomly selected phage plaques after each round of panning, and DNA sequences of the inserts from the fifth round of panning were determined and compared using BLAST analysis (<http://www.ncbi.nlm.nih.gov/BLAST>). Panning yielded three clones: ribosomal protein L7, a known autoantigen often cloned by others, and two genes of unknown function (Table 1 and Table 2). To further prove that these clones have been specifically selected, each one was mixed back with 300-fold excess of wild-type phage and panned against postvaccine IgG from the same patient. As shown in Fig. 2A–B, three rounds of panning were sufficient to completely enrich (more than 80%) with the selected clones, thus, suggesting that the cloning was indeed specific.

Next, the clones were tested for specificity to sera from normal donors or patient sera before and after Id-vaccination, with prevaccine and postvaccine IgG, respectively. All three clones bound patient IgG slightly better (up to 3-fold), than normal donor AB serum (Fig. 2C–F). However, binding percentages of postvaccine and prevaccine sera from the same patient were not significantly different (Fig. 2C–D). These data suggest that sera from patients with FL lymphoma have antibodies specific to autologous antigens, and a phage-displayed expression library can be used to simplify and streamline the SEREX-based screening.

4.2. Cloning of B cell lymphoma patient antigens using T7 phage display approach

Next, we constructed a T7 phage expression cDNA library from a tumor biopsy isolated from a FL patient, who responded to Id-vaccinations. The library, containing 1.7×10^6 independent phage clones with the insert sizes between 250 and 1250 bp, presumably expressed the fragments in all three reading frames since modified *EcoRI* adapters were used (see Materials and methods). To ensure a preferential selection for tumor-associated antigens binding postvaccine IgG, the phage library was panned initially 5 times in 96-well plates coated with postvaccine IgG, and depleted by passing through columns coupled with sera from a normal donor (AB serum) and prevaccine serum (see “Depletion step”, Fig. 1). The enriched phage library was plated on *E. coli* lawn and randomly picked 50 plaques were analyzed by PCR sequencing for a single nucleotide reaction (T-track). The majority of selected clones (>80%) contained a single gene fragments, the 3'-end of hypothetical gene designated in GenBank as MGC9515 (BC036263, Fig. 3C). The sequence comparisons of deduced peptide suggest that patient antibody recognized multiple repeats, such as “KTPPLATQEAEKPRKPKRQRAAEMEPPPE” and “PKRRR-VGDVEPSRKPKRRRAADVEPS LPE”, or a unique sequence “PKRRLS” (Fig. 3C). The clones have been specifically selected by patient IgG, since they could be easily enriched back after being diluted to 1000-fold with wild-type

phage (Fig. 3A). However, despite extensive attempts to deplete with normal and prevaccine IgG, the selected MGC9515 clones bound prevaccine and postvaccine and normal IgG equally well (Fig. 3B). Thus, these data suggest MGC9515 may represent one of numerous strongly immunogenic autoantigens.

Next, the remaining 16 clones were pooled and extensively depleted by passing over columns coupled with IgG from normal and prevaccine sera. Then, the clones were mixed with 1:1000 diluted postvaccine serum and panned using anti-human IgG coated magnetic beads. All procedures were performed in the presence of UV-killed wild-type phage to reduce non-specific binding (see Materials and methods). Surprisingly, the majority of selected clones again contained various overlapping fragments of MGC9515 (Fig. 3C). Overall, these results suggest that the selection procedure we used was favorable for the preferential cloning of strongly immunogenic autoantigens. Therefore, these strongly immunogenic autoantigens may presumably mask or hamper binding of tumor-specific antigens that usually generate only weaker immune responses. To overcome this obstacle, we speculate that these antigens may enable us to clone weakly immunogens, if used as blocking and depleting reagents.

4.3. Preferential cloning of an antigen specific to postvaccine patient serum

Next, to increase chances for TAAs, we have introduced several modifications. For example, instead of a single patient IgG, we used IgG pooled from five follicular lymphoma patients. The pooled IgG were extensively depleted from MGC9515 binders by passing through beads coupled with lysate from FL-aa-4-2 phage, which expressed an immunogenic fragment of MGC9515. In addition, formaldehyde-killed FL-aa-4-2 phage was included in all procedures, including the blocking step. The phage library, which was initially negatively selected on prevaccine IgG, was panned against the pooled postvaccine IgG. After three rounds of panning, individual phage plaques were randomly selected and T-track sequenced as previously described. Among 16 different gene fragments cloned, 2 genes, designated FL-aa-5 and FL-aa-6, were best represented, 26% and 21%, respectively (Table 1 and Table 2).

The selected clones were then tested for their specificity to pooled prevaccine and postvaccine sera from patients. An equal amount of each phage clone (1×10^8 pfu, 50 μ L) was mixed with various sera and bound phage were captured in wells coated with rabbit anti-human IgG. After an extensive washing step, bound phage were eluted and quantified by plating on *E. coli* lawn. As shown in Fig. 4A, some clones, such as FL-aa-5, FL-aa-6, FL-aa-9, FL-aa-11, FL-aa-15 and FL-aa-19 bound both prevaccine and postvaccine sera equally well. In contrast, two clones, namely, FL-aa-7, and FL-aa-18, reacted preferentially with postvaccine sera and had a minimal cross-reactivity to prevaccine sera. When binding to individual patient sera was assessed, FL-aa-18 was specific to postvaccine serum from patient #7 (Fig. 4B and D) and FL-aa-7 reacted mostly with the patient #38 postvaccine serum (Fig. 4C and E). DNA sequencing has revealed that FL-aa-7 (FL-aa-7-1 and FL-aa-7-2) contained overlapping fragments of 5'-untranslated region (UTR) of the same gene, human GTPase, IMAF family member (NM_153236, Fig. 5A) (Tolpin et al., 1980). In contrast, FL-aa-18 contained alternative frame of U1-snRNP 70K gene (NM_003089), but a deduced peptide sequence expressed on a phage was in a different reading frame (Fig. 6A).

To prove that the deduced peptides were expressed on the phage surface and detected with patient sera, we made synthetic peptides, respectively, designated FL-aa-7-1 peptide and FL-aa-18 peptide. Both synthetic peptides FL-aa-7-1 (Fig. 5A) and FL-aa-18 (Fig. 6A) could specifically bind postvaccine serum from patients #7 and #38, respectively. In contrast, control peptides, such as influenza peptide (Flu, Fig. 5B and Fig. 6B) and Ig CDR3 peptide from the lymphoma patient (CDR3, Fig. 5B and Fig. 6B) failed to bind the sera. Furthermore, both FL-aa-7-1 and FL-aa-18 peptides could also specifically inhibit binding of their respective FL-aa-18 and FL-aa-7-1 phage clones to the postvaccine sera in dose dependent way (respectively,

Fig. 5C and Fig. 6C). As expected, control peptides Flu (Fig. 5D and Fig. 6D) and CDR3 (Fig. 5E and Fig. 6E) failed to inhibit binding of FLaa-7-1 (Fig. 5D and E) or FLaa-18 (Fig. 6D and E) phages, respectively. Thus, these data suggest that Id-vaccinated patients #7 and #38 generated specific antibodies to peptides FLaa-18 and FLaa-7-1, expressed by phage clones FLaa-18 and FLaa-7-1, respectively.

In order to evaluate whether the genes for FLaa-7 and FLaa-18 peptides are expressed in FL tumor, we compared mRNA expression levels of the putative genes of origin, hGIMAP, and U1-snRNP 70K in primary lymphoma samples (five patients), one lymphoma cell line (RL) and normal B cells (two healthy donors) using semi-quantitative RT-PCR. There was no clear over-expression of hGIMAP mRNA in tumor, compared with normal B cell samples (data not shown). However, U1-snRNP 70K mRNA was expressed in at least two of the five primary tumors but not in the single lymphoma cell line or normal B cells tested. (Fig. 7). The data are in concordance with the observation that postvaccine sera from patient #7 also contained significant amounts of specific antibodies to FLaa-18 peptide. It is also necessary to acknowledge that these peptides may be structurally similar to peptides in expressed, but unrelated proteins other than hGIMAP or U1-snRNP 70K.

To further test the specificity of FLaa-18 and FLaa-7-1 peptides, we tested their binding with prevaccine and postvaccine sera from 32 different patients with B cell lymphoma and 10 sera from healthy donors. As expected, FLaa-7-1 (Fig. 7A) and FLaa-18 (Fig. 7B) peptides bound preferentially postvaccine sera from patients #7 and #38, respectively. However, the peptides also recognized sera from a number of other, but not every patient. Postvaccine sera from patients #24 and #7 recognized both FLaa-18 and FLaa-7 peptides, FLaa-18 cross-reacted with postvaccine, but not prevaccine, sera from patients 24, 23, 26, and 36 (Fig. 7B), and, similarly, FLaa-7 could bind postvaccine sera from patients 6, 7, 14, 17 and 24 (Fig. 7A). In contrast, sera from 10 healthy donors did not react with either of the peptides. Overall, these data indicate that Id-vaccinated B cell lymphoma patients mounted antibody responses specifically for FLaa-7-1 and FLaa-18 peptides, which may represent a potential immunotherapeutic tumor antigen. The clinical and biological relevance of the response is to be elucidated (Fig. 8).

5. Discussion

It has been hypothesized that Id-vaccinated patients with follicular lymphoma may elicit clinically relevant immune responses to antigens unrelated to Id by a mechanism of “epitope spreading” (Butterfield et al., 2003; Disis et al., 2004). To test this hypothesis and to identify new lymphoma-associated antigens, we used sera from a patient who had been actively immunized with an Id vaccine formulation that had successfully induced CD4⁺ and CD8⁺ T cells and anti-Id antibody responses to screen a phage expression cDNA library from the follicular lymphoma lymph node biopsy. However, we also assumed that these patients would have, at least, low levels of antibody against potential lymphoma-associated antigens to be utilized for cloning of TAAs. Most reported tumor-associated antigens were cloned using the SEREX approach. The method requires large amounts of patient sera and biopsy materials, and the selection process is subjective, based on interpretation of individual researchers. SEREX was used to clone B cell lymphoma antigens in most cases without much success (Huang et al., 2002). In our hands, the strategy yielded too many positive clones when patient sera was used to screen SEREX library from the LN biopsies of follicular lymphoma patients, thus complicating the search. In contrast, expression of the library on a phage surface allowed us to streamline and simplify the cloning procedure, and to properly use limited amounts of sera from vaccinated patients. However, our main concern was to select phage clones specific only for postvaccine, but not preimmune, sera. Initially, most of the cloned fragments belonged to a set of known autoantigens, which reacted with normal and patient sera equally well. This

preferential cloning of autoantigens is speculated to be due to the presence of strong anti-autoantigen reactivity in human sera, which could mask and hamper cloning of other tumor associated antigens, presumed to be a weak immunogens. Therefore, to overcome this problem, we used these strong autoantigens as blocking and depleting reagents. The similar strategy also was utilized by others to block a residual serum binding (Tsui et al., 2002).

Consistent with our hypothesis, we identified two immunogenic peptides (FL-aa-7 and FL-aa-18), unrelated to idiotype, which were preferentially recognized by postvaccine sera. Importantly, the peptides did not have significant binding to sera from healthy donors or prevaccine IgG. Although the chemotherapy intervention alone could induce generation of humoral responses to tumor antigens, we have deliberately set a relatively high threshold to consider clones positive only when their binding differences between postvaccine and prevaccine sera were greater than two-fold. Interestingly, both peptides were presumed to be derived from either the 5'-untranslated region (5'-UTR) or an alternative ORF.

The DNA sequence of FL-aa-7 was perfectly matched to the recently cloned hGIMAP7 gene encoding a novel GTPase family member (GIMAP family member 7, NM_153236), which has been shown to be expressed in various tissues and localized in the ER and Golgi. Diverse cancer tissue and cell lines, including leukemias and lymphomas, expressed the gene at albeit very low levels (Krucken et al., 2004). In our case, we did not detect FL-aa-7 mRNA in any of the tumor samples. The translated fragment of FL-aa-7 corresponded to an alternative reading frame of hGIMAP7 of the locus such as TCR γ alternative reading frame protein (TARP) (Wolfgang et al., 2000). The fact that leukemia cells often contain deletions or translocation breakpoints within a region on chromosome 7q35–7q36.1 that also contains the hGIMAP gene cluster (Dohner et al., 1998), suggests that the FL-aa-7 peptide might be a product of chromosomal rearrangement with genes such as low density lipid receptor (LDLR) (Wang et al., 1999), although it is derived from an unknown gene, expressed specifically by follicular B cell lymphomas.

The sequence of the second peptide, FL-aa-18, matched to alternative frame of U1-snRNP 70K (NM_003089) (Theissen et al., 1986), but its deduced peptide sequence was in a different reading frame (Fig. 6A). Out of frame peptides are known to be cloned when antibody-displayed phage library were used by others (Mintz et al., 2003; Wang et al., 2005). The U1-snRNP 70K is a common target for autoantibodies in lupus and scleroderma-spectrum disorders, including mixed connective tissue disease (St Clair et al., 1990).

However, it is unclear whether the peptide is derived from the translated 5'-UTR of the genes, or from a yet to be discovered antigen. We are currently screening a human cDNA library to elucidate this question. Regardless, our data indicate that the deduced peptides sequences from the cloned T7 phages are indeed recognized by postvaccine sera only, and suggesting their potential immunotherapeutic role.

In summary, the data presented support our central hypothesis that patients who responded to Id vaccinations may generate immune responses to other unrelated to Id antigens by a mechanism of epitope spreading. Moreover, the differential use of the sera from the vaccinated patients, with the exclusion of non-specific binding autoantigen to the patient preimmune sera, enabled us to clone two peptides preferentially recognized by postvaccine sera. Overall, the approach we developed is simple and potent, and allows its use either in combination with SEREX, or alone as a phage displayed expression cDNA library.

Acknowledgment

We thank Alison Woo for editorial assistance. This work was supported by a grant from the Ladies Leukemia League.

References

- Bendandi M, Gocke CD, Kobrin CB, Benko FA, Sternas LA, Pennington R, Watson TM, Reynolds CW, Gause BL, Duffey PL, Jaffe ES, Creekmore SP, Longo DL, Kwak LW. Complete molecular remissions induced by patient-specific vaccination plus granulocyte-monocyte colony-stimulating factor against lymphoma. *Nat. Med* 1999;5:1171. [PubMed: 10502821]
- Brandle D, Brasseur F, Weynants P, Boon T, Van den Eynde B. A mutated HLA-A2 molecule recognized by autologous cytotoxic T lymphocytes on a human renal cell carcinoma. *J. Exp. Med* 1996;183:2501. [PubMed: 8676070]
- Brichard V, Van Pel A, Wolfel T, Wolfel C, De Plaen E, Lethe B, Coulie P, Boon T. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med* 1993;178:489. [PubMed: 8340755]
- Butterfield LH, Ribas A, Disette VB, Amarnani SN, Vu HT, Oseguera D, Wang HJ, Elashoff RM, McBride WH, Mukherji B, Cochran AJ, Glaspy JA, Economou JS. Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. *Clin. Cancer Res* 2003;9:998. [PubMed: 12631598]
- Chen YT, Scanlan MJ, Sahin U, Tureci O, Gure AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc. Natl. Acad. Sci. U. S. A* 1997;94:1914. [PubMed: 9050879]
- Disis ML, Goodell V, Schiffman K, Knutson KL. Humoral epitope-spreading following immunization with a her-2/neu peptide based vaccine in cancer patients. *J. Clin. Immunol* 2004;24:571. [PubMed: 15359116]
- Dohner K, Brown J, Hehmann U, Hetzel C, Stewart J, Lowther G, Scholl C, Frohling S, Cuneo A, Tsui LC, Lichter P, Scherer SW, Dohner H. Molecular cytogenetic characterization of a critical region in bands 7q35-q36 commonly deleted in malignant myeloid disorders. *Blood* 1998;92:4031. [PubMed: 9834205]
- Huang S, Preuss KD, Xie X, Regitz E, Pfreundschuh M. Analysis of the antibody repertoire of lymphoma patients. *Cancer Immunol. Immunother* 2002;51:655. [PubMed: 12439611]
- Jager E, Chen YT, Drijfhout JW, Karbach J, Ringhoffer M, Jager D, Arand M, Wada H, Noguchi Y, Stockert E, Old LJ, Knuth A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J. Exp. Med* 1998;187:265. [PubMed: 9432985]
- Kao H, Marto JA, Hoffmann TK, Shabanowitz J, Finkelstein SD, Whiteside TL, Hunt DF, Finn OJ. Identification of cyclin B1 as a shared human epithelial tumor-associated antigen recognized by T cells. *J. Exp. Med* 2001;194:1313. [PubMed: 11696596]
- Krucken J, Schroetel RM, Muller IU, Saidani N, Marinovski P, Benten WP, Stamm O, Wunderlich F. Comparative analysis of the human GIMAP gene cluster encoding a novel GTPase family. *Gene* 2004;341:291. [PubMed: 15474311]
- Mandrizzato S, Brasseur F, Andry G, Boon T, van der Bruggen P. A CASP-8 mutation recognized by cytolytic T lymphocytes on a human head and neck carcinoma. *J. Exp. Med* 1997;186:785. [PubMed: 9271594]
- Mintz PJ, Kim J, Do KA, Wang X, Zinner RG, Cristofanilli M, Arap MA, Hong WK, Troncso P, Logothetis CJ, Pasqualini R, Arap W. Fingerprinting the circulating repertoire of antibodies from cancer patients. *Nat Biotechnol* 2003;21:57. [PubMed: 12496764]
- Prezzi C, Nuzzo M, Meola A, Delmastro P, Galfre G, Cortese R, Nicosia A, Monaci P. Selection of antigenic and immunogenic mimics of hepatitis C virus using sera from patients. *J. Immunol* 1996;156:4504. [PubMed: 8666827]
- Rosenberg SA. Progress in the development of immunotherapy for the treatment of patients with cancer. *J. Intern. Med* 2001;250:462. [PubMed: 11902815]
- Sahin U, Tureci O, Schmitt H, Cochlovius B, Johannes T, Schmits R, Stenner F, Luo G, Schobert I, Pfreundschuh M. Human neoplasms elicit multiple specific immune responses in the autologous host. *Proc. Natl. Acad. Sci. U. S. A* 1995;92:11810. [PubMed: 8524854]

- St Clair EW, Query CC, Bentley R, Keene JD, Polissson RP, Allen NB, Caldwell DS, Rice JR, Cox C, Pisetsky DS. Expression of autoantibodies to recombinant (U1) RNP-associated 70K antigen in systemic lupus erythematosus. *Clin Immunol. Immunopathol* 1990;54:266. [PubMed: 2104788]
- Theissen H, Etzerodt M, Reuter R, Schneider C, Lottspeich F, Argos P, Luhrmann R, Philipson L. Cloning of the human cDNA for the U1 RNA-associated 70K protein. *Embo J* 1986;5:3209. [PubMed: 3028775]
- Tolpin MD, Starr SE, Arbeter AM, Plotkin SA. Inactivated mouse cytomegalovirus vaccine: preparation, immunogenicity, and protective effect. *J. Infect. Dis* 1980;142:569. [PubMed: 6255043]
- Tsui P, Tornetta MA, Ames RS, Silverman C, Porter T, Weston C, Griego S, Sweet RW. Progressive epitope-blocked panning of a phage library for isolation of human RSV antibodies. *J. Immunol. Methods* 2002;263:123. [PubMed: 12009209]
- Wang RF. The role of MHC class II-restricted tumor antigens and CD4+ T cells in antitumor immunity. *Trends Immunol* 2001;22:269. [PubMed: 11323286]
- Wang RF, Wang X, Rosenberg SA. Identification of a novel major histocompatibility complex class II-restricted tumor antigen resulting from a chromosomal rearrangement recognized by CD4 (+) T cells. *J. Exp. Med* 1999;189:1659. [PubMed: 10330445]
- Wang X, Yu J, Sreekumar A, Varambally S, Shen R, Giacherio D, Mehra R, Montie JE, Pienta KJ, Sanda MG, Kantoff PW, Rubin MA, Wei JT, Ghosh D, Chinnaiyan AM. Auto-antibody signatures in prostate cancer. *N. Engl. J. Med* 2005;353:1224. [PubMed: 16177248]
- Wolfgang CD, Essand M, Vincent JJ, Lee B, Pastan I. TARP: a nuclear protein expressed in prostate and breast cancer cells derived from an alternate reading frame of the T cell receptor gamma chain locus. *Proc. Natl. Acad. Sci. U. S. A* 2000;97:9437. [PubMed: 10931945]
- Xie X, Wacker HH, Huang S, Regitz E, Preuss KD, Romeike B, Parwaresch R, Tiemann M, Pfreundschuh M. Differential expression of cancer testis genes in histological subtypes of non-Hodgkin's lymphomas. *Clin. Cancer Res* 2003;9:167. [PubMed: 12538465]

Abbreviations

TAA, tumor-associated antigens (TAA); SEREX, serological identification of recombinantly expressed genes; Id, idiotype; PBMC, peripheral blood mononuclear cells (PBMC); CT, cancer/testis; PBS, Phosphate buffered saline; HPLC, High performance liquid chromatography; CDR3, Complementarity determining region 3..

Fig. 1.

Schema of biopanning and depletion steps. A cDNA library was constructed from fresh tumor, cloned into T7 phage vector, and expressed recombinant protein on the surface of phage. Specific clones were selected by repeated binding, elution, and infection (panning) with patient serum IgG coated on 96-well plates. For the depletion step, the selected phage library on postvaccine sera or IgG was passed through columns coated with IgG from healthy donor or prevaccine sera. Individual phage plaques were tested by DNA sequence analysis.

Fig. 2.

The selected clones can be enriched after dilution with wild-type phage. The experiments were performed using a mixture of FL-aa-1 clone and control phage (vector phage) at a ratio of 1:300. The phage mixture was incubated with postvaccine IgG. Numbers of pannings and percentage (%) of PCR positive FL-AA-1 (open circle) or wild-type phage (closed circle) are shown (A). After each round of selection randomly picked plaques were monitored by PCR analysis (B). Numbers represent number of before (#0), after the first (#1) and second (#2) pannings. The FL-aa-1 phage was enriched approximately 70% after one round of selection, and almost all of the phage contained the FL-aa-1 gene after two rounds of selection. The reactivity of clones FL-aa-1 (C), FL-aa-2 (D) and FL-aa-3 (C) and wild-type phage (D) were tested in 96-well plates coated with IgG from prevaccine (Pre IgG) and postvaccine (Post IgG). The IgG was from sera of patients #1 and 35, or from healthy donors (Nor). Control binding was performed in 5% dry milk in PBS (Milk).

Fig. 3.

FL-aa-4 clone phage is a strongly immunogenic noncancer-related autoantigen. (A) Selection of FL-aa-4 phage after dilution with a 1000-fold excess of vector phage. The phage mixture was incubated with post-Id vaccine serum IgG. Number of panning rounds is shown, as first, second, third, and fourth. Percentage (%) of selected FL-aa-4 phage (open circle) or wild-type phage (closed circle) tested by PCR are shown. (B) Binding titers (pfu/mL) to prevaccine, postvaccine IgG or normal donor serum IgG for two clones, FL-aa-4-1 and FL-aa-4-2, are shown. Sequences of 4 selected clones were aligned and the common fragments are in boldface letters, which represent various pieces of the carboxyl terminus of human hypothetical protein MGC9515 (BC036263). (C) Comparison of the inserts suggests that patient contained antibodies to multiple repeats “KTPPLATQEAEEKPRKPKRQRAAEMEPPPE” or “PKRRRVGDVEPSRKPKRRRAADVEPSLPE” and a unique region sequence “PKRRRLS”.

Fig. 4.

FL-aa-7 and 18 phages specific to postvaccine patient serum. (A) Reactivity of individual phage clones or wild-type phage (V) to prevaccine (open bar) and postvaccine (filled bar) serum diluted 1:100 was tested. Equal amounts of phage clones (1×10^8 pfu/50 μ L)-sera (1:100) complexes were incubated in 96-well plates coated with rabbit anti-human IgG (10 mg/ μ L). Binding of phage clones FL-aa-18 (B) or FL-aa-7 (C) was performed in wells coated with prevaccine and postvaccine sera from different patients (#1, 7, 31, 35, 37 and 38, or mixture of several patient sera (Mix), or normal donor sera (Nor). Titration of the binding of phage clones FL-aa-18 (D) and FL-aa-7 (E) was tested using wells coated with patient #7 (D) and #38 (E) sera. The binding of wild-type phage (vector) is also shown. (Pre), prevaccine sera; (Post), postvaccine patient sera, (Nor), normal donor sera. Data are representative of three independent experiments.

Fig. 5. Nucleotide and amino acid sequence for inserted fragments of phage clones FL-aa-7 and FL-aa-7 peptide specificity. The FL-aa-7 antigenic peptide derived from the 5'-untranslated region of the hAIN7 gene (A). The peptides were deduced from the in-frame reading of the fused capsid protein of T7 phage. Amino acid sequences of the capsid protein gene are in lower case letters. Antigenic peptide and coding regions are highlighted. Postvaccine sera (Post) specifically bind specifically synthetic peptides FL-aa-7 (B). No binding is detected with prevaccine sera (Pre). Control peptides, influenza peptide (Flu) and FL patient-derived CDR3 peptide (CDR3) failed to bind the sera. Furthermore, FL-aa-7 peptides specifically inhibited the binding of parental phage clones FL-aa-7 (C) to postvaccine sera (closed bar). In contrast, control Flu (D) and CDR3 (E) peptides failed to inhibit binding of FL-aa-7 phage. The experiment was repeated at least 5 times. Amount of peptide (µg/mL) used in the inhibition assay is shown on abscissa.

Fig. 6.

Nucleotide and amino acid sequence for inserted fragments of phage clones FL-aa-18 and FL-aa-18 peptide specificity. The FL-aa-18 deduced peptide sequence derived from an alternative reading frame of u1-snRNP 70K gene (A). The peptides were deduced from the in-frame reading of the fused capsid protein of T7 phage. Amino acid sequences of the capsid protein gene are in lower case letters. Antigenic peptide and coding regions are highlighted.

Postvaccine sera (Post) specifically bind specifically synthetic peptides FL-aa-18 (B). No binding is detected with prevaccine sera (Pre). Control peptides, influenza peptide (Flu) and FL patient-derived CDR3 peptide (CDR3) failed to bind the sera. Furthermore, FL-aa-18 peptides specifically inhibited the binding of parental phage clones FL-aa-18 (C) to postvaccine sera (closed bar). In contrast, control Flu (D) and CDR3 (E) peptides failed to inhibit binding of FL-aa-18 phage. The experiment was repeated at least 5 times. Amount of peptide (μg/mL) used in the inhibition assay is shown on abscissa.

Fig. 7.

Expression of FL-aa-18 mRNA in FL tumors from five patients and B cells from two healthy donors. FL tumor cells were harvested from 5 patients for total RNA preparation. Gene expression was measured for FL-aa-18 by semi-quantitative RT-PCR. Gene expression data were normalized to β -actin expression as described in Materials and methods. RL: RL follicular lymphoma cell line. N1 and N2: normal B cells.

Fig. 8.

Reactivity of individual FL patient prevaccine and postvaccine sera ($n=38$) or sera from 10 normal donors were tested for binding with peptides FL-aa-7 and FL-aa-18. Serum samples from normal donors ($n=10$), and patients with FL ($n=38$) were analyzed by ELISA at a dilution of 1:100 in 96-well plates coated with FL-aa-7 (A) or FL-aa-18 peptide (B). The insert is representative data tested in triplicate. Postvaccine sera from the patient (black bar), prevaccine sera (open bar), and normal donor sera (grey bar) are shown.

Table 1
Identified clones and recognition antigens with pre, postvaccine serum

Clone name	GenBank no.	Identity	Antibody responses		Correct reading frame
			Pre	Post	
FL-aa-1	AF201950	<i>Homo sapiens</i> kiaa-iso protein mRNA	+	+	Y
FL-aa-2	AK024436	FLJ00026 protein mRNA	+	+	Y
FL-aa-3	BC008850	Ribosomal protein L7	+	+	Y
FL-aa-4	BC036263	Unknown (protein for MGC:9515) cDNA	+++	+++	Y
FL-aa-5	BF035815	#	+	++	N
FL-aa-6	Not found		+++	+++	N
FL-aa-7	NM_153236	<i>Homo sapiens</i> GTPase, IMAP family member 7 (<i>gimap7</i>)	-	++	N
FL-aa-8	Not found		+	++	
FL-aa-9	Not found		+++	+++	
FL-aa-11	A1284640	#	+++	++	N
FL-aa-15	Not found		+	+	
FL-aa-17	Not found		++	+	
FL-aa-18	NM_003089	<i>Homo sapiens</i> small nuclear ribonucleoprotein 70 kDa polypeptide (RNP antigen) (SNRP70)	-	+++	N
FL-aa-19	Not found		++	++	

No homologous sequences found. Similarity in the overlapping sequences was always above 98%.
-, negative: <10 pfu/μL; +, weakly positive: <50 pfu/μL; ++, positive: <150 pfu/μL; +++, strongly positive: >200 pfu/μL phage eluted titer.

Table 2

The amino acid sequences of selected clones

Clone name	The amino acid sequences of selected clones
FL-aa-1	PEFLGMSVAFVPDWLRGKAEVNQETIQRLLLEENDQLIRCIVEYQNKGRGNECVQYQHVLHRNLIYLATIADASPTSTSKAME [*]
FL-aa-2	FIMRCGEAVEKNKRLITADQREYQQLKKNYNKLENLKRMIERKIPELYKPIFRR [*]
FL-aa-3	EGVEEKKKEVPAVPETLKKKRRNFAELKIKRLRKKFAQKMLRKARRKL
FL-aa-4	ARGKTPAERLRGPLPPSADDNLKTPSERQLTPLPPSAPPSADDNIKTPAERLRGPLPPSADDNLKTPPLATQEAEAEKPRKPKRQRAAEMEPPPEPKRQV
FL-aa-5	IKKRNDIKG [*]
FL-aa-6	PQWLMPVVPALWEAETGGS [*]
FL-aa-7	QKQQLKQPPWRKPENSTCSREGLVRA9 [*]
FL-aa-8	SRRRGSPARVGGGRKAPPPRPVGGSR [*]
FL-aa-9	ERAMGRRIAMW [*]
FL-aa-11	TPLIPALWEAEMGGSRGQEIETILANTVKPHFH [*]
FL-aa-15	WKQRDNMKEITQDTWKK [*]
FL-aa-17	FNQKRKHPTWKL [*]
FL-aa-18	GQRQPRHVHGV [*]
FL-aa-19	RSKRASGGQARWLRPVPALSEAEVGGSRGQEMETILANTVKN [*]

* Stop codon.