

Cloning and expression in *E. coli* of a functional Fab fragment obtained from single human lymphocyte against *anthrax toxin*

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Abstract

Human lymphocytes derived from the blood of a donor immunized with anthrax vaccine were isolated and enriched for B-cells by Nycoprep density centrifugation. Individual anti-anthrax protective antigen (PA) B-cells were isolated by fluorescence activated cell sorting with fluorescence-labeled recombinant PA (rPA). The RNA from sorted single B-cells was extracted using plant total RNA as the carrier prior to purification by Nanoprep RNA isolation columns and then cDNA was prepared. Donor specific human Fab primer sets were developed based on rapid amplification of 5'-complementary DNA end results. Heavy chain and light chain of human Fab were amplified from the donor single B-cells by PCR. The amplified heavy and light chain were then cloned into the expression vector pASK-IBA2 and expressed in *Escherichia coli* (*E. coli*). The chains combined *in vivo* to form a functional Fab which was then purified as one protein. The human Fab antibodies produced by this technique were functional when tested in Western blots where the rPA was the target as well as in ELISA. This approach allowed us to obtain human Fab that retained the natural heavy and light chain pairing, which is supposed to have a high antigen-binding affinity.

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1. Introduction

Anthrax is an infectious disease caused by a spore forming bacterium, *Bacillus anthracis*. The *anthrax toxin* is formed by a complicated process, where the protective antigen (PA) of the toxin combines with the edema factor (EF) to produce edema toxin (ET). The ET, in turn, combines with the lethal factor (LF) to produce the lethal toxin (LT). The PA provides the cell binding function of the *anthrax toxin* (Kimple et al., 1992). Neutralizing the PA activity is best achieved by producing antibodies to the recombinant protective antigen (rPA). The antibodies, which are a component of the immune system, play an important role in the protection of the human body against toxic agents and pathogenic organisms. Passive immunization of individuals has shown to increase chances of survival when individuals

encounter biological toxins and/or pathogenic organisms in a hostile confrontation (Greenfield and Bronze, 2004; Giannasca and Warny, 2004). The use of truly human antibodies for passive immunization is the ideal option (Giannasca and Warny, 2004; Subbarao et al., 2004; Vollmers and Brandlein, 2002). Current strategies to obtain human monoclonal hybridoma cells involve the use of human immunocytes in severe combined immunodeficient (SCID) mice or transgenic mice with human immunoglobulin IgG gene loci used by Medarex Inc., GenPharm International (Jakobovits, 1995; Longberg and Huszar, 1995), but these technologies remain expensive and are not readily accessible.

An alternative approach is the cloning of human monoclonal antibodies obtained from the messenger RNA (mRNA) of the activated B-lymphocyte population found in peripheral blood of volunteers. Obtaining blood from recently vaccinated human volunteers may increase the yield of reactive B lymphocytes in terms of both number of cells available and the diversity of novel antibody sequences. In this approach, large complex combinatorial IgG light chain (Lc) and heavy chain (Hc) libraries

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were generated without the use of “*in vitro*” selection by biopanning using phage display technology (McCafferty et al., 1990; Vaughan et al., 1990; Orum et al., 1994; Crameri et al., 1996; Roguska et al., 1994; Vollmers and Brandlein, 2002). Cloning of genes from lymphocytes can also be achieved by other methods such as anchor PCR technique (Bertinetti et al., 2006), and by cell sorting (Meijert et al., 2006).

Individual lymphocytes displaying *de novo* selected antibody to fluorescence-labeled antigen were isolated by fluorescence activated cell sorting (FACS) and cloned as human Fab antibodies. Technical challenges of cloning Lc and Hc gene fragments from a single-cell source were overcome with this new approach that may become the premiere source of human antibodies for passive immunization and medical therapy.

2. Materials and methods

2.1. Isolation of human lymphocytes

Human lymphocytes were isolated from volunteers who were previously vaccinated with anthrax vaccine adsorbed, BiothraxTM (Emergent BioSolutions, Gaithersburg, Maryland USA). Blood (45 ml) was collected in Vacutainers (Becton-Dickinson) containing sodium heparin. Plasma was collected by centrifugation. Cells were resuspended in two blood volumes of Dulbecco Modified Eagle Medium (DMEM) and layered over Nycoprep 1.077 density gradient centrifugation media (Nycomed). Tubes were centrifuged at $1000 \times g$ for 15 min with no brake. Buffy coats at the medium interface, containing mononuclear cells, were collected and washed once in DMEM. The rPA was labeled with fluorescein-5-isothiocyanate (FITC) according to the manufacturer recommendation. Briefly, *B. anthracis* rPA was prepared at 0.1–0.4 mg/ml in Phosphate buffered saline (PBS) with dye to protein ratio of 0.5:1 (w/v). FITC-rPA was desalted into DMEM medium by Sephadex G-50 spin column chromatography. Cells were re-suspended in FITC-rPA in DMEM for 30 min at room temperature, followed by washing once in DMEM. The cells were centrifuged and re-suspended in DMEM medium to 1×10^6 /ml. FITC-rPA in PBS (dye:protein 0.7–1.28) was added to a final concentration of 1:10 to 1:50 (v/v) and incubated for 30–40 min at room temperature. Cells were washed three times in DMEM and re-suspended in 200 μ l PBS. To validate the presence of fluorescent cells, 10 μ l aliquots were spotted onto pre-cleaned glass microscope slides and wet-mounted using a cover slip. Slides were examined by visible and fluorescent microscopy at $100\times$ and $400\times$ magnification using a Nikon 100 eclipse fluorescent microscope equipped with FITC excitation and emission filters. Cells were sorted into 96 well plates and mRNA was extracted from each individual cell. Batch sorting was also done to isolate 5000 cells for rapid amplification of 5' complementary DNA ends (5' RACE).

2.2. 5' RACE

Total RNA was isolated from human B cells from two donors by using the TRIzol reagent (Invitrogen, Burlington,

ON) according to the procedures recommended by the manufacturer. Using a GeneRacer kit (Invitrogen), 1 μ g of RNA from B cells was dephosphorylated with calf intestinal phosphatase and then decapped with tobacco acid pyrophosphatase. GeneRacer RNA oligo was ligated to the 5' end of decapped mRNA and reverse-transcribed with GeneRacer Oligo dT primer by reverse transcriptase. Antibody IgG heavy chain cDNA library (IgG-cDNA) and kappa light chain (κ Lc) cDNA library (kappa-cDNA) were constructed. Amplification of 5'-end of IgG heavy chain or kappa light chain genes was conducted using touch-down PCR with GeneRacer 5' primer and IgG heavy chain constant region gene reverse specific primer (5'-ACC TTG GTG TTG CTG GGC TTG TGA T-3') or kappa light chain constant region gene reverse specific primer (5'-GGG CGT TAT CCA CCT TCG ACT GTA-3'). The PCR products were then cloned into PCR 2.1 TA TOPO vector (Invitrogen), and transformed into TOP10 chemically competent *Escherichia coli* (*E. coli*) cells (Invitrogen). About thirty IgG heavy chain clones and 30 kappa light chain clones from each donor were sequenced using the plasmid-specific M13 primers. The sequencing reactions were performed using CEQTM DTCS Quick Start Kit (Beckman, Fullerton, CA). The reaction products were purified by Centri-SepTM columns (Princeton Separations, Adelphia, NJ), and then run on an on a CEQTM 8000 Genetic Analysis System (Beckman). Sequences were assembled and analyzed using Lasergene DNA software (DNASTAR Inc., Madison, WI). The multiple sequence alignment from different clones was carried out using the MegAlign program of the Lasergene Software package (DNASTAR Inc.) to produce the primer sets used in the PCR cloning of the Fab fragment.

2.3. Cloning and expression

Five micrograms of carrier plant RNA was used as carrier and was added to each well containing a single lymphocyte. The RNA was extracted from the single lymphocytes using Absolutely RNA Nanoprep Kit (Stratagene, CA) according to the manufacturer's instructions, following the addition of 5 μ g of total RNA extracted from plant cells that were used as a carrier. Reverse transcription (RT) was done on 10 μ l of each RNA extract placed in a 0.25 ml tube containing 2 μ l of 0.5 μ g/ μ l Oligo dT primer. The mixture was heated at 70 °C for 10 min, after which the tubes were cooled on ice for 2 min. This was followed by the addition of 10 μ l of 5 $\times$ First Strand Buffer, 2 μ l of 10 mM dNTP mixture, 5 μ l of 0.1 M dithiothreitol, 19 μ l of water, 2 μ l RNase out (Invitrogen) and 1 μ l (200 U) of superscript III reverse transcriptase (Invitrogen). The tubes were incubated for 1 h at 42 °C then placed on ice. RNA complementary to the cDNA was digested by the addition of 0.5 μ l RNase H (1 unit/ μ l) and incubated at 37 °C for 20 min. PCR reactions were performed in a volume of 50 μ l, containing 1 $\times$ easy-A buffer (Stratagene), 5 μ l cDNA (1–2 ng), 5 μ M primer mix, 0.2 mM dNTPs and 2.5 U of Easy A *Taq* polymerase (Stratagene) using the Mastercycler (Eppendorf). The cDNA was amplified using the following program: 95 °C for 2 min then 35 cycles consisting of 95 °C for 40 s, 58 °C for 30 s, and 72 °C for 70 s with one cycle of 72 °C for 10 min (Saiki et al., 1988).

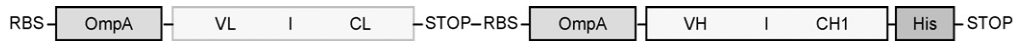


Fig. 1. Schematic diagram showing the construction of the human Fab fragment in a single vector. The ribosomal binding site (RBS) upstream from the secretion signal (OmpA), followed by the variable region and the constant region of the light chain (VL and VC), and a stop codon. The construction of the heavy chain cassette was the same as the light chain with the exception of the addition of the His tag at the C terminal.

The amplicons were then cloned into TOPO-TA pCR2.1 and sequenced.

Based on the sequence data obtained from the 5' RACE, light chains were amplified by PCR using the following forward (LCED1) primer: 5'-**GAA TTC** TCA ATG ATG ATG ATG ATG ATG GCA TTC TAG ACT ATT ATG AAC ATT CTG TAG GGG C-3' containing a 6-Histidine tag (His tag), a stop codon and an *EcoR*-1 restriction site (in bold), and a reverse primer (HPSR1): 5'-**CTG CAG** CTG CAC AGG GTC CTG GGC CGA GCT CGT GGT GAC TCA-3', containing a *Pst*-1 restriction site (in bold). The amplified products were cloned into the pASK-IBA2 plasmid. Similarly, the Fab segment of the heavy chain was amplified by PCR using the following forward and reverse primers: HCED-1 primer: 5'-**GAA TTC** 5'-AGC ATC ACT AGT ACA AGA TTT GGG CTC-3' containing an *EcoR*-1 restriction site (in bold), and HCHR – 5'-**AAG CTT** TCA ATG ATG ATG ATG ATG ATG AGG TVC AAC TGC TCG AGT CKG G-3' containing a His tag, a stop codon and a *Hind*III restriction site (in bold).

To construct the bicistronic expression plasmid, PCR was performed on the heavy chain to add an ompA secretion signal and a ribosomal binding site using the following forward primer: 5'-**CTG CAG** GAG GGC AAA AAA TG-3', containing an *EcoR*I restriction site, and the reverse primer: 5'-**AAG CTT** TCA ATG ATG ATG ATG ATG ACA AGA TTT GGG CTC AAC TC-3' containing a His tag, a stop codon, and a *Hind* III restriction site (Fig. 1). This was then ligated into the pASK-IBA2 vector, which was used previously by Skerra (1994). The plasmid construct (pAAhuFab) was then used to transform *E. coli* cells (BL21 DE3) for expression. The expressed protein was detected by Western blot analysis using anti-human Fab antibody.

2.4. Electrophoresis and Western blots

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on 12% polyacrylamide (29.2:0.8 acrylamide-bisacrylamide) vertical gels with 3% stacking gel using the Miniprotein II system (Bio-Rad, Richmond, CA), and the Laemmli method (1970). The recombinant protein in the induced cell pellet was released from the periplasmic space by adding 1 ml per 100 ml culture volume of 100 mM Tris-HCl, pH 8.0, 500 mM sucrose, 1 mM EDTA and incubating on ice for 30 min. Spheroplasts were removed by centrifugation (13,000 × *g* for 5 min at 4 °C) and the resulting supernatant was used for Ni-NTA affinity chromatography. Sample preparation consisted of mixing three parts sample in one part of loading buffer (0.06 M Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.025% bromophenol blue). The mixture was heated for 5 min at 95 °C and a 10 µl sample loaded onto the gel. Pre-stained molecular weight marker was used (pre-stained low range molecular weight marker, Bio-Rad,

catalogue number 161–0305). Electrophoresis was performed for 70 min at 140 V. The resolved proteins were blotted onto PVDF membrane (Bio-Rad). The membranes were blocked for 1 h in TBS (0.05% Tween 20) containing 5% nonfat milk. The blots were developed using AP conjugate substrate kit (Bio-Rad)

2.5. Enzyme-linked immunosorbent assay (ELISA)

The reactivity of human Fab to rPA was examined by an ELISA. Nunc maxisorp™ flat bottomed 96 well plates (Canadian Life Technologies, Burlington, ON) were coated overnight at 4 °C with rPA at a fixed concentration of 10 µg/ml in carbonate bicarbonate buffer, pH 9.6, containing 0.02% sodium azide. The plates were washed five times with phosphate buffered saline containing 0.01 Tween-20 (PBST) and then blocked in 2% bovine serum albumin for 2 h. After five washes with PBST, plates were incubated for 1.5 h with various concentrations of 0.01–100 µg/ml. Following five washes with PBST, plates were incubated for 1.5 h with HRP-donkey anti-human IgG antibodies diluted 1:3000 in PBST. Finally, the plates were washed five times with PBST and developed for 30 min with a substrate consisting of 2,2'-azino-di-(3-ethyl-benzthiazoline-sulfonic acid) diammonium salt (ABTS) and hydrogen peroxide (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The reactions were read at an absorbance of 405 nm by a microplate auto reader (Molecular Devices, Sunnyvale, CA).

3. Results

5' RACE was applied to analyze 5'-end sequences of human antibody IgG heavy and kappa light chains from the two donors in order to design forward primers to amplify Fab genes from human single B cells. Approximate 30 clones of each chain and from each donor were randomly picked up for DNA sequencing. The DNA fragments of 5' RACE of IgG heavy chain and kappa light chain transcripts were in the length of 300–400 bp. Sequence analysis showed that there were three kinds of dominant IgG heavy chains and four kinds of dominant kappa light chains. Base on the sequence results, three IgG heavy chain (HC) forward primers and three kappa light chain (κ Lc) forward primers were designed (Table 1).

FACS analysis consistently showed a population of 1–3% of the total B cells that were reactive against FITC-rPA. Cells exhibiting the strongest fluorescent signal were sorted into PCR plates for single-cell RT-PCR and for batch sorting. Fifteen cells were selected for RT-PCR. The products were analyzed by agarose gel electrophoresis. Nine cells were found to contain a PCR product of molecular weight consistent with the projected target size of Lc. Five of these cells were found to contain the complementary Hc sequence. Two cells were found to contain the Hc only. We were not able to produce any product from

Table 1
The results obtained from 5' RACE showing the different dominant sequences of variable regions of the light and heavy chains

Kappa light chain variable region	
DRESKL1:	5'-ATGGACATGAGGGTCCC(C/T)GCTCAG-3'
DRESKL2:	5'-ATGAGGCTCCCTGCTCAGCTCCTG-3'
DRESKL3:	5'-ATGGAAACCCAGCGCAGCTTCTC-3'
DRESKL4:	5'-ATGGTGTTGCAGACCCAGGTCTTC-3'
Heavy chain variable region	
VHA	5'-AGG TGC AGC TGC TCG AGT CTG G-3'
VHB	5'-AGG TGC AGC TGC TCG AGT CGG G-3'
VHD	5'-AGG TGC AAC TGC TCG AGT CGG G-3'

four cells, which could be due to incompatibility between the primers and the IgG sequences, even though degenerate primers were used to amplify the genes.

Interestingly almost all the anti-anthrax Lc sequences obtained from one donor were γ Lc rather than κ Lc. This was unexpected as κ Lc sequences occur more frequently in the human population than γ Lc sequences (approximately 70% of all human Lc are κ chains). Random sequencing of total RNA from this donor showed expression of primarily γ chains as well.

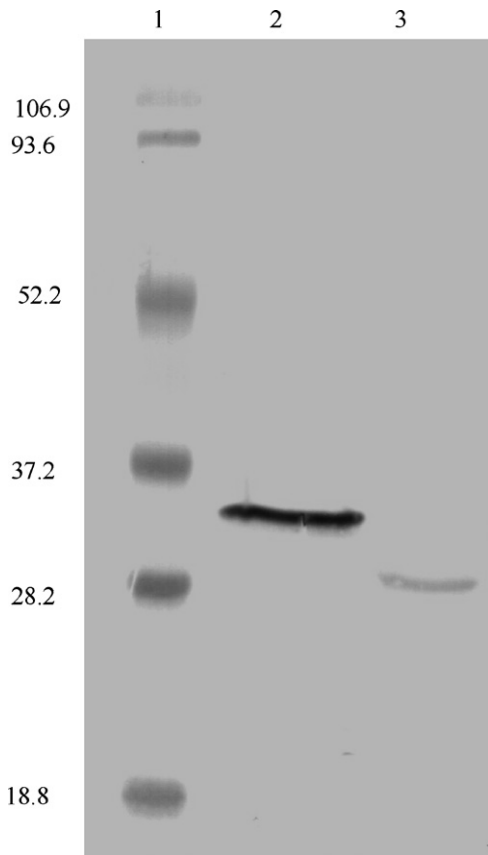


Fig. 2. Western blots analysis on the expressed and purified light and heavy chains. Lane 1: low mass pre-stained protein marker in kDa (Bio-Rad), lane 2: expressed light chain purified on the IMAC column and detected by goat-antihuman anti Fab monoclonal antibodies and lane 3: expressed heavy chain purified on the IMAC column and detected by goat anti-human Fab monoclonal antibody.

The light and the heavy chains of the anti-anthrax human Fab (pAAhuFab) antibody were expressed separately in *E. coli* as soluble proteins and purified by affinity chromatography on immobilized metal affinity resin (IMAC). The solubilized protein fractions collected from the IMAC columns, were purified and analyzed by SDS-PAGE (12%) and stained with Coomassie brilliant blue. A protein product was detected at the approximate expected size of 28 kDa (data not shown). The expression was confirmed by Western blot analysis using anti human Fab antibodies. The results showed a protein band of about 28 kDa, which is consistent with the projected protein size (Fig. 2).

To determine the antigen binding activity of the expressed antibody, the purified proteins were tested on Western blots; the separate chains produced no activities against rPA protein.

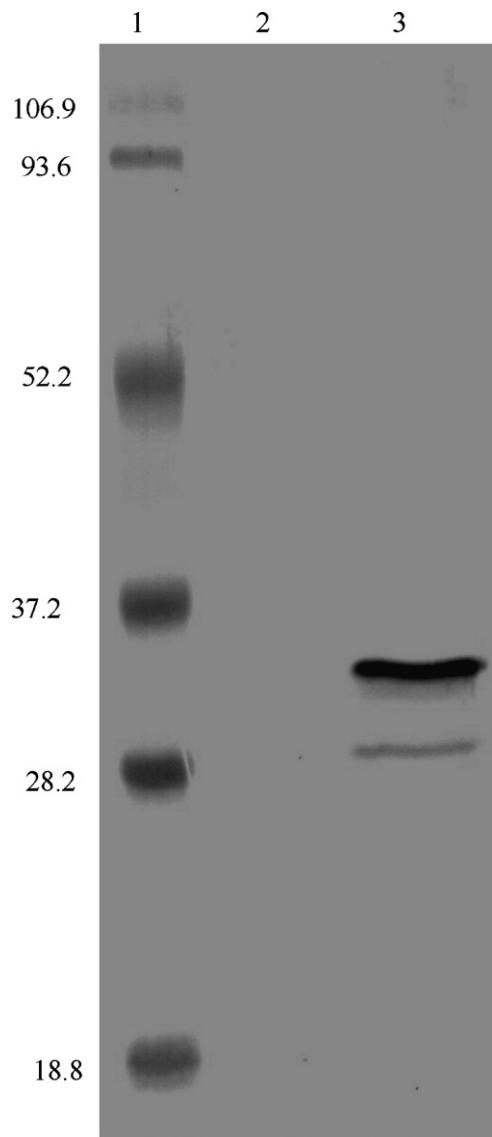


Fig. 3. Western blots analysis on the expressed and Fab fraction. Lane 1: low mass pre-stained protein marker in kDa (Bio-Rad), lane 2: protein lysate obtained from *E. coli* as a negative control and lane 3: expressed Fab fragment obtained from the total soluble protein expressed in *Escherichia coli*, and detected by goat anti-human Fab monoclonal antibodies.

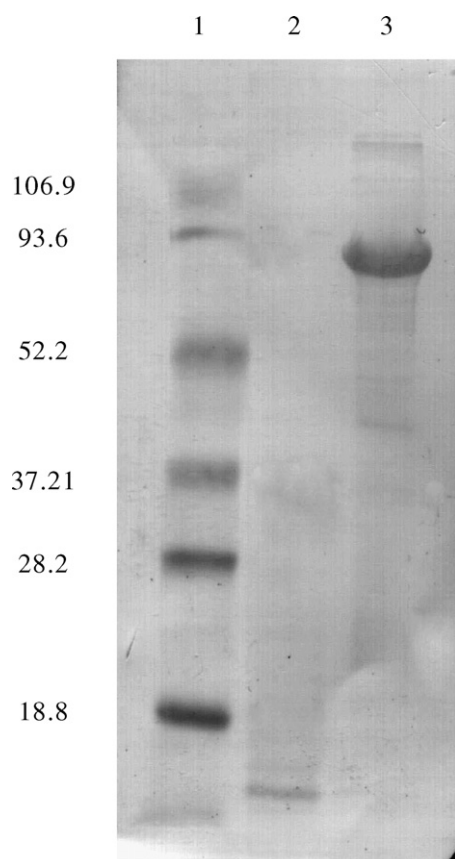


Fig. 4. Western blot detection of the rPA by expressed human Fab. The activity of the expressed Fab was detected by goat anti-human Fab monoclonal antibody. Lane 1: molecular weight marker in kDa (Bio-Rad), lane 2: protein lysate obtained from *E. coli* as a negative control and lane 3: purified rPA protein.

When the periplasmic cell fraction of the expressed protein obtained from the *E. coli* strain BL21 DE3 harbouring the bicistronic plasmid pAAhuFab was applied to the IMAC column, a single protein fraction was eluted with an imidazole gradient. The eluted protein was analyzed on SDS PAGE. Two protein bands were detected with molecular weights of 28 and 30 kDa representing the heavy chain and the light chain, respectively (Fig. 3). The antigen-binding activity was determined by Western blots and in an ELISA test. The purified protein showed a very high affinity to rPA protein in both Western blots (Fig. 4) as well as ELISA (Fig. 5).

4. Discussion

Anthrax infection initially manifests itself due to the toxin-mediated immune dysfunction, which permits the anthrax bacteria to evade immune surveillance and thus disseminate throughout the body and produce high level of toxins. The high levels of toxins later in the infection result in massive organ failure, followed finally by death. Since PA plays a critical role in virulence, it has become the main target for development of therapeutic antibodies. Human antibodies have been suggested as the optimal molecules for passive immunotherapy. Cloning human antibody genes from human B-cells to create reactive antibodies

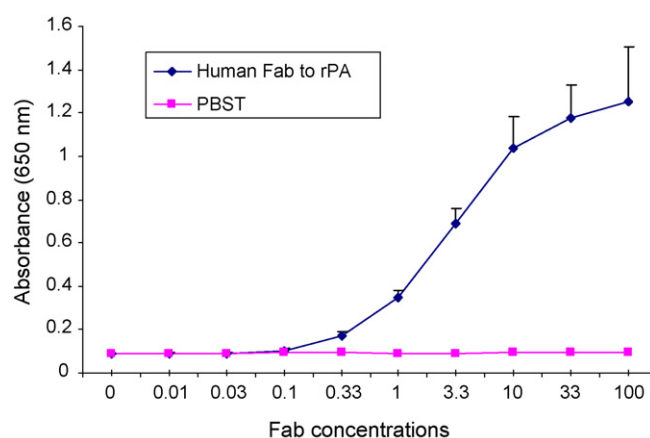


Fig. 5. Detection of rPA by ELISA test using expressed human Fab.

or Fab antibodies is one of common methods to develop therapeutic antibodies.

Circulating B cells from peripheral blood provide a ready source of starting material for cloning human antibodies. B cells comprise approximately 8% of the total mononucleated leukocyte fraction in peripheral blood. Within the B cell population, about 1–2% of the B cells are able to produce antibodies that are reactive against any particular antigen given sufficient stimulation. This number increases to 3–5% after the subject is vaccinated with a specific antigen, as the vaccination stimulates differentiation of antigen-specific B cells to antibody-producing plasma cells. However, even these antigen-specific B cells are a polyclonal population, with a wide range of affinities and specificities. Most existing approaches for antibody gene amplification do not provide nature paired heavy chain and light chain since amplified antibody genes are from a batch of B-cells, not one-single cell. The amplified antibody genes do not contain the cognate heavy and light chains. Therefore, they do not represent *in vivo* specificity and affinities. The primary difficulty encountered in cloning antibody genes from one-single cell is low mRNA template amount. We overcame this problem by introducing plant RNA as a carrier in purification of mRNA from single B-cells and successfully developed human anti-rPA Fab antibody from immunized donor. The antibody was efficiently expressed and purified in bacteria using a bicistronic expression plasmid. This antibody was demonstrated to bind to rPA in both ELISA and Western blots detection. This human anti-rPA Fab antibody deserves further evaluation as an immunotherapeutic agent.

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References

- Bertinetti, C., Simon, F., Zirlik, K., Heining-Mikesch, K., Pfeifer, D., Osterroth, F., Rosenthal, F.M., Veelken, H., 2006. Cloning of idiotype immunoglobulin genes in B cell lymphomas by anchored PCR and production of individual

- recombinant idiotype vaccines in *Escherichia coli*. Eur. J. Haematol. (Ahead of print).
- Crameri, A., Cwirla, S., Stemmer, W.P.C., 1996. Construction and evolution of antibody-phage libraries by DMA shuffling. Nat. Med. 2, 100–102.
- Giannasca, P.J., Warny, M., 2004. Active and passive immunization against *Clostridium difficile* diarrhoea and colitis. Vaccine 22, 848–856.
- Greenfield, R.A., Bronze, M.S., 2004. Current therapy and the development of therapeutic options for the treatment of diseases due to bacterial agents of potential biowarfare and bioterrorism. Curr. Opin. Investig. Drugs 5, 135–140.
- Jakobovits, A., 1995. Production of fully human antibodies by transgenic mice. Curr. Opin. Biotech. 6, 561–566.
- Kimple, K.R., Molloy, S.S., Thomas, G., Leppla, S.H., 1992. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc. Natl. Acad. Sci. U.S.A. 89, 10277–10281.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.
- Longberg, N., Huszar, D., 1995. Human antibodies from trans-genic mice. Int. Rev. Immunol. 13, 65–93.
- McCafferty, J., Griffiths, A.D., Winter, G., Chiswell, D.J., 1990. Phage antibodies: filamentous phage displaying antibody variable domains. Nature 348, 552–554.
- Meijert, P., Andersent, P.S., Hansen, M., Steinaa, L., Jensen, A., Lantto, J., Oleksiewicz, M., Kaja Tengbjerg, K., Poulsen, T., Coljee, V.W., Bregenholt, S., Haurum, J.S., Nielsen, L.S., 2006. Isolation of human antibody repertoires with preservation of the natural heavy and light chain pairing. J. Mol. Biol. 358, 764–772.
- Orum, H., Andersen, P.S., Oster, A., Johansen, K.L., Riise, E., Bjørnvad, M., Svendsen, I., Engberg, J., 1994. Efficient method for constructing comprehensive murine Fab antibody libraries displayed on phage. Nucl. Acids Res. 21, 4491–4498.
- Roguska, M.A., Pedersen, J.T., Keddy, C.A., Henry, A.H., Searle, S.J., Lambert, J.M., Goldmacher, V.S., Blattler, W.A., Rees, A.R., Guild, B.C., 1994. Humanization of murine monoclonal antibodies through variable domain resurfacing. Proc. Natl. Acad. Sci. U.S.A. 91, 969–973.
- Saiki, K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 219, 487–489.
- Skerra, A., 1994. A general vector, pASK84, for cloning, bacterial production, and single-step purification of antibody Fab fragments. Gene 141, 79–84.
- Subbarao, K., McAuliffe, J., Vogel, L., Fahle, G., Fischer, S., Tatti, K., Packard, M., Shieh, W.J., Zaki, S., Murphy, B., 2004. Prior infection and passive transfer of neutralizing antibody prevent replication of severe acute respiratory syndrome corona virus in the respiratory tract of mice. J. virol. 78, 3572–3577.
- Vaughan, T.J., Williams, A.I., Pritchard, K., Osbourn, J.K., Pope, A.R., Earnshaw, I.C., McCafferty, I., Hodits, R.A., Wilton, J., Johnson, K.S., 1990. Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. Nat. Biotechnol. 14, 309–314.
- Vollmers, H.P., Brandlein, S., 2002. Nature's best weapons to fight cancer: revival of human monoclonal IgM antibodies. Hum. Antibod. 11, 131–142.