

# Generation and selection of immunized Fab phage display library against human B cell lymphoma

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The approval of using monoclonal antibodies as a targeted therapy in the management of patients with B cell lymphoma has led to new treatment options for this group of patients. Production of monoclonal antibodies by the traditional hybridoma technology is costly, and the resulting murine antibodies often have the disadvantage of triggering human anti-mouse antibody (HAMA) response. Therefore recombinant Fab antibodies generated by the phage display technology can be a suitable alternative in managing B cell lymphoma. In this study, we extracted total RNA from spleen cells of BALB/c mice immunized with human B lymphoma cells, and used RT-PCR to amplify cDNAs coding for the  $\kappa$  light chains and Fd fragments of heavy chains. After appropriate restriction digests, these cDNA fragments were successively inserted into the phagemid vector pComb3H-SS to construct an immunized Fab phage display library. The diversity of the constructed library was approximately  $1.94 \times 10^7$ . Following five rounds of biopanning, soluble Fab antibodies were produced from positive clones identified by ELISA. From eight positive clones, FabC06, FabC21, FabC43 and FabC59 were selected for sequence analysis. At the level of amino acid sequences, the variable heavy domains ( $V_H$ ) and variable light domains ( $V_L$ ) were found to share 88-92% and 89-94% homology with sequences coded by the corresponding murine germline genes respectively. Furthermore, reactivity with membrane proteins of the B cell lymphoma was demonstrated by immunohistochemistry and western blotting. These immunized Fab antibodies may provide a valuable tool for further study of B cell lymphoma and could also contribute to the improvement of disease therapy.

**Keywords:** B cell lymphoma, Fab, phage display library, pComb3H-SS vector

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## Introduction

The effects of various available treatments against malignant lymphoma, including conventional chemotherapy, radiotherapy, bone marrow transplantation and peripheral blood stem cell transplantation, are usually not completely satisfactory. With the discovery of hybridoma technology, monoclonal antibody (mAb)-based targeting anticancer therapy has provided an alternative approach to treating this malignant disease [1]. However, early clinical trials using murine mAbs suffered from human anti-mouse antibody

response (HAMA), especially when multiple infusions were required to obtain therapeutic efficacy [2,3]. These host responses considerably altered the pharmacokinetic profile of the antibody, which led to rapid clearance of mAb and prevented repeated dosing [4]. Although many patients with B cell lymphoma have compromised immune systems, HAMA reactions are still problematic. Therefore, techniques to ameliorate the HAMA response by developing less immunogenic mAbs are required, including chimerization, humanization and development of human antibodies from phage display libraries [5-7]. Several examples of clinical antibody humanization have been described. The human anti-murine antibody response could be minimized by replacing murine sequences with homologous sequences derived from the human framework. Rituximab used in the therapy of B cell lymphoma is an

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example of a chimeric antibody and has produced some striking effects [8-10]. The construction and selection of phage antibody libraries represent an additional solution to the problem of HAMA response. In this approach, Fab fragments can be generated by a random combination of Fd chains (comprising the variable region ( $V_H$ ) and the first constant region ( $C_{H1}$ ) of the heavy chain) and full-length light chains (comprising the variable region ( $V_L$ ) and the constant region ( $C_L$ ) of the light chain). Compared with the conventional hybridoma technology, this technique is relatively simple, and a considerable amount of antibodies could be obtained through a procedure called biopanning. Furthermore, in this approach, only Fab fragments of the antibodies are produced. It was suggested that antibody fragments had potential advantages over whole antibodies for some therapeutic purposes, since they are smaller and can thus better penetrate organs and tissues [11,12]. Therefore combinatorial phage display technology is now considered a very useful tool for the generation of both diagnostic and therapeutic mAbs [13,14]. Here we describe the construction of Fab antibodies against B cell lymphoma by the phage display technology. In addition, we have utilized a simple whole-cell-based biopanning procedure to isolate a panel of specific B cell lymphoma Fab antibodies.

## Materials and Methods

### *Vector, E. coli and helper phage*

The vector pComb3H-SS [15,16] (kindly provided by the Scripps Research Institute, La Jolla, CA, USA), a modified version of the original pComb3H phagemid vector [17], about 4915 base pairs, contains ampicillin resistance (Amp) gene, start sites of plasmid COIE1 and F1 phage replication. The expression of inserting fragments is controlled by the same *LacZ* promoter on the upstream sequence of vector pComb3H-SS. *E. coli* XL1-Blue contains tetracycline resistance (Tet) gene on its gene type of Tn10. Helper phage (VCSM13) contains kanamycin resistance (Kana) gene with valency of  $10^{12}$  pfu/L. It is amplified in SOC culture medium and preserved at 4 °C.

### *Immunization of animals with B cell lymphoma strain*

Human B lymphoma cell strain Raji and Daudi were cultured in RPMI-1640 medium supplemented with 10% FCS and antibiotics.  $2 \times 10^7$ /ml cells were used for the immunization of four BALB/c mice (referred to as No.1, No.2, No.3 and No.4) by subcutaneous injection. The mice were given three boosts of the Raji cells and Daudi cells in a four week interval. Serum samples were titrated by indirect Enzyme-Linked Immunosorbent Assay (ELISA). Following the demonstration of sufficient antibody titer, the mice were given a final immunization with  $8 \times 10^7$ /mL cells. 3 days later, the mice were euthanized, exsanguinated, and the spleens were harvested.

### *RNA isolation and RT-PCR amplification of heavy chain Fd and $\kappa$ light chain*

Total RNA of the harvested mice spleen was extracted with Trizol

Reagent according to the manufacturer's procedure (Invitrogen, USA). The purity and concentration were determined by measuring the absorbance (*A*) at 260 nm and 280 nm ( $A_{260}/A_{280}$ ). To generate first-strand cDNA, an oligo(dT)18 was used as primer, and 30  $\mu$ g RNA was reverse-transcribed in the light of MMLV First Strand cDNA Synthesis Kit Protocols (Fermentas, USA). PCR amplification of heavy chain Fd and whole  $\kappa$  light chain genes were performed with Taq polymerase and primers specific to murine Fd and Kappa light chain described in Table 1. PCR productions of amplified  $V_H$ - $C_{H1}$  and  $V_L$ - $C_L$  from cDNA synthesis were gel purified according to QIAEXII Agarose Gel Extraction Protocols (Qiagen, USA), and then digested with excess restriction enzymes as detailed below.

### *Library construction*

The  $\kappa$  light chain products of PCR were digested with an excessive restriction enzymes *SacI* and *XbaI* (Biolabs, USA), and about 4.0  $\mu$ g was ligated overnight with 40  $\mu$ g of *SacI/XbaI*-linearized pComb3H-SS vector (purified by agarose gel extraction) in a total volume of 200  $\mu$ l with 60 units of T4 DNA ligase (Promega, USA) at 16 °C. Following ligation, the recombinant DNA was precipitated, washed and dissolved in 15  $\mu$ l of distilled water. 1  $\mu$ l of recombinant DNA was transformed by electroporation [18] into 40  $\mu$ l of *E. coli* XL1-Blue every time. After transformation, 1 ml SOC medium was added and the culture was shaken for 1 h at 37 °C, and then 10 ml of SB containing 20  $\mu$ g/ml ampicillin and 10  $\mu$ g/ml tetracycline was cultivated for an additional hour at 37 °C on a shaker. At this point, culture aliquots were plated on LB agar/ampicillin to titer library size, which was calculated by counting the number of ampicillin-resistant colonies. The insertion of target  $\kappa$  fragments was detected by digestion with *SacI* and *XbaI*. The culture was added to 100 ml of SB containing 50  $\mu$ g/ml ampicillin and 10  $\mu$ g/ml tetracycline and was cultivated overnight. Phagemids containing the  $\kappa$  light chains were prepared from this overnight culture and named pComb3H-S+ $\kappa$ . For cloning the heavy chain Fd fragments, the Fd products of PCR and Phagemids pComb3H-S+ $\kappa$  were digested with excessive restriction enzymes *XbaI* and *SpeI*. The ligation, transformation, *E. coli* amplification and determination of the library were performed as described above. The insertion of target Fd fragments was detected by digestion with *XhoI* and *SpeI* from the plasmids extracted from several random XL1-Blue monoclonal. The Fab fragments' insertion was detected by digestion with *SacI* and *SpeI*. The Phagemids with Fd and  $\kappa$  were named pComb3H+ $\kappa$ +Fd. Approximately VCSM13 ( $10^{12}$  pfu) helper phage as described by de Alboran *et al.* [19] was added to XL1-Blue samples containing Fab gene libraries and incubated for 3 h at 37 °C with shaking. 70  $\mu$ g/ml Kanamycin was added to and the culture was shaken overnight at 37 °C. The cells were centrifuged at 4000 rpm for 15 min at 4 °C. The supernatant was mixed with 5 ml 20% PEG 8000/2.5 M NaCl and incubated on ice for 30 min, and then phages were precipitated by centrifugation at 8000 rpm for 20 min at 4 °C. The supernatant was discarded and the pellet drained. The phages were resuspended in 1.5 ml PBS/1% BSA, vortexed and centrifuged at 13000 rpm for 5 min to pellet debris. The supernatant was stored at 4 °C or used directly for the next round of biopanning.

### *Biopanning of the primer Fab phage antibody library*

6-well plate (Nuncclon™, Denmark) was coated by  $5 \times 10^6$  cells with equal Raji and Daudi cells, and then blocked overnight with 2% BSA/PBS at 4 °C. 1 ml of phage library was added to each well

**Table 1** Primers for PCR amplification of murine Fd and Kappa light chain genes

Primers	Sequences
Heavy Chain Fd 3' primers	
IgG 1	5'-AGG CTT <u>ACT AGT</u> ACA ATC CCT GGG CAC AAT-3'
IgG 2a	5'-GTT CTG <u>ACT AGT</u> GGG CAC TCT GGG CTC-3'
IgG 3	5'-GGG GGT <u>ACT AGT</u> CTT GGG TAT TCT AGG CTC-3'
Heavy Chain Fd 5' primers	
Hc 1	5'-AGG TCC AGC TGC <u>TCG AGT</u> CTG G-3'
Hc 2	5'-AGG TCC AGC TGC <u>TCG AGT</u> CAG G-3'
Hc 3	5'-AGG TCC AGC TTC <u>TCG AGT</u> CTG G-3'
Hc 4	5'-AGG TCC AGC TTC <u>TCG AGT</u> CAG G-3'
Hc 5	5'-AGG TCC AAC TGC <u>TCG AGT</u> CTG G-3'
Hc 6	5'-AGG TCC AAC TGC <u>TCG AGT</u> CAG G-3'
Hc 7	5'-AGG TCC AAC TTC <u>TCG AGT</u> CTG G-3'
Hc 8	5'-AGG TCC AAC TTC <u>TCG AGT</u> CAG G-3'
Hc 9	5'-AGG T I I A I C T I C <u>TCG AGT</u> C(TA)G G-3'
κ light chain 3' primer	5'-GCG CCG <u>TCT AGA</u> ATT AAC ACT CAT TCC TGT TGA A-3'
κ light chain 5' primers	
Lc 1	5'-CCA GTT CCG <u>AGC TCG</u> TTG TGA CTC AGG AAT CT-3'
Lc 2	5'-CCA GTT CCG <u>AGC TCG</u> TGT TGA CGC AGC CGC CC-3'
Lc 3	5'-CCA GTT CCG <u>AGC TCG</u> TGC TCA CCC AGT CTC CA-3'
Lc 4	5'-CCA GTT CCG <u>AGC TCC</u> AGA TGA CCC AGT CTC CA-3'
Lc 5	5'-CCA GAT GTG <u>AGC TCG</u> TGA TGA CCC AGA CTC CA-3'
Lc 6	5'-CCA GAT GTG <u>AGC TCG</u> TCA TGA CCC AGT CTC CA-3'
Lc 7	5'-CCA GTT CCG <u>AGC TCG</u> TGA TGA CAC AGT CTC CA-3'

Footnote: Restriction enzymes recognition sites are underlined, with A|CTAGT for *SpeI*, C|TCGAG for *XhoI*, T|CTAGA for *XbaI* and GAGCTC for *SacI*.

and incubated at 37 °C for 2 h. Unbound phages were washed 1, 5, 10, 10, 10 times with TBS/0.5% Tween 20 (TBST) for rounds 1, 2, 3, 4 and 5 respectively. Bound phages were eluted by adding elution buffer (0.1 M HCL, adjusted to pH 2.2 with solid glycine and containing 0.1% BSA) and the eluted solution was neutralized with 2 M Tris buffer, pH 8.0. Amplification procedures with VCSM13 helper phage were followed as described above, while input and output phages were titrated on LB-ampicillin plates. Phage binding, elution, and amplification steps were performed 5 rounds over consecutive days.

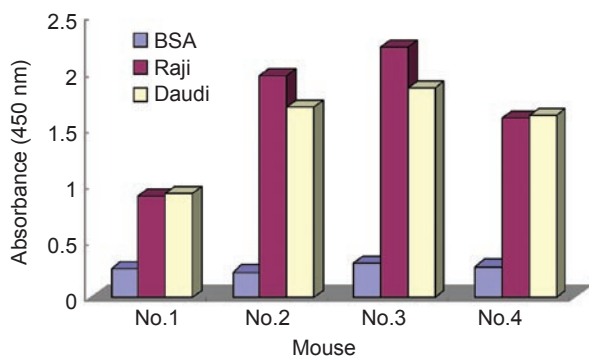
#### Soluble expression of the Fab

Phagemids pComb3H+κ+ Fd (about 200 ng) prepared from last biopanning rounds were extracted and digested with the restriction enzymes *NheI* and *SpeI* to remove gene III fragments. The self-ligated phagemids without gene III fragments were transformed into XL1-Blue cells. Transformants were grown on LB plates containing ampicillin. Clones were examined for the removal of gene III fragments by *SacI/NotI* digestion, which yielded a 2.0 kb fragment. Clones were grown in 10 ml SB containing 20 mM MgCl<sub>2</sub> and

50 µg/ml ampicillin at 37 °C until the culture reached OD600=1.0. At this point, Isopropyl-β-D-thiogalactopyranoside (IPTG) was added up to the final concentration of 1 mM and then the culture was incubated overnight at 30 °C. The bacteria pelleted by centrifugation were resuspended in 1 ml PBS and lysed by freezing and thawing six times. The debris were pelleted and centrifuged at 15 000 rpm at 4 °C for 15 min. The supernatant containing soluble Fab was used directly for ELISA analysis or stored at -20 °C.

#### Analysis of soluble Fab reactivity by ELISA

Each Fab clone solution (100 µl) was incubated for 2 h at 37 °C in triplicate wells of an ELISA plate that had been coated with Raji cells, Daudi cells, Patu8988 cells (pancreatic cancer cell strain) and A549 cells (lung cancer cell strain) respectively and blocked with 2% BSA as described above. After five times of washing with PBS/0.05% Tween20, 50 µl horseradish peroxidase (HRP)-conjugated goat anti-mouse Fab antibody (Sigma, USA) was added (1:2 000 in PBS/2%(v/v) BSA) and incubated for 1.5 h at 37 °C. Following further ten times of washing, color was developed with 100 µl 3,3',5,5'-tetramethylbenzidine (TMB) substrate, and the reaction was stopped



**Figure 1** The titers of the antisera from BALB/c mice immunized with Raji cells and Daudi cells were analyzed by ELISA.

with 50  $\mu$ l of 2 M  $H_2SO_4$ . Clones were considered positive when the OD was more than 3 times the signal seen in wells not coated with Raji cells or Daudi cells. In each ELISA, a negative control without Fab was used to assess background signals.

#### Sequence analysis

The sequences of Fd and  $\kappa$  light chain DNA fragments including clones FabC06, FabC21, FabC43 and FabC59 from the specific positive clones were determined by Shanghai Invitrogen Biotechnology Co.Ltd and their homology with murine Ig was analyzed according to GenBank. Primers used for sequencing the  $\kappa$  light chain were 5'-GGAATTGTGAGCGGATAA-3' and 5'-CAGCGAG-TAATAACAATCCAG-3'. Primers used for sequencing the Fd fragment were 5'-AGCCGCTGGATTGTTATT-3' and 5'-GAACCGCCTCCCTCAGA-3'. The nucleotide sequences of  $V_H$  and  $V_L$  were translated into the sequences of amino acid, and their complementary determining regions (CDRs) and framework regions (FRs) were located according to Immunoglobulin BLAST (available from <http://www.ncbi.nlm.nih.gov/BLAST/>).

#### Western blot analysis

To determine the molecular weight of the soluble Fab antibodies expressed by specific positive clones, the soluble Fab antibodies from FabC06 separated by 12% SDS-PAGE in reducing conditions

(with  $\beta$  ME) as well as in non-reducing conditions (without  $\beta$  ME /DTT) were transferred to nitrocellulose membrane (Invitrogen, USA). After strips were blocked with 5% fat-free milk powder for 1 h at room temperature, the Fab was detected by HRP conjugated goat anti-mouse Fab antibody (1:5 000). After being washed, the immunoreactive bands were visualized with ECL Reagent (Pufei Bio-tech, China) and exposed to x-ray film. An equal volume of pComb3H-SS plasmid transformed into XL1-Blue cell lysates served as the negative control for the Western blot experiment.

To investigate the specificity of soluble Fab antibodies toward the membrane antigens of B cell lymphoma, one million Raji cells and Daudi cells were lysed respectively in 100  $\mu$ l Cell Lysis Buffer (Beyotime Biotechnology, China). Protein products were separated by 12% SDS-PAGE under reducing conditions, transferred onto nitrocellulose membrane, probed with soluble Fab antibodies from FabC06 and FabC21, and followed by HRP conjugated goat anti-mouse Fab antibody (1:5 000). Bands were developed with ECL substrate and exposed to x-ray film.

#### Analysis of Fab reactivity by immunohistochemistry

In order to perform immunohistochemistry, 5  $\mu$ m-thick paraffin-embedded B cell lymphoma tissue sections were cut, and subsequently dewaxed, re-hydrated, and then subjected to antigen retrieval heated in 10 mM citrate buffer in a microwave for 15 min. The sections were cooled, treated with 3%  $H_2O_2$ , blocked with 10% goat serum, and then incubated overnight at 4  $^{\circ}C$  with the respective soluble Fab antibodies from FabC06, FabC21, FabC43 and FabC59. Negative controls were incubated with an equivalent volume of diluent solution alone. After 3 washes with PBS, the standard streptavidin-biotin-peroxidase complex technique by means of sequential 20 min incubation with biotinylated goat anti-mouse Fab (Sigma, USA) and peroxidase-labeled streptavidin (Invitrogen, USA) was performed. 3, 3'- diaminobenzidine (DAB) was used as a substrate chromogen solution for the development of peroxidase activity. Hematoxylin was used for nuclear counterstaining, and then the sections were mounted and cover-slipped.

## Results

#### Characterization of serum and selection of immunized mice

Three days after the final booster injection with Raji and Daudi cells, sera from immunized mice were collected and analyzed by ELISA for the antibody response to tumor

**Table 2** Selective enrichment of phages

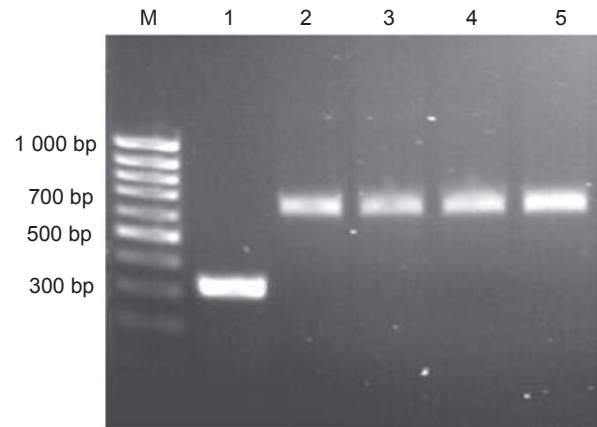
Rounds of panning	Input Phages (cfu)	Eluted Phages (cfu)	Yield (%)	Washing times
First	$4.00 \times 10^{11}$	$3.00 \times 10^5$	$7.50 \times 10^{-5}$	1
Second	$1.00 \times 10^{11}$	$1.33 \times 10^5$	$1.33 \times 10^{-5}$	5
Third	$7.50 \times 10^{10}$	$3.75 \times 10^5$	$5.00 \times 10^{-4}$	10
Fourth	$4.00 \times 10^{10}$	$5.40 \times 10^5$	$1.35 \times 10^{-3}$	10
Fifth	$1.50 \times 10^{10}$	$2.70 \times 10^6$	$1.80 \times 10^{-2}$	10

Footnote: Yield (%) = (No. of eluted phages)  $\times$  100 / (No. of input phages)

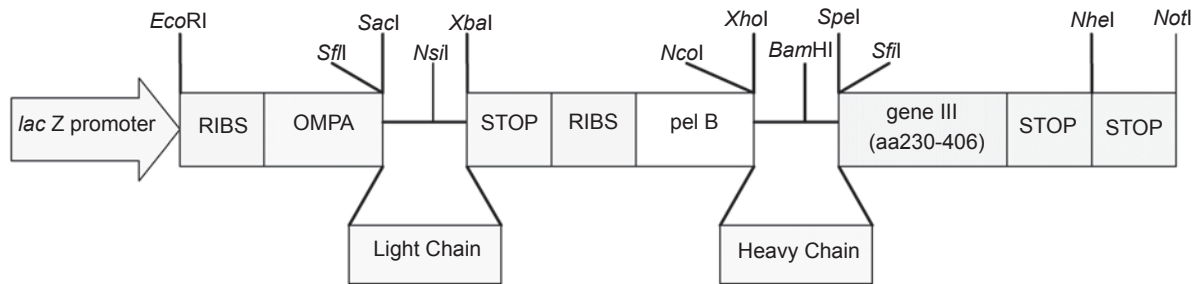
cells, with BSA coated wells serving as the negative control. The results showed that three of the four mice had a strong antibody response to Raji cells and Daudi cells (Figure 1). The mice with the highest (No.3) and the second highest titers (No.2) were killed for Fab phage display library construction.

*RT-PCR amplification of heavy chain Fd and κ light chain*

Total RNAs were isolated from spleen cells from the immunized mice (No.2 and No.3). The integrity of RNAs was checked by alkaline denaturing agarose gel electrophoresis. The  $A_{260}/A_{280}$  was 2.09. cDNAs coding for the immunoglobulin Fd and κ chains were amplified by a variety of primer combinations designed to amplify a majority of the known mouse antibody sequences, with suitable restriction sites for later cloning into the pComb3H-SS vector. The size of the amplified fragments of Fd and κ was about 680 bp (Figure 2).



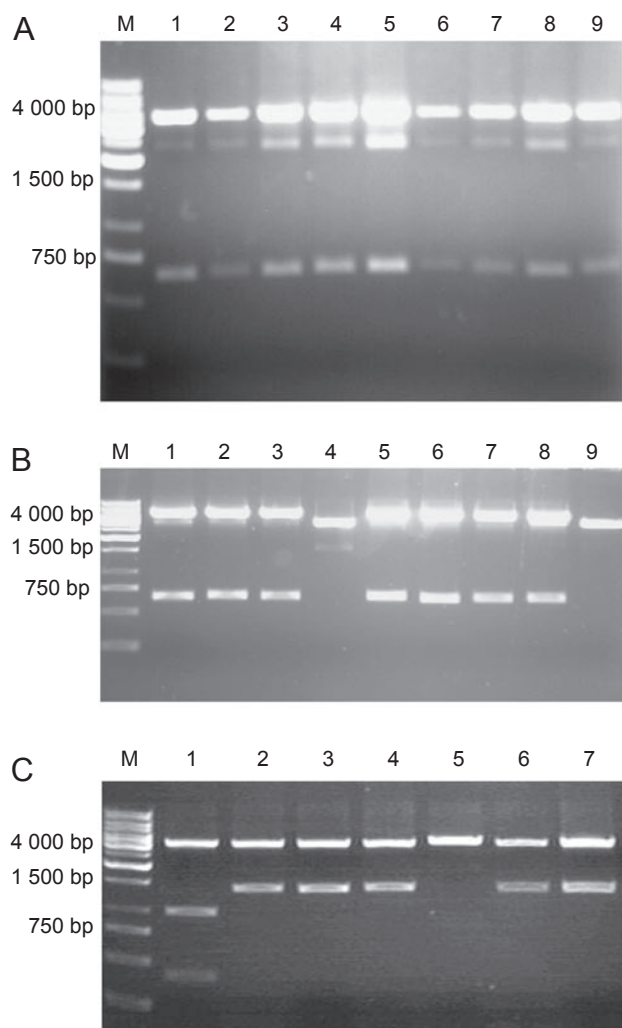
**Figure 2** Amplification of cDNAs coding for the κ light chains and the heavy chain Fd fragments by PCR. Lane M: 100 bp DNA marker; Lane 1: β-actin; Lane 2: Mixed κ light chains; Lane 3: Mixed Fd fragments of heavy chains IgG1; Lane 4: Mixed Fd fragments of heavy chains IgG2a; Lane 5: Mixed Fd fragments of heavy chains IgG3.



**Figure 3** Structure of the phagemid vector pComb3H-SS. The vector is provided only in the pComb3H-SS form where the SS designation is used for the vector with stuffer fragments. The heavy chain stuffer is 300 bp and the light chain stuffer is 1200 bp of the sequence. The *SacI* and *XbaI* restriction sites are used for cloning of the light chain fragments whilst the Fd fragments are inserted at *XhoI* and *SpeI* sites. Cleavage of pComb3H by *SpeI* (A|CTAGT) and *NheI* (G|CTAGT) would produce compatible cohesive ends that can be re-ligated, resulting in the removal of the gene III product of VCSM13 producing soluble Fabs. The *lacZ* promoter drives the synthesis of the light chain and Fd/gene III transcript. The leader peptides *ompA* and *pelB* target these two polypeptides to the bacterial periplasm where the soluble light chain fragment and the membrane-bound Fd fragment associate via a disulfide bond.

**Table 3** ELISA detection of reactivity between positive Fabs and various cells (mean±SD,  $A_{450}$  value)

positive clones	Raji cells	Daudi cells	Patu 8988	A549	BSA
FabC02	1.693±0.087	0.497±0.038	0.273±0.045	0.294±0.052	0.263±0.038
FabC06	1.886±0.059	1.522±0.081	0.249±0.028	0.371±0.039	0.271±0.031
FabC21	1.732±0.061	1.542±0.092	0.290±0.026	0.342±0.045	0.203±0.027
FabC36	1.519±0.033	0.627±0.023	0.298±0.031	0.284±0.035	0.297±0.032
FabC43	1.610±0.034	1.763±0.044	0.306±0.037	0.345±0.020	0.301±0.036
FabC48	1.536±0.054	1.489±0.064	0.364±0.031	0.296±0.049	0.276±0.038
FabC59	1.558±0.048	1.792±0.036	0.291±0.034	0.327±0.027	0.261±0.040
FabC65	1.597±0.037	1.184±0.051	0.323±0.030	0.284±0.041	0.317±0.022



**Figure 4** Fab library construction. **(A)** Identification of the  $\kappa$  insert by *SacI/XbaI* digestions. The  $\kappa$  light chain PCR products were cloned between the *SacI* and *XbaI* sites of the pComb3H-SS vector and the  $\kappa$  inserts were identified by *SacI/XbaI* digestions. Lane M: 1 kb DNA marker; Lane 1 to lane 9: nine randomly selected clones which contained the expected  $\sim$ 680 bp insert. **(B)** Fd insert identification by *XhoI/SpeI* digestions. Phagemids containing the  $\kappa$  light chain repertoires were named as pComb3H-S+ $\kappa$ . The heavy chain Fd PCR products digested with *XhoI/SpeI* were cloned between *XhoI* and *SpeI* sites of pComb3H-S+ $\kappa$ . Lane M: 1 kb DNA marker; Lane 1 to lane 9 show that 7 of the 9 randomly selected clones contained the  $\sim$ 680 bp Fd fragment. **(C)** Identification of Fab inserts by *SacI/SpeI* digestions. Lane M: 1 kb DNA marker; Lane 1 to lane 7 show that 5 of the 7 examined clones contained the  $\sim$ 1 500 bp Fab ( $V_H C_H1-V_L C_L$ ) fragment.

#### Library construction

The Fab library was constructed in two steps. In the first step, the  $\kappa$  light chain fragments were digested by restric-

tion enzymes *SacI/XbaI*, gel-purified, and ligated to the phagemid vector pComb3H-SS that had been cut with the same enzymes. The schematic structure of pComb3H-SS is shown in Figure 3 [17]. Following precipitation and washing, the  $\kappa$  ligation mixture was electroporated into *E. Coli* XL1-Blue to form the  $\kappa$  light chain gene library. The total number of transformants was about  $4.5 \times 10^7$ . Examination of plasmids from nine randomly selected XL1-Blue clones revealed that all nine clones contained the expected 680 bp insert (Figure 4A). Thus the size of the  $\kappa$  light chain library was  $\sim 4.5 \times 10^7$ . In the second step, the heavy chain Fd fragments were digested with *XhoI/SpeI* and cloned into the vector already containing the  $\kappa$  light chain repertoires. After transforming XL1-Blue, the Fab library was formed and the total number of transformants reached  $3.5 \times 10^7$ . Examination of plasmids from nine randomly selected XL1-Blue clones showed that seven of these contained the expected insert of 680 bp (Figure 4B). By digestion with *SacI/SpeI*, five of these seven plasmids released the expected insert of  $\sim$ 1500 bp (Figure 4C). Thus we estimated that the actual diversity of our Fab library was  $\sim 1.94 \times 10^7$  ( $3.5 \times 10^7 \times 78\% \times 71\%$ ).

#### Phage Enrichment

After five rounds of biopanning, the percentage of phage yield increased from  $7.50 \times 10^{-5}$  to  $1.80 \times 10^{-2}$ , representing an enrichment of about 200 fold (Table 2) as a result of successive removal of low affinity and nonspecifically bound phages.

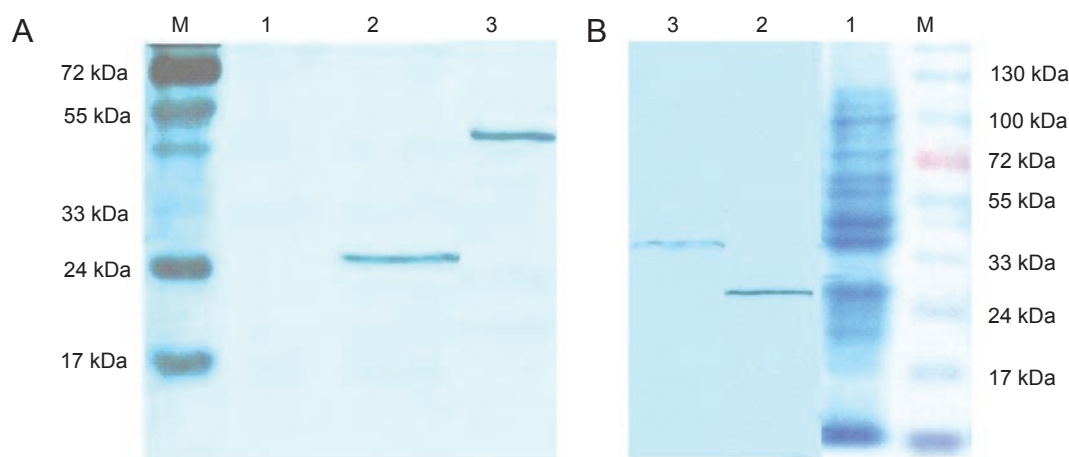
#### Selection of soluble Fab antibodies against *B lymphoma cell lines* by ELISA

After the final round of biopanning, 65 clones were tested by ELISA against Raji cells, Daudi cells and BSA for the production of soluble Fab fragments. Finally, eight clones that showed high reactivity against Raji or Daudi cells with low background reaction against BSA were selected as positive clones. Among the eight positive clones, six showed high reactivity to both Raji and Daudi cells, and two only to Raji cells but not to Daudi cells. The eight positive Fabs were also tested against the pancreatic cancer cell line Patu 8988 and lung cancer cell line A549, and none of these clones demonstrated significant cross-reactivity with these two cell lines (Table 3).

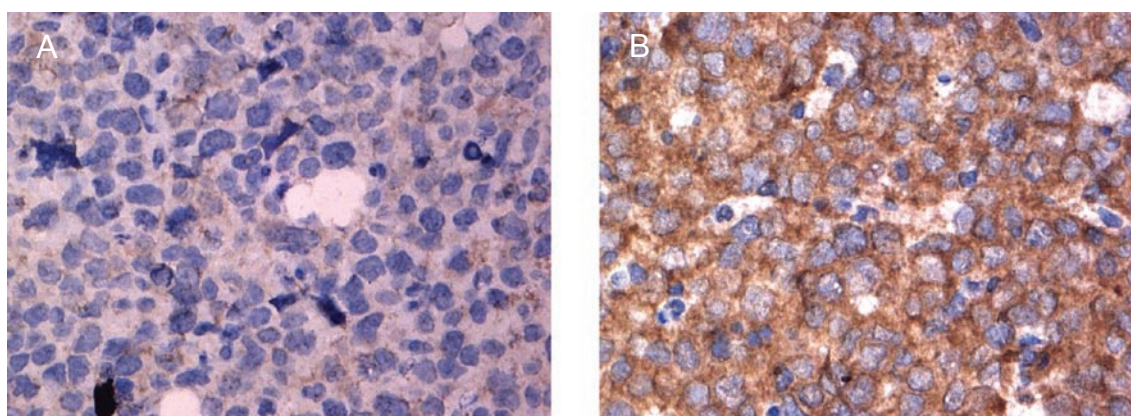
#### Sequence analysis of specific positive clones

From the eight positive clones, FabC06, FabC21, FabC43 and FabC59 were selected for sequence analysis. The results of a homology search showed that the  $V_H$  chains of FabC06, FabC21, FabC43 and FabC59 share 92%, 89%, 88% and 92% homology in amino acid sequences with the closest mouse Ig heavy chain variable region germline





**Figure 6** Western blot analysis. **(A)** Profile of the Fab antibody from FabC06. Lane M: Protein marker; Lane 1: Negative control of pComb3H-SS transformed bacterial cell lysates; Lane 2: Fab under reducing conditions; Lane 3: Fab under non-reducing conditions. **(B)** The specificity of Fab antibodies toward membrane antigens of the B lymphoma cells analyzed by western blot. Lane M: Protein marker; Lane 1: Protein profile of Raji cells analyzed by reducing SDS-PAGE; Lane 2: Western blot of Raji cell proteins with the Fab antibody from FabC21; Lane 3: Western blot of Raji cell proteins with the Fab antibody from FabC06.



**Figure 7** Immunohistochemical analysis of B lymphoma section with a soluble Fab antibody. **(A)** Negative control (original magnification:  $\times 400$ ); **(B)** The positive reaction of FabC06 was found on the membranes of B lymphoma cells (original magnification:  $\times 400$ ).

sequence (Accession No. P01807) respectively.  $V_L$  chains of FabC06, FabC21, FabC43 and FabC59 share 89%, 94%, 81% and 94% homology in amino acid sequences with the closest mouse Ig kappa variable region germline sequence (Accession No. AAA38888.1) respectively. None of the  $V_H$  and  $V_L$  sequences of the four clones were identical to each other. The three complementary determining regions (CDRs) and four framework regions (FRs) were marked for  $V_H$  chains and  $V_L$  chains in Figure 5. As for the CDRs, CDR3 of the heavy variable domain showed the most frequent nucleotide and amino acid substitutions. On the other hand, only minor substitutions occurred in FRs. These

results suggest that CDR3 of the heavy variable domain is associated with Fab function, which is the capability to recognize different antigens on the membrane of B lymphoma cells.

#### *Analysis of Fab antibodies by SDS-PAGE and western blot*

To determine the molecular weight of Fab antibodies, soluble Fab antibodies from FabC06 were resolved by SDS-PAGE under both reducing and non-reducing conditions, and were subsequently detected by western blot analysis. As shown in Figure 6A, under non-reducing conditions, the



soluble Fab appeared as a heterodimer (about 50 kDa) consisting of Fd and the light chain. Under reducing conditions, both Fd and the light chain were detected in monomeric form (about 25 kDa). These results indicated that the light chain and the heavy chain Fd were correctly linked by disulfide-bonding. The soluble Fab antibodies from FabC06 reacted mainly with a band at approximately 33 kDa on the membrane of Raji cells, while soluble Fab antibodies from FabC21 reacted with a band at approximately 25 kDa (Figure 6B). These results showed that the two soluble Fab antibodies from FabC06 and FabC21 recognized different membrane antigens of B lymphoma cells. Similar results were obtained for Daudi cells (data not shown).

#### *Analysis of Fab reactivity by immunohistochemistry*

Under the described condition for immunohistochemistry analysis, strong immunoreactivity for the Fabs was observed on the membrane of B lymphoma cells (Figure 7). However, no immunoreactivity was observed in the wider parts of the cytoplasm or in the nucleoli of B lymphoma cells.

## **Discussion**

The emergence of phage display technique presents a new approach in tumor immunotherapy. Several phage antibody libraries against tumors have been constructed and screened, such as those against melanoma, osteosarcoma and prostate cancer [7, 20, 21]; and some of these phage antibodies showed high affinity and specificity for tumor antigens. As for lymphoma, so far only two studies have reported the generation of specific phage display library. Bruenke *et al.* [22] generated bispecific single-chain Fv antibody (bscFv) from hybridomas 3G8 and F3.3 using the phage display library, which could mediate specific lysis of malignant human B-lymphoid cell lines. In another report, Dong *et al.* [23] isolated two Fab clones against the viral capsid antigen (VCA) of Epstein-Barr virus (EBV) from a patient with marginal zone B cell lymphoma.

In this study, we extracted total RNA from spleen cells of BALB/c mice immunized with Raji and Daudi cells. cDNAs coding for the  $\kappa$  light chains and heavy chain Fd fragments were amplified by RT-PCR, and the amplified products were then used to construct the phage display library. It has been shown that when the diversity of an antibody phage display library reaches  $1 \times 10^7$  individual clones, it will contain 99% of all antibody molecules. The size of antibody libraries that have been constructed so far generally ranges between  $10^6$  and  $10^8$  clones [24, 25]. The size of our library for B cell lymphoma is  $\sim 1.94 \times 10^7$ , which is of medium size and should satisfy the needs for covering the diversity of antibodies [26]. Following several

rounds of biopanning, phages producing specific antibody molecules could be enriched [27]. Our study showed that the phage Fab libraries were enriched by about 200 fold after five rounds of biopanning with Raji cells and Daudi cells. ELISA analysis showed that the phage displaying Fab had significant binding activity with antigens associated with B cell lymphoma. Finally, eight clones that showed high reactivity with Raji cells or Daudi cells were selected as positive clones. To verify the origin of the Fab antibody genes and to estimate the extent of sequence diversity, amino acid sequences from four positive clones were aligned and compared with their closest murine germline V regions.  $V_H$  and  $V_L$  domains were found to share 88-92% and 89-94% homology with the closest murine germline V regions respectively. As the  $V_H$  domain of an antibody is known to play a major role in antigen recognition and binding [28], and also as somatic mutations in V genes during B cell maturation are indispensable for rearranged antibodies to obtain high specificity and affinity [29], we further analyzed the  $V_H$  sequences of the four positive clones. We found that frequent amino acid substitutions occurred in CDR3, but only minor substitutions occurred in FRs. Western blot analysis showed that different Fab antibodies reacted with different membrane proteins of B lymphoma cells (Figure 6). It is likely that differences in CDR3 sequences may underlie the ability of respective Fab antibodies to recognize different antigens.

Today, mAbs are generated either by the hybridoma technology or from antibody libraries [30]. For practical reasons, the hybridoma technology is always confined to mice, thus making it difficult to use the resulting monoclonal antibodies for therapeutic purposes. Such antibodies may trigger human anti-mouse antibody (HAMA) response, especially when multiple infusions are required to obtain therapeutic efficacy [2, 3]. Antibody libraries allow the generation of mAbs from virtually any species whose immunoglobulin genes are known. In this way, potential problems associated with the hybridoma approach, such as HAMA, low fusion, instability and low antibody production, will be resolved. Antibody libraries have been used to exploit large naive or synthetic antibody repertoires, or combinations of both, for the generation of human mAbs [31, 32]. In contrast to antibodies derived from large naive or synthetic repertoires, however, antibodies from immunized animals are subjected to *in vivo* selection and thus are more likely to selectively recognize a given antigen without cross-reactivity to another antigen [7]. In this study, we chose spleen cells of mice immunized with B lymphoma cells to construct phage display library. Though this method can reduce the diversity of antibodies against the target antigen, it should increase the proportion of specific antibodies and enhance the possibility of obtaining high affinity

antibodies in a relatively small-size library. However, we can not directly construct human antibody phage library from lymphoma patients, because patients with lymphoma always have defects in humoral immunity, rendering it difficult to construct an effective phage antibody library against lymphoma.

In order to ensure the diversity of our phage antibody library, we took the following measures: firstly, total RNA was extracted from spleen of immunized mice to reduce the chance of RNA degradation; secondly, among the primers of Scripps Research Institute, twenty primers were designed, which included nine pairs of primers for subset IgG1, IgG2a and IgG3 respectively and seven pairs of primers for murine kappa light chain; thirdly, since the V<sub>H</sub> domain of an antibody is known to play a major role in antigen recognition and binding [28], the light chain was cloned first, followed by the heavy chain; and finally, the library size was improved by increasing the electroporation efficiency and repeating electroporation.

Standard phage display techniques depend upon panning of Fab-producing phages against purified antigen immobilized on substrates. These conditions may not be applicable if the antigen is not available in purified form or if it changes conformation under immobilization conditions. There are other cases, such as autoimmunity, in which the antigen itself may be unknown. In all of these cases, it would be difficult or impossible to apply traditional panning techniques for the identification of reactive antibodies of interest. This situation was encountered in our study, because the screening of our phage antibody library needed to be performed against membrane-bound antigens on B lymphoma cells. Some authors have successfully applied the phage display technology in screening, isolation and identification of tumor-associated cell surface antigens, such as those of melanomas, prostate cancer and colorectal carcinomas [7, 33, 34]. Various differentiation antigens are exposed on the membrane of B cell lymphoma, such as CD20, CD19 and CD22. It is worth noting that 95% of B lymphoma patients have high levels of CD20 on the membranes of tumor cells [35], while there is no exposure on cells of the normal tissue and multipotent stem cells. Thus, in our present study, we developed a method of cell-based panning to screen the Fab phage library, using six well plates coated by Raji and Daudi cells. After five rounds of panning, the enrichment was about 200 fold and ELISA assays identified eight positive clones.

In conclusion, we have successfully constructed a phage display antibody library for B cell lymphoma with fairly good sensitivity and specificity. These immunized Fab antibodies would lay a valuable experimental foundation for further study of B cell lymphoma and may also contribute to the improvement in the therapy of this disease.

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